Genes and environment underlying lung health

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Kim de Jong

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General introduction

K. de Jong

GENERAL INTRODUCTION

COPD

Chronic obstructive pulmonary disease (COPD) is a prevalent disease associated with a large burden of morbidity and mortality worldwide. The global burden of COPD is still increasing and the disease is expected to become the 3rd leading cause of death by 2030[1]. In the Netherlands, about 360,000 (2%) individuals had COPD and 6,353 individuals died due to COPD in 2011, corresponding to 4% of all deaths that year[2].

COPD is characterized by persistent and often progressive airflow obstruction caused by an abnormal inflammatory response to noxious particles and gases. This inflammatory response leads to structural changes and increased mucus production in the central airways (chronic bronchitis), inflammation and remodeling in the peripheral airways (bronchiolitis, small airways disease), and loss of lung tissue in the lung parenchyma (emphysema). Chronic bronchitis, small airways disease and emphysema often co-exist, and the predominant phenotype varies from person to person.

Chronic bronchitis may precede or coincide with airway narrowing but may also be present in patients without COPD[3]. An increased number of mucus producing cells and enlargement of mucus glands results in increased secretion of mucus, which is normally secreted as part of a normal biological mechanism protecting the airways and lung tissue against noxious particles and gases. Overproduction of mucus (chronic mucus hypersecretion) is seen in individuals with and without COPD.

Many noxious particles and gases, such as from tobacco smoking affect both the small airways and the large airways. Loss and narrowing of the small airways is seen in patients with mild COPD even before the onset of emphysematous destruction and this becomes increasingly evident in severe COPD[4].

Classification of COPD

Spirometry is the most commonly available and reproducible test for the diagnosis and classification of COPD[5]. The diagnosis of COPD is based on post-bronchodilator forced expiratory volume in one second (FEV_1) and the ratio of FEV_1 to forced vital capacity (FVC). Airflow obstruction is defined as an $FEV_1/FVC<70\%$. According to the guidelines of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) until 2012, disease severity is classified in four stages, determined by the level of FEV_1 as percentage of predicted given gender, age and height (table 1). Whereas the spirometric parameters FEV_1 and the FEV_1/FVC ratio are used to indicate obstruction of predominantly the large airways, the forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}) is used to measure small airways obstruction. This parameter is however largely dependent on the level of FVC.

Lung function is age-dependent. Lung function increases during lung development and growth *in utero* and during childhood. The plateau phase is reached around the age of 20 years. After the age of 30, lung function starts to decline as part of the normal aging process. Abnormally accelerated decline of lung function is seen in COPD and may occur at several stages of life[6,7].

Table 1. Severity of COPD classified in stages according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) until 2012.

Stage	FEV_1/FVC	FEV_1	Classification
I	<70%	≥80%	Mild
II	<70%	≥50-80%	Moderate
III	<70%	≥30-50%	Severe
IV	<70%	<30%	Very severe

COPD clinically manifests itself predominantly after the age of 40 years, yet development of the disease starts long before and may even have its origins in childhood[8]. Complex interactions between environmental and genetic factors may affect lung development *in utero*, reduce lung growth in childhood and accelerate the decline of lung function during adulthood. People with a lower level of lung function and/or an accelerated lung function decline are more prone to experience respiratory symptoms and limitations in exercise capacity, and are at increased risk to develop COPD later in life (figure 1).

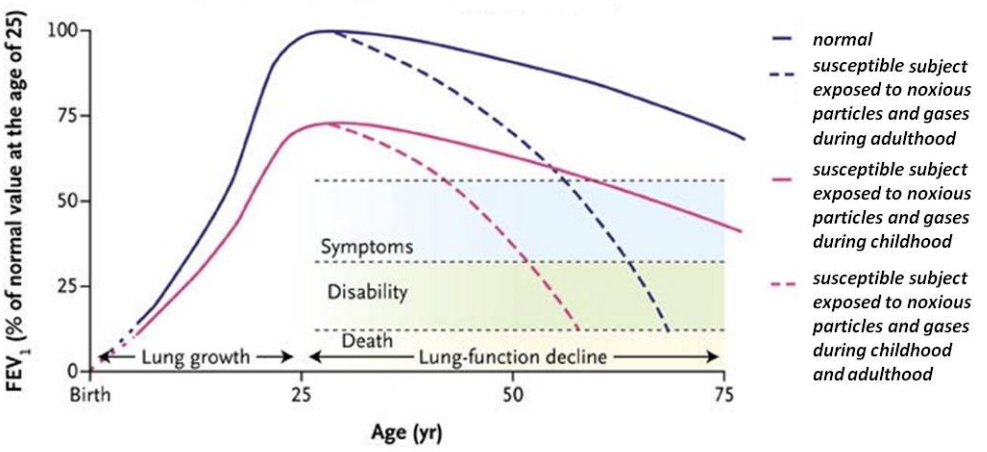


Figure 1. Level of lung function and decline during the life-span (adapted from Brusselle, 2009)[9].

Non-smoking COPD

Development of COPD likely results from complex interactions between (multiple) environmental exposures and genetic factors. In the developed world, smoking is regarded to be the most important risk factor for COPD. There are, however, two important aspects to consider. First, not all smokers develop COPD[10,11], suggesting that there is inter-individual difference in susceptibility to exposure to tobacco smoke. Secondly, the population-attributable fraction (PAF) of tobacco smoke as a cause for COPD reported in literature ranges from 10 to 98%, most studies however show PAFs of less than 80%, suggesting that other environmental risk factors exist[12]. Various studies in developed countries have shown that about 25 to 45% of all COPD patients never smoked (figure 2)[13,14]. In the developing countries, the proportion non-smoking COPD is often higher, which may be caused by indoor air pollution from biomass fuel used for cooking and heating[1,13].

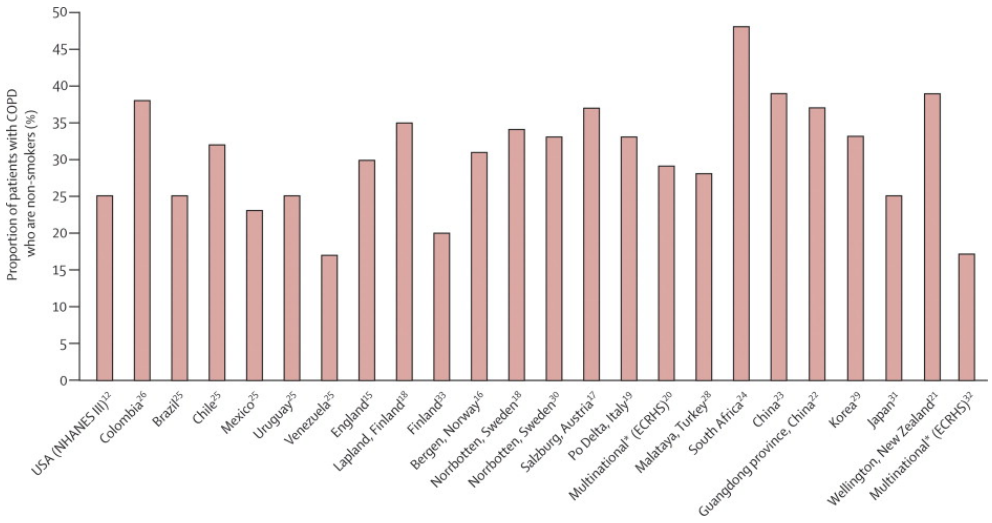


Figure 2. Prevalence of non-smoking COPD across different studies worldwide (Salvi and Barnes, the Lancet, 2009)[13].

In the developed countries, other environmental risk factors for COPD apart from personal smoking include environmental tobacco smoke (ETS), occupational exposures and ambient air pollution. Like active tobacco smoking, ETS exposure induces inflammation and oxidative stress in the lungs and has been associated with reduced levels of lung function at birth[15,16] and in adulthood[17,18], as well as with respiratory symptoms[19,20] and increased COPD risk[21,22]. Occupational exposures such as organic and inorganic dusts, chemical agents and fumes are other and underappreciated risk factors for COPD[5,23-25]. It has been estimated that about 15-20% of all COPD cases are work related[26,27], with proportions up to 30% in never smokers[27]. Finally, ambient (outdoor) air pollution, a mixture of hundreds of pollutants originating from industry, traffic, heating, and other sources, can induce airway oxidative stress, pulmonary and

systemic inflammation and has been associated with reduced lung function levels, accelerated lung function decline and an increased risk for COPD[12,28,29].

It needs to be determined which factors drive COPD development in non-smokers and which biological pathways are underlying these associations. Furthermore it should be studied whether similar or differential biological pathways underlie smoking and non-smoking COPD. As for tobacco smoking, there is likely inter-individual difference in genetic susceptibility to ETS, occupational exposures and ambient air pollution.

Genetic susceptibility

The human genome consists of deoxiyribonucleic acid (DNA) that is made up of nucleotides composed of a backbone (deoxyribose), a phosphate group and a base. There are four different types of bases; adenine (A), thymine (T), cytosine (C) and guanine (G). In the double helix structure of DNA, adenine always pairs with thymine (A-T) and cytosine always pairs with guanine (C-G). Although more than 99% of all DNA is similar between individuals, certain base pairs differ. These differences are called single nucleotide polymorphisms (SNPs) (figure 3). There are millions of SNPs in the human genome. Yet the functionality is only known for a small proportion of these variants. Functional SNPs may cause altered gene expression (eQTL), altered protein structure or altered splice variants.

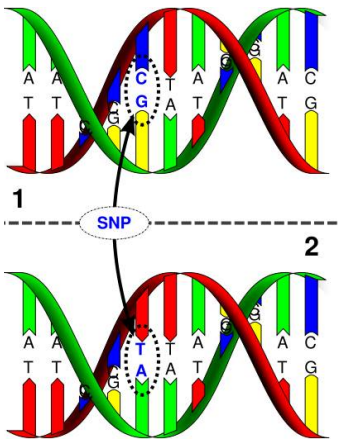


Figure 3. Single nucleotide polymorphism (SNP), a single base-pair difference between individuals.

About 1-2% of all individuals with COPD suffer from alpha-1-antitripsin-deficiency (AAT-deficiency), a disorder resulting from a mutation in a single gene causing early onset emphysema. More commonly, COPD results from complex interactions between multiple genes and multiple environmental exposures. This is illustrated by the fact that only a relatively small proportion of 15-25% of all smokers eventually develop COPD[10,11]. Studying genetic susceptibility to COPD is important since it may provide novel insights into biological pathways leading to disease development.

Until recently, candidate gene approaches have been used to investigate several SNPs in candidate genes or whole pathways of genes chosen a-priori based on a hypothetical biological mechanism. The main focus of these candidate gene approaches has been on the so-called pathogenic trait of COPD: oxidative stress, proteases-antiprotease imbalance and persistent inflammation, that may essentially cause goblet cell metaplasia and hyperplasia, mucus hypersecretion,

airway wall fibrosis, alterations in smooth-muscle cells and extracellular matrix, with associated destruction and loss of lung tissue[30].

Since the Human Genome Project has been published in 2003 and genotyping costs have decreased, genome-wide genotyping of large samples has become possible. In contrast to candidate gene approaches where several plausible candidates are tested, genome-wide association (GWA) studies are hypothesis-free approaches testing hundreds of thousands genetic markers across the entire genome. This approach aims to identify novel loci associated with disease risk. Essentially this may provide novel insights in (novel) biological pathways associated with disease development. The first GWA study on the level of lung function, published in 2007, amongst others identified a SNP in *GSTO2*, a gene involved in the oxidative stress response, that was associated with both the level of FEV_1 and FVC[31]. Since 2007 more GWA studies have been performed and several novel genetic loci associated with the level of lung function have been identified[32-35]. The first GWA study on COPD, published in 2009, reported two loci associated with the disease, HHIP and CHRNA3/5 (nicotinic receptor). Later studies suggested that the nicotinic receptor gene is associated with smoking habits rather than COPD itself[36]. Thus far these GWA studies have disregarded the environment, although it seems likely that genetic variation is of importance when there are environmental exposures triggering the development of the disease.

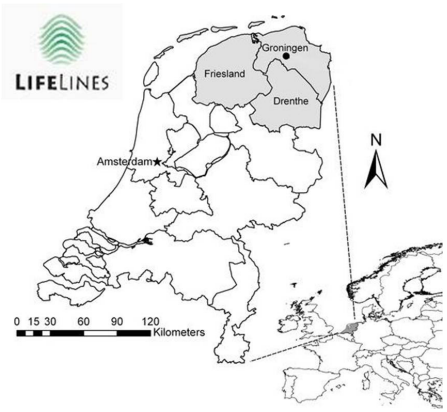
The number of published genome-wide interaction (GWI) studies, that aim to identify loci associated with a disease given a certain exposure, is limited. GWI studies are difficult to establish. First, in addition to acquiring detailed phenotypic and genotypic information like in GWA studies, extensive exposure assessment has to be performed. Second, a main limitation of testing gene-environment interactions on a genome-wide scale is its limited power[37]. Cohorts for genome-wide studies need to be large in order to have sufficient power to overcome the multiple testing penalty (Bonferroni corrected p-value = 0.05/number of tests). Introducing an interaction term in the model may both decrease the power and increase the error due to noise in the exposure assessment. Different strategies have been proposed to analyze the data with maximal power. The optimal strategy largely depends on the outcome of interest, i.e. studying cases versus controls or studying a quantitative trait such as level of lung function.

AIMS OF THE THESIS

Aims: To assess whether environmental exposures are associated with the level of lung function and the prevalence of COPD, and to assess inter-individual differences in genetic susceptibility to the effects of these exposures.

STUDY POPULATIONS

As mentioned before, studying interactions between exposures (i.e. smoking and occupational exposures) or genes and exposures in genome-wide studies requires large datasets with extensive characterization of genotype, phenotype, and exposure. With the LifeLines and Vlagtwedde-Vlaardingen population-based cohorts, well characterized clinical, genetic and exposure data is available for a large number of subjects.



The LifeLines cohort study

LifeLines is an observational follow-up study in a large representative sample of the population of the Northern provinces of the Netherlands covering three generations. The LifeLines cohort study started in 2006 and the total number of 165,000 individuals included will be followed for 30 years. LifeLines is designed to study genes, exposures and their interactions in the aetiology of complex (multifactorial) diseases and healthy aging[38]. All subjects undergo extensive medical examination, including spirometry, and detailed information on environmental exposures is acquired. Most of the work presented in this thesis is performed on data from the first and second release of the LifeLines cohort study including approximately 13,000 unrelated individuals selected for genome-wide genotyping.

The Vlagtwedde-Vlaardingen cohort study

The Vlagtwedde-Vlaardingen cohort is a general population-based cohort including subjects from a rural area in the North-East of the Netherlands (Vlagtwedde) and subjects from an urban area in the South-West of the Netherlands (Vlaardingen). The cohort started in 1965 and was followed for 25 years, measurements were performed every 3 years. The study aimed to obtain knowledge on the prevalence of chronic airway diseases as well as to gain deeper insight in determinants of these diseases, i.e. endogenous factors such as age, sex, allergy and bronchial hyperresponsiveness, and exogenous factors such as tobacco smoking and air pollution[39]. Genome-wide genotyping has been performed on blood samples from a subset of subjects included in the last survey (1989/1990). Genotypes are available for approximately 1,500 subjects.

OUTLINE OF THE THESIS

In **Chapter 2** we assessed associations of various occupational exposures with the level of lung function and the prevalence of COPD in the Dutch general population, and secondly whether these associations were different between never and ever smokers and between males and females.

In **Chapter 3** we assessed if occupational exposures were associated with obstruction of the small airways, as measured with forced expiratory flow at 25 to 75% of FVC ($FEF_{25-75\%}$), and whether these associations were different between never and ever smokers.

In **Chapter 4** we assessed if occupational exposures that were associated with the level of lung function in two Dutch general population based cohorts (chapter 3), were additionally associated with the longitudinal decline of lung function, and whether these associations were different between never and ever smokers.

In **Chapter 5** we assessed risk factors (active smoking, exposure to environmental tobacco smoke exposure and occupational exposures) for chronic mucus hypersecretion (CMH) in subjects with and without COPD.

In **Chapter 6** we used a candidate-gene approach to assess whether associations between ETS exposure during different periods throughout the life-span and the level of lung function during adulthood were modified by genetic variation in *Gluthatione-S-Transferases Omega 1* and *2* (*GSTO*), genes that are involved in oxidative stress reactions and detoxification of xenobiotic substances.

In **Chapter 7** we used a genome-wide approach to identify novel genetic loci and pathways underlying individual susceptibility to the effects of ETS exposure on the level of FEV_1.

In **Chapter 8** we used a genome-wide approach to identify novel genetic loci that affect individual susceptibility to the effects of common occupational exposures, i.e. biological dust, mineral dust and gases and fumes, on the level of FEV_1. Secondly we assessed whether newly identified loci were cis-acting expression quatitive trait loci (cis-eQTLs) in lung tissue.

In **Chapter 9** we used a genome-wide approach to identify novel genetic loci that affect individual susceptibility to the effects of exposure to pesticides on the level of FEV_1.

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2

Pesticides and other occupational exposures are associated with airway obstruction: the LifeLines cohort study

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ABSTRACT

Background

Occupational exposures are important and possibly modifiable contributors to the global burden of COPD. Exposure to vapors, gases, dusts and fumes (VGDF) has been associated with a 2-3 fold higher COPD risk. Less is known about effects of occupational exposure to pesticides and solvents. In the current study we assessed if VGDF, pesticides and solvents are associated with the level of lung function and the prevalence of airway obstruction in the general population.

Methods

We included 11,851 subjects aged 18-89 years from the LifeLines cohort study. Regression models assessing associations between occupational exposures (no/low/high), level of lung function (pre-bronchodilator FEV_1, FEV_1/FVC), mild and moderate/severe airway obstruction were adjusted for sex, age, height, weight, current/ex smoking and packyears. Additionally we stratified by smoking status and gender and tested for interaction. A second general population cohort (n = 2,364) was used to verify our initial findings.

Results

Occupational exposure to VGDF and pesticides was associated with a lower level of FEV_1 and FEV_1/FVC and with a higher prevalence of mild and moderate/severe airway obstruction in the two general populations investigated. There were no associations with exposure to solvents.

Conclusions

Occupational exposure to both VGDF and pesticides is associated with airway obstruction in the general population.

BACKGROUND

Worldwide, millions of people suffer from chronic obstructive pulmonary disease (COPD). About 3 million people died due to COPD in 2005[1]. The morbidity and mortality associated with the disease causes an enormous economic burden; health care costs of COPD in the USA alone were estimated to be 50 billion dollar in 2010[2]. The global burden of COPD is still increasing and the disease is expected to become the third leading cause of death by 2030[3,4]. Yet the cellular and molecular pathways driving COPD are still not fully understood[5]. Tobacco smoking is considered to be the main risk factor for COPD, although a substantial proportion of 15-20% of all COPD cases has been attributed to occupational exposures,[6] with proportions up to 30% in never smokers[7].

Occupational exposure to broadly defined categories like vapors, gases, dusts, fumes and their composite measure (VGDF) have been shown in several studies to increase COPD risk 2-3 fold[8-11]. Joint exposure with smoking was shown to increase the risk even 14-fold[9]. Epidemiological studies investigating effects of more specific occupational exposures, like pesticides and solvents are scarce. Since the agricultural sector employs more than 1.1 billion workers worldwide (about 34% of the global working force),[12] adverse health effects associated with occupational exposure to pesticides can have a large public health impact. This is especially true in highly exposed populations, such as agricultural workers in developing countries who often use pesticides with insufficient protective equipment and training[13]. Like pesticides, solvents are widely used agents in every day practice, such as degreasing, cleaning and painting. Possible adverse health effects, for instance due to their volatile and irritable properties, might therefore apply to millions of people worldwide.

Because occupational exposures are common, yet also potentially modifiable contributors to the global burden of COPD, it is important to determine which occupational factors drive the development of COPD. Although COPD clinically manifests predominantly after age 40, it is relevant to study early phenotypes that may be associated with development of COPD later in life, such as decreased level of lung function and early signs of airway obstruction. In the current study we assessed the associations of occupational exposure to gases and fumes, mineral dust, biological dust, their composite measure VGDF, pesticides in general, herbicides and insecticides specifically, and various types of solvents on level of lung function and the prevalence of mild and moderate/severe airway obstruction in a general population cohort. Additionally, differential associations for never and ever smokers and males and females were investigated. A second general population cohort from the same area, i.e. the Vlagtwedde-Vlaardingen cohort, was used to verify our initial findings.

METHODS

Study sample

We included individuals from the LifeLines cohort study, a multi-disciplinary prospective population-based cohort study examining health and health-related behavior of persons living in the Northern region of The Netherlands[14]. Subjects were recruited via general practitioners. In the current study we included 13,301 subjects from the second data release of the LifeLines cohort. All LifeLines participants received a medical examination and questionnaires at baseline. The medical examination included pre-bronchodilator spirometry (FEV_1, FEV_1/FVC) using a Welch Allyn Version 1.6.0.489, PC-based SpiroPerfect with Ca Workstation software. The questionnaires included questions regarding personal characteristics, smoking habits, job title and description of current or last held job.

Occupational exposure

Job title and description were coded according to the International Standard Classification of Occupations version 1988 (ISCO-88)[15]. These four-digit classification codes were used to estimate job-specific exposures to VGDF (subcategories gases and fumes, mineral dust and biological dust), pesticides (subcategories herbicides and insecticides), and various types of solvents (aromatic, chlorinated, other) using the ALOHA+ Job Exposure Matrix (JEM)[8]. The ALOHA+ JEM classifies subjects based on the ISCO-88 job codes into no, low and high exposure categories (0/1/2). In case a participant had two different jobs simultaneously, exposures of both jobs were averaged and rounded to the nearest integer (0.5 = 1 and 1.5 = 2).

Statistical analysis

Associations of the specific occupational agents with pre-bronchodilator level of lung function (FEV_1 and FEV_1/FVC), mild and moderate/severe airway obstruction were assessed using linear and logistic regression adjusted for sex, age, height, weight, current/ex smoking and packyears (log packyears+1), all at enrolment. Mild obstruction was defined as pre-bronchodilator FEV_1/FVC<70%. To assess associations with more severe obstruction, we defined moderate/severe obstruction as having pre-bronchodilator FEV_1/FVC<70% and FEV_1<80%predicted and no obstruction as having pre-bronchodilator FEV_1/FVC≥70% and FEV_1≥80%predicted.[16] Subjects with mild obstruction (pre-bronchodilator FEV_1/FVC<70% and FEV_1≥80%predicted) or with possible other pathology, like restrictive lung disease (pre-bronchodilator FEV_1/FVC≥70% and FEV_1<80%predicted) were excluded from this analysis (LifeLines n = 1,517 (13%) and Vlagtwedde-Vlaardingen n = 436 (18%)).

Because of substantial co-exposure between the specific occupational agents (supplementary table 1 in the online supplement) we additionally adjusted the models with exposure to VGDF, gases, fumes, mineral dust and biological dust for exposure to pesticides, whereas the models with exposure to pesticides, herbicides and insecticides were additionally

adjusted for exposure to VGDF. Since subjects with high exposure to pesticides always were highly exposed to VGDF, it was not possible to formally test for interaction between the two exposures.

In additional analyses we stratified by smoking status (never/ever) and by gender. A subject was defined as ever smoker when being either a current or ex-smoker. Interactions between the exposures and smoking or gender were tested by including their interaction terms in the unstratified models (i.e. low exposure*ever smoker; high exposure*ever smoker, and low exposure*gender; high exposure*gender, respectively). P-values <0.05 were considered statistically significant. All analyses were performed in SPSS version 20.0 (IBM Corporation, USA).

Table 1. Characteristics of the included study populations from the LifeLines and Vlagtwedde-Vlaardingen cohorts.

	LifeLines	Vlagtwedde-Vlaardingen
N with non-missing data	11851	2364
Males, n (%)	4878 (41)	1265 (54)
Age (yrs), median (min-max)	47 (18-89)	52 (35-79)
Never smokers, n (%)	5091 (43)	760 (32)
Ever smokers, n (%)	6760 (57)	1604 (68)
Ex smokers, n (%)	4267 (36)	753 (32)
Current smokers, n (%)	2493 (21)	851 (36)
Packyears in ever smokers, median (25-75th percentiles)	10 (5-19)	19 (9-31)
Lung function, mean (sd)		
FEV_1%predicted[a]	103 (14)	93 (16)
FEV_1/FVC (%)[b]	76 (7)	74 (9)
Airway obstruction		
No (FEV_1/FVC≥70%), n (%)	10097 (85)	1725 (73)
FEV_1%predicted, mean (sd)	105 (13)	98 (13)
Mild (FEV_1/FVC<70%), n (%)	1754 (15)	639 (27)
FEV_1%predicted, mean (sd)	89 (15)	79 (16)
Moderate/severe[c], n (%)	458 (4)	314 (13)
FEV_1%predicted, mean (sd)	70 (9)	67 (11)

[a]FEV_1%predicted is FEV_1 as percentage predicted based on reference equations by Quanjer et al[16]. [b]Vlagtwedde-Vlaardingen: FEV_1/IVC.
[c]Moderate/severe obstruction: subjects with moderate/severe obstruction = pre-broncho-dilator FEV_1/FVC<70% and FEV_1<80%, subjects without obstruction = pre-broncho-dilator FEV_1/FVC≥70% and FEV_1≥80%. Subjects with mild obstruction (pre-broncho-dilator FEV_1/FVC<70% and FEV_1≥80% predicted) or pre-bronchodilator FEV_1/FVC≥70% and FEV_1<80%predicted were excluded from this analysis (LifeLines n = 1517 (13%) and Vlagtwedde-Vlaardingen n = 436 (18%)).

Verification cohort

Subjects that participated in the last survey (1989/1990) of the Vlagtwedde-Vlaardingen cohort were used to verify our initial findings (table 1). The Vlagtwedde-Vlaardingen cohort is a general population based cohort that has started in 1965 and has been followed for 25 years. During each survey information was collected by questionnaires and spirometry, using a slow inspiratory manoeuvre, was performed with a water-sealed spirometer (Lode instruments, Groningen, the

Netherlands). We used current job, or the last held job in case of current unemployment (e.g. retirement) that was reported at the last survey (1989/1990). Job coding, exposure assessment and statistical analyses were performed according to the same protocol as in the LifeLines cohort.

RESULTS

Characteristics of the LifeLines study population and prevalence of exposure

From the initial LifeLines sample of 13,301 subjects, a total of 1,450 subjects were excluded because of insufficient quality of spirometry (n = 725) or lacking information on covariates (n = 725). Characteristics of the in- and excluded subjects can be found in the supplementary file (supplementary table 2). Table 1 shows the characteristics of 11,851 LifeLines participants that were included in the final sample. High level of occupational exposure (category 2) to the broadly defined category VGDF was quite common (11%) (for an overview of the type of workers with high exposure to VGDF see supplementary table 3). Only a small number of people had high exposure to pesticides (1%) (for numbers and prevalence of each exposure see table 2). Males were more often exposed, and had more often high exposure (category 2) than females.

VGDF exposure

Occupational exposure to VGDF in general (figure 1a), and the subcategory gases and fumes, was associated with lower levels of FEV_1 (table 2) and FEV_1/FVC (table 3), and a higher prevalence of mild and moderate/severe airway obstruction (table 4), with the strongest associations for the groups with high exposure. The negative association of high exposure to VGDF and gases and fumes with level of FEV_1 was significantly stronger in ever smokers than never smokers (i.e. p-values for interaction <0.05), the associations with FEV_1/FVC were not significantly different between never and ever smokers. Exposure to mineral dust was associated with a lower level of FEV_1 and with a higher prevalence of mild and moderate/severe airway obstruction, whereas the association with level of FEV_1/FVC was less consistent. The association between low exposure to mineral dust and level of FEV_1 was significantly stronger in ever compared to never smokers. Exposure to biological dust was not significantly associated with level of FEV_1 (table 2) and FEV_1/FVC (table 3) or airway obstruction (table 4). There were no significant differences in the associations between occupational exposures and level of FEV_1 or FEV_1/FVC between males and females.

Pesticide exposure

Exposures to pesticides (figure 1b), and the subcategories herbicides and insecticides were associated with significantly lower levels of FEV_1 in an exposure-dependent way (table 2). Exposure to pesticides was also associated with a lower level of FEV_1/FVC (table 3) and a higher prevalence of mild and moderate/severe airway obstruction (table 4), yet these associations only reached significance for exposure to herbicides. The association between low exposure to pesticides

and level of FEV_1 was significantly stronger in the ever compared to the never smokers (p-value for interaction <0.05). There was no difference between ever and never smokers for high exposure to pesticides. Associations of exposure to pesticides with FEV_1/FVC were not significantly different between ever and never smokers or males and females.

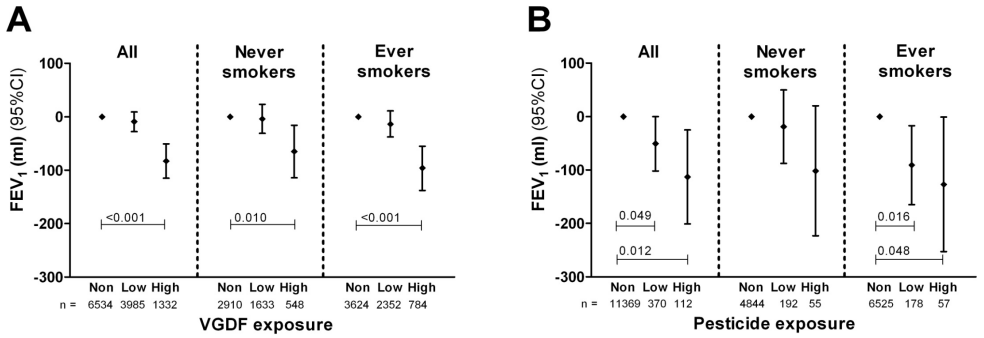

Figure 1. The association between occupational exposure (no/low/high) to VGDF (A) and to pesticides (B) and the level of FEV_1 in the whole group and stratified by smoking status (never/ever).

Solvent exposure

Low exposure to aromatic solvents was associated with a marginally lower level of FEV_1/FVC (supplementary table 4) and a higher prevalence of mild airway obstruction (supplementary table 5). There were no associations between low exposure to aromatic solvents and level of FEV_1 or moderate/severe airway obstruction and no associations between high exposure and level of FEV_1, FEV_1/FVC or prevalence of airway obstruction. There were no associations between exposure to chlorinated and other types of solvents and level of FEV_1, FEV_1/FVC (supplementary table 4) or prevalence of airway obstruction (supplementary table 5).

Verification of associations in the Vlagtwedde-Vlaardingen cohort

Full data on all covariates was available for 2,364 subjects participating in the last survey of the Vlagtwedde-Vlaardingen cohort. These subjects were slightly older, more often male, more often ever smoker, had a lower level of lung function and more often had airway obstruction than subjects from the LifeLines cohort (table 1). Exposure to high levels of VGDF (33%) and pesticides (12%) was more common than in the LifeLines cohort (for the prevalence of all exposures in the Vlagtwedde-Vlaardingen cohort, see supplementary table 6).

The associations of VGDF and the subcategory gases and fumes on level of FEV_1 (supplementary table 6), FEV_1/FVC, (supplementary table 7) and the prevalence of mild and moderate/severe airway obstruction (table 4) in the Vlagtwedde-Vlaardingen cohort were comparable with the LifeLines cohort. Contrary to findings in the LifeLines cohort the associations with level of lung function were not stronger in the ever smokers. Moreover, the associations of

exposure to mineral dust with level of FEV_1 and prevalence of airway obstruction could not be replicated. Associations between occupational exposure to pesticides and a lower level of FEV_1/FVC (supplementary table 7) and a higher prevalence of mild and moderate/severe airway obstruction (table 4) in the Vlagtwedde-Vlaardingen cohort were comparable with associations in the LifeLines cohort. The negative associations between exposure to pesticides and level of FEV_1 was replicated in the ever smokers only (supplementary table 6).

The marginal association between low exposure to aromatic solvents and a lower level of FEV_1/FVC in the LifeLines cohort was replicated in the Vlagtwedde-Vlaardingen cohort (supplementary table 8).

DISCUSSION

Main findings

Occupational exposure to VGDF and pesticides was associated with a lower level of FEV_1 and FEV_1/FVC and a higher prevalence of airway obstruction. There were no consistent associations with exposure to solvents.

Results in relation to other studies

In line with previous findings in the literature we showed that occupational exposure to VGDF was clearly associated with lower levels of FEV_1 and FEV_1/FVC as well as with a higher prevalence of airway obstruction in both our general populations investigated[8-11,17,18]. Associations in our study were exposure-dependent. In the European Community Respiratory Health Survey (ECRHS) study high exposure to VGDF was associated with a 61 ml lower FEV_1 in current smokers, whereas in our study we found a 96 ml lower FEV_1 associated with high exposure to VGDF in ever smokers. Contrary to our findings there was no association in never smokers from the ECRHS study[18]. These differences might relate to the lower average age of the ECRHS population which consisted mainly of young adults (range 20-44 years) compared to LifeLines (18-89 years). We found consistent associations with the subcategory gases and fumes in both cohorts. The association between exposure to mineral dust, lower level of FEV_1 and higher prevalence of airway obstruction was present in the LifeLines cohort but not in the Vlagtwedde-Vlaardingen cohort. This might be due to differences between both cohorts, for example regarding exposure intensity within the exposed. In general, findings in both cohorts confirm that occupational exposure to VGDF is associated with lower levels of lung function and a higher prevalence of airway obstruction.

To our knowledge this is the first study showing associations of exposure to pesticides, including herbicides and insecticides, with a lower level of lung function and a higher prevalence of airway obstruction in two general populations from a westernized country. Exposure to specific types of pesticides has been associated with chronic bronchitis in U.S. farmers[19] and their spouses[20] enrolled in the Agricultural Health Study.

Table 2. Associations between occupational exposures and level of FEV_1 (ml) in the LifeLines cohort, adjusted for sex, age, height, weight, current, ex smoking, (log) packyears smoked and co-exposure to VGDF/pesticides. Stratification according to smoking status (never/ever) and gender is shown.

LifeLines												
	FEV_1 (ml)											
	All		Never smokers			Ever smokers		Males			Females	
Exposure[a]	b (95% CI)	N (%)	b (95% CI)	N		b (95% CI)	N	b (95% CI)	N		b (95% CI)	N
VGDF												
Non-exposed	Ref	6534 (55)	Ref	2910		Ref	3624	Ref	2580		Ref	3954
Low	-9 (-28 ; 9)	3985 (34)	-4 (-31 ; 23)	1633		-14 (-38 ; 11)	2352	-23 (-61 ; 15)	1167		-5 (-23 ; 13)	2818
High	**-83 (-115 ; -51)*** **	1332 (11)	**-65 (-114 ; -16)** **	548	#	**-96 (-138 ; -55)*** **	784	**-83 (-125 ; -40)*** **	1131		-65 (-138 ; 8)	201
Gases/Fumes												
Non-exposed	Ref	7007 (59)	Ref	3154		Ref	3853	Ref	2702		Ref	4305
Low	-13 (-31 ; 5)	4159 (35)	-4 (-31 ; 23)	1692		-21 (-45 ; 4)	2467	-34 (-71 ; 2)	1566	#	-3 (-22 ; 16)	2593
High	**-73 (-110 ; -35)*** **	685 (6)	-35 (-95 ; 24)	245	#	**-96 (-144 ; -47)*** **	440	**-76 (-125 ; -27)** **	610		-36 (-122 ; 51)	75
Mineral dust												
Non-exposed	Ref	9389 (79)	Ref	4121		Ref	5268	Ref	3366		Ref	6023
Low	**-28 (-52 ; -5)***	1924 (16)	-9 (-45 ; 26)	754	#	**-45 (-75 ; -14)** **	1170	**-56 (-96 ; -17)** **	1037	#	-4 (-31 ; 24)	887
High	**-65 (-111 ; -18)** **	538 (5)	**-79 (-151 ; -7)***	216		-57 (-118 ; 4)	322	**-65 (-125 ; -5)***	475		-69 (-186 ; 48)	63
Biological dust												
Non-exposed	Ref	8127 (69)	Ref	3514		Ref	4613	Ref	3729		Ref	4398
Low	-7 (-26 ; 13)	3256 (28)	-10 (-39 ; 18)	1355		-4 (-30 ; 22)	1901	-3 (-47 ; 41)	783		-11 (-30 ; 8)	2473
High	-35 (-85 ; 16)	468 (4)	-7 (-80 ; 65)	222	#	-58 (-128 ; 12)	246	-46 (-113 ; 22)	366		29 (-75 ; 133)	102
All pesticides												
Non-exposed	Ref	11369 (96)	Ref	4844		Ref	6525	Ref	4523		Ref	6846
Low	**-51 (-102 ; 0)***	370 (3)	-19 (-88 ; 50)	192	#	**-91 (-165 ; -17)***	178	-54 (-127 ; 18)	264		-26 (-103 ; 50)	106
High	**-113 (-201 ; -25)***	112 (1)	-102 (-223 ; 20)	55		**-127 (-253 ; -1)***	57	-94 (-213 ; 24)	91		-110 (-274 ; 54)	21
Herbicides												
Non-exposed	Ref	11680 (99)	Ref	5008		Ref	6672	Ref	4746		Ref	6934
Low	-59 (-140 ; 22)	132 (1)	-34 (-146 ; 77)	64		-82 (-197 ; 33)	68	-38 (-148 ; 72)	104		-81 (-223 ; 61)	28
High	**-204 (-350 ; -58)** **	39 (0.3)	-175 (-376 ; 26)	19		**-241 (-450 ; -32)***	20	-172 (-379 ; 35)	28		-191 (-416 ; 34)	11
Insecticides												
Non-exposed	Ref	11425 (96)	Ref	4870		Ref	6555	Ref	4576		Ref	6849
Low	-50 (-105 ; 5)	315 (3)	-1 (-75 ; 73)	166		**-109 (-190 ; -28)** **	149	-56 (-137 ; 25)	212		-21 (-99 ; 56)	103
High	**-109 (-197 ; -21)***	111 (1)	-98 (-219 ; 24)	55		-124 (-251 ; 4)	56	-91 (-210 ; 28)	90		-109 (-273 ; 55)	21

[a] Non-exposed subjects were assigned as reference category (Ref); VGDF = Vapors, Gases, Dust, Fumes; *p<0.05; **p<0.01; ***p<0.001. # Significantly different for never and ever smokers or males and females (i.e. p-value for interaction < 0.05).

Table 3. Associations between occupational exposures and level of FEV_1/FVC (%) in the LifeLines cohort, adjusted for sex, age, height, weight, current, ex smoking, (log) packyears smoked and co-exposure to VGDF/pesticides. Stratification according to smoking status (never/ever) and gender is shown.

LifeLines			FEV_1/FVC (%)									
	All		Never smokers		Ever smokers		Males			Females		
Exposure[a]	b (95% CI)	N (%)	b (95% CI)	N	b (95% CI)	N	b (95% CI)	N		b (95% CI)	N	
VGDF												
Non-exposed	Ref	6534 (55)	Ref	2910	Ref	3624	Ref	2580		Ref	3954	
Low	**-0.3 (-0.6 ; -0.1)****	3985 (34)	-0.1 (-0.5 ; 0.3)	1633	**-0.5 (-0.9 ; -0.2)****	2352	-0.5 (-0.9 ; 0)	1167		-0.3 (-0.6 ; 0)	2818	
High	**-0.7 (-1.2 ; -0.3)****	1332 (11)	-0.6 (-1.3 ; 0.1)	548	**-0.8 (-1.4 ; -0.2)****	784	**-0.8 (-1.3 ; -0.2)****	1131		-0.7 (-1.9 ; 0.5)	201	
Gases/Fumes												
Non-exposed	Ref	7007 (59)	Ref	3154	Ref	3853	Ref	2702		Ref	4305	
Low	**-0.4 (-0.6 ; -0.1)****	4159 (35)	-0.1 (-0.5 ; 0.2)	1692	**-0.5 (-0.9 ; -0.2)****	2467	-0.4 (-0.9 ; 0)	1566		**-0.3 (-0.6 ; 0)***	2593	
High	**-0.7 (-1.3 ; -0.2)****	685 (6)	-0.6 (-1.4 ; 0.3)	245	**-0.8 (-1.6 ; -0.1)***	440	**-0.8 (-1.4 ; -0.1)***	610		-0.6 (-2.1 ; 0.8)	75	
Mineral dust												
Non-exposed	Ref	9389 (79)	Ref	4121	Ref	5268	Ref	3366		Ref	6023	
Low	**-0.4 (-0.8 ; -0.1)***	1924 (16)	-0.4 (-0.9 ; 0.1)	754	**-0.5 (-1.0 ; -0.1)***	1170	**-0.6 (-1.1 ; -0.1)***	1037		-0.2 (-0.7 ; 0.2)	887	
High	-0.1 (-0.8 ; 0.5)	538 (5)	-0.3 (-1.3 ; 0.7)	216	-0.1 (-0.9 ; 0.8)	322	-0.4 (-1.1 ; 0.4)	475		1.2 (-0.7 ; 3.2)	63	
Biological dust												
Non-exposed	Ref	8127 (69)	Ref	3514	Ref	4613	Ref	3729		Ref	4398	
Low	-0.1 (-0.4 ; 0.2)	3256 (28)	0.1 (-0.3 ; 0.5)	1355	-0.2 (-0.6 ; 0.1)	1901	0.4 (-0.2 ; 1.0)	783	#	-0.3 (-0.6 ; 0)	2473	
High	-0.2 (-0.9 ; 0.5)	468 (4)	-0.3 (-1.3 ; 0.7)	222	-0.1 (-1.1 ; 0.9)	246	-0.2 (-1.0 ; 0.7)	366		0.6 (-1.1 ; 2.4)	102	
All pesticides												
Non-exposed	Ref	11369 (96)	Ref	4844	Ref	6525	Ref	4523		Ref	6846	
Low	-0.4 (-1.1 ; 0.3)	370 (3)	-0.5 (-1.5 ; 0.5)	192	-0.3 (-1.4 ; 0.7)	178	-0.3 (-1.3 ; 0.6)	264		-0.5 (-1.7 ; 0.8)	106	
High	-1.1 (-2.3 ; 0.2)	112 (1)	-0.7 (-2.4 ; 1.0)	55	-1.4 (-3.3 ; 0.4)	57	-0.7 (-2.2 ; 0.8)	91		-2.5 (-5.2 ; 0.2)	21	
Herbicides												
Non-exposed	Ref	11680 (99)	Ref	5008	Ref	6672	Ref	4746		Ref	6934	
Low	-0.3 (-1.5 ; 0.8)	132 (1)	0.3 (-1.3 ; 1.8)	64	-0.9 (-2.6 ; 0.8)	68	0 (-1.4 ; 1.4)	104		-1.5 (-3.8 ; 0.9)	28	
High	**-2.8 (-4.8 ; -0.7)****	39 (0.3)	-2.7 (-5.5 ; 0.1)	19	-2.7 (-5.7 ; 0.3)	20	**-2.9 (-5.5 ; -0.3)***	28		-2.4 (-6.1 ; 1.4)	11	
Insecticides												
Non-exposed	Ref	11425 (96)	Ref	4870	Ref	6555	Ref	4576		Ref	6849	
Low	-0.4 (-1.2 ; 0.3)	315 (3)	-0.5 (-1.6 ; 0.5)	166	-0.4 (-1.6 ; 0.8)	149	-0.4 (-1.4 ; 0.6)	212		-0.5 (-1.8 ; 0.8)	103	
High	-1.1 (-2.3 ; 0.2)	111 (1)	-0.7 (-2.4 ; 1.0)	55	-1.5 (-3.3 ; 0.4)	56	-0.7 (-2.2 ; 0.8)	90		-2.5 (-5.2 ; 0.2)	21	

[a] Non-exposed subjects were assigned as reference category (Ref); VGDF = Vapors, Gases, Dust, Fumes; *p<0.05; **p<0.01; ***p<0.001. #Significantly different for males and females (i.e. p-value for interaction < 0.05).

Table 4. Associations between occupational exposures and airway obstruction adjusted for sex, age, height, weight, ever smoking (no/yes), (log) packyears smoked and co-exposure to VGDF/pesticides in the LifeLines and Vlagtwedde-Vlaardingen cohorts.

	LifeLines						Vlagtwedde-Vlaardingen					
Exposure [a]	**Mild obstruction (FEV_1/FVC<70%)**			**Moderate/severe obstruction** [b]			**Mild obstruction (FEV_1/IVC<70%)**			**Moderate/severe obstruction** [b]		
	OR (95% CI)	**N** [c]	**P**	**OR (95% CI)**	**N** [c]	**P**	**OR (95% CI)**	**N** [c]	**P**	**OR (95% CI)**	**N** [c]	**P**
VGDF												
Non-exposed	Ref	917		Ref	233		Ref	196		Ref	76	
Low	1.08 (0.96 ; 1.22)	578	0.199	1.05 (0.84 ; 1.31)	145	0.662	1.22 (0.95 ; 1.59)	167	0.124	**1.82 (1.27 ; 2.60)**	89	**0.001**
High	**1.41 (1.16 ; 1.70)**	259	**<0.001**	**1.39 (1.00 ; 1.92)**	80	**0.047**	1.29 (0.95 ; 1.74)	276	0.101	**1.85 (1.23 ; 2.79)**	149	**0.003**
Gases/Fumes												
Non-exposed	Ref	985		Ref	251		Ref	227		Ref	97	
Low	1.10 (0.98 ; 1.24)	631	0.116	1.03 (0.83 ; 1.28)	164	0.787	1.11 (0.87 ; 1.42)	334	0.401	**1.45 (1.04 ; 2.01)**	182	**0.028**
High	**1.44 (1.16 ; 1.80)**	138	**0.001**	**1.47 (1.02 ; 2.11)**	43	**0.039**	1.32 (0.92 ; 1.89)	78	0.130	1.55 (0.95 ; 2.53)	35	0.081
Mineral dust												
Non-exposed	Ref	1317		Ref	328		Ref	348		Ref	165	
Low	**1.19 (1.03 ; 1.37)**	331	**0.018**	1.22 (0.95 ; 1.56)	91	0.128	1.16 (0.87 ; 1.54)	104	0.318	1.29 (0.88 ; 1.90)	50	0.199
High	**1.46 (1.11 ; 1.92)**	106	**0.007**	**1.76 (1.15 ; 2.72)**	39	**0.010**	1.10 (0.76 ; 1.58)	187	0.619	0.99 (0.61 ; 1.61)	99	0.972
Biological dust												
Non-exposed	Ref	1196		Ref	314		Ref	324		Ref	141	
Low	1.04 (0.92 ; 1.18)	481	0.502	0.99 (0.79 ; 1.24)	121	0.916	**1.34 (1.04 ; 1.73)**	243	**0.022**	**1.74 (1.24 ; 2.43)**	137	**0.001**
High	0.91 (0.66 ; 1.25)	77	0.550	0.77 (0.45 ; 1.33)	23	0.345	1.25 (0.84 ; 1.84)	72	0.271	1.54 (0.92 ; 2.55)	36	0.098
All pesticides												
Non-exposed	Ref	1667		Ref	428		Ref	478		Ref	227	
Low	0.83 (0.60 ; 1.14)	59	0.241	1.15 (0.69 ; 1.93)	20	0.595	1.33 (0.87 ; 2.01)	52	0.185	1.08 (0.61 ; 1.90)	23	0.791
High	1.28 (0.79 ; 2.09)	28	0.322	1.95 (0.92 ; 4.13)	10	0.083	**1.48 (1.04 ; 2.10)**	109	**0.029**	**1.78 (1.14 ; 2.79)**	64	**0.011**
Herbicides												
Non-exposed	Ref	1711		Ref	444		Ref	511		Ref	241	
Low	1.04 (0.65 ; 1.67)	27	0.872	1.11 (0.51 ; 2.45)	8	0.791	1.29 (0.85 ; 1.98)	50	0.235	1.60 (0.93 ; 2.76)	28	0.090
High	**2.11 (1.03 ; 4.30)**	16	**0.040**	**3.56 (1.28 ; 9.88)**	6	**0.015**	1.36 (0.93 ; 2.00)	78	0.112	**1.66 (1.02 ; 2.69)**	45	**0.040**
Insecticides												
Non-exposed	Ref	1675		Ref	430		Ref	494		Ref	237	
Low	0.81 (0.58 ; 1.15)	51	0.241	1.21 (0.70 ; 2.09)	18	0.497	1.04 (0.64; 1.70)	36	0.867	0.66 (0.33 ; 1.33)	13	0.249
High	1.32 (0.81 ; 2.16)	28	0.268	2.05 (0.96 ; 4.35)	10	0.062	1.39 (0.98 ; 1.98)	109	0.067	**1.62 (1.04 ; 2.52)**	64	**0.033**

[a] Non-exposed subjects were assigned as reference category (Ref); VGDF = Vapors, Gases, Dust, Fumes; [b] Moderate/severe airway obstruction = pre-bronchodilator FEV_1/FVC<70% and FEV_1<80%, without obstruction = pre-bronchodilator FEV_1/FVC≥70% and FEV_1≥80%. Subjects with mild obstruction (pre-bronchodilator FEV_1/FVC<70% and FEV_1≥80%predicted) or pre-bronchodilator FEV_1/FVC≥70% and FEV_1<80%predicted were excluded from this analysis (LifeLines n = 1517 (13%) and Vlagtwedde-Vlaardingen n = 436 (18%)). For Vlagtwedde-Vlaardingen: FEV_1/IVC instead of FEV_1/FVC. [c] Number of subjects with obstruction.

To date, few studies showed that pesticide exposed farming or manufacturing workers had lower lung function levels than non-exposed workers,[21] whereas others found no associations[22-24]. These studies were all performed in specific subgroups, like plantation or pesticide factory workers in developing countries. In contrast, we investigated a general population and occupational exposure to pesticides appeared to be associated with a significant loss of FEV_1 and FEV_1/FVC, especially in smokers. For example, if mean life-time exposure to pesticides would be about 20 years, a total loss of 200 ml FEV_1 for exposure to herbicides implies a loss of 10 ml per year. This corresponds to smoking of one package of cigarettes per day for one year, which was associated with an 11 ml loss of FEV_1 per packyear smoked in our study.

Moreover, we showed that workers exposed to pesticides had an almost 2-times higher prevalence of moderate/severe airway obstruction than non-exposed workers. These associations were similar in both populations investigated. Subjects that were highly exposed to pesticides included gardeners, field-grown crop and vegetable growers, and mixed crop and animal farmers. Associations in the LifeLines sample remained present when each of these three main occupational groups with high exposure to pesticides was excluded one-by-one, yet associations were clearly strongest in the field crop and vegetable growers. Between 1985 and today about 90% of agriculture in the northern Dutch provinces consisted of arable crops, on average ~30% potatoes, ~30% cereals, ~15% beets and ~15% maize (personal communication, M. Brouwer, University of Utrecht). In terms of pesticide use this means that mainly herbicides have been applied (cereals, beets, maize) and substantial fungicide use on potatoes (mainly dithiocarbamate fungicides) (for more specific information see supplementary file: additional information 1).

In a global perspective, the agricultural sector employs a large share of the population worldwide, especially in developing countries where workers often use pesticides with insufficient protective equipment and training[13]. Therefore, interventions to reduce exposure levels in this occupational sector could contribute to lowering the global burden of COPD.

Strengths and limitations

A limitation of this study was the incomplete occupational history within the LifeLines cohort study. However, since we believe that people are more likely to move from so called blue-collar to white-collar occupations, for example due to symptoms or objective disease, than the other way around, we hypothesize that with using current or last held job we rather have under- than over-estimated the association between occupational exposures and lung function level. Secondly, we have used pre-bronchodilator spirometry to define airway obstruction and assessed associations in a sample including subjects below 40 years of age. However, the associations did not change when we restricted our analysis to subjects aged 40 years and older. When the analyses on level of lung function were stratified by age (<40 and ≥40 years) we found that the association between exposure to pesticides and level of lung function remained only in the group with older age, which may be due to a longer time of exposure or the use of better protective equipment

nowadays. The associations between lung function and exposure to vapors, gases, dusts and fumes and level of lung function remained in both age groups. Finally, we have assessed associations with actual ml-values FEV_1, with extensive adjustment for individual predictors of lung function level rather than percentage predicted values using an external reference population. However, associations did not change when we used FEV_1 as percentage predicted instead of the actual level of FEV_1 in ml.

Because the considerable sample size and inclusion of subjects from rural parts of the Netherlands we were able to assess associations of exposures with low prevalence, like pesticides, and additionally the interaction between occupational exposures and smoking. Secondly, findings were verified in a second independent cohort. Another strength of the study was the use of the ALOHA+ job exposure matrix (JEM), that was built specifically for use in general population studies. In general, JEM-based exposure estimates are less likely to be affected by recall bias and differential misclassification of exposure compared to self-reported exposures[25,26]. Finally we have adjusted our models to overcome possible confounding resulting from co-exposure to pesticides/VGDF. Unadjusted models (not shown) yielded considerably stronger associations, suggesting that workers in occupations exposed to both VGDF and pesticides might be at higher risk than suggested by the adjusted associations that were shown in the current paper.

Conclusions

To conclude, we showed large and clinically relevant losses of lung function level signified by airway obstruction in individuals occupationally exposed to vapors, gases, dusts and fumes, and pesticides within two general populations. Interventions to reduce exposure levels at the workplace could therefore significantly contribute to lowering the global burden associated with COPD.

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2

SUPPLEMENTARY MATERIAL

Supplementary table 1. Spearman's rank correlations between the exposures as no, low, and high exposure (0/1/2) in the LifeLines cohort.

	VGDFs				Pesticides			Solvents		
Spearman's Rank Correlation	VGDF	Gases and Fumes	Mineral Dust	Biological Dust	All Pesticides	Herbicides	Insecticides	Aromatic	Chlorinated	Other
VGDF		0.92	0.64	0.72	0.33	0.20	0.32	0.41	0.34	0.56
Gases and Fumes			0.63	0.61	0.22	0.14	0.21	0.40	0.39	0.52
Mineral Dust				0.37	0.38	0.26	0.37	0.35	0.27	0.03
Biological Dust					0.33	0.20	0.36	0.15	0.02	0.45
All Pesticides						0.60	0.94	0.12	-0.05	-0.08
Herbicides							0.60	0.24	-0.03	-0.07
Insecticides								0.13	-0.05	-0.07
Aromatic Solvents									0.72	0.48
Chlorinated Solvents										0.52
Other Solvents										

Supplementary table 2. Characteristics of the included and excluded subjects from the LifeLines cohort.

	Included	Excluded	Test	p-value
N with non-missing data	11851	1450		
Males, n (%)	4878 (41)	679 (47)	Chi-square	<0.001
Age (yrs), median (min-max)	47 (18-89)	51 (22-88)	MWU	<0.001
Ever smokers, n (%)	6760 (57)	1068 (80)	Chi-square	<0.001
Packyears in ever smokers, median (25-75th	10 (5-19)	12 (5-22)	MWU	0.002
Lung function, mean (sd)				
FEV_1%predicted[a]	103 (14)	100 (16)	t-test	<0.001
FEV_1/FVC (%)	76 (7)	74 (8)	t-test	<0.001

[a] FEV_1%predicted is FEV_1 as percentage predicted based on reference equations by Quanjer et al (1993).

Supplementary table 3. Overview of the number of workers (%) with high exposure to VGDF in the LifeLines cohort.

N	%	ISCO code	Occupation
159	11.9	6121	Dairy and livestock producers
102	7.7	8324	Heavy truck and lorry drivers
100	7.5	7124	Carpenters and joiners
77	5.8	7231	Motor vehicle mechanics and fitters
67	5.0	7212	Welders and flame cutters
64	4.8	7233	Agricultural- or industrial-machinery mechanics and fitters
63	4.7	9333	Freight handlers
57	4.3	7136	Plumbers and pipe fitters
50	3.8	6113	*Gardeners, horticultural and nursery growers
50	3.8	9313	Building construction labourers
41	3.1	7141	Painters and related workers
33	2.5	6111	*Field crop and vegetable growers
31	2.3	7411	Butchers, fishmongers and related food preparers
23	1.7	6130	*Market-oriented crop and animal producers
22	1.7	7412	Bakers, pastry-cooks and confectionery makers
22	1.7	8332	Earth-moving and related plant operators
22	1.7	9312	Construction and maintenance labourers: roads, dams and similar constructions
21	1.6	3141	Ships' engineers
20	1.5	8251	Printing-machine operators
18	1.4	3227	Veterinary assistants
18	1.4	6100	Market-oriented skilled agricultural and fishery workers: not specifically classifiable
15	1.1	9133	Hand-launderers and pressers
14	1.1	2452	Sculptors, painters and related artists
14	1.1	7123	Concrete placers, concrete finishers and related workers
219	17.2	Other	Other

* workers with additional high exposure to pesticides.

Supplementary table 4. Associations between exposure to various types of solvents and level of FEV_1 and FEV_1/FVC adjusted for sex, age, height, weight, current, ex smoking and (log) packyears in the LifeLines cohort. Stratification according to smoking status (never/ever) and gender is shown.

LifeLines										
	FEV_1 (ml)									
Solvent Exposure[a]	All b (95% CI)	N	Never smokers b (95% CI)	N		Ever smokers b (95% CI)	N	Males b (95% CI)	N	Females b (95% CI)	N
Aromatic solvents										
Non-exposed	Ref	10812	Ref	4668		Ref	6144	Ref	4012	Ref	6800
Low	-4 (-36 ; 27)	957	22 (-24 ; 69)	400		-23 (-66 ; 20)	557	-5 (-47 ; 37)	793	23 (-36 ; 81)	164
High	-36 (-136 ; 65)	82	-81 (-262 ; 101)	23		-26 (-148 ; 97)	59	-23 (-151 ; 105)	73	-12 (-259 ; 235)	9
Chlorinated solvents										
Non-exposed	Ref	10980	Ref	4757		Ref	6223	Ref	4262	Ref	6718
Low	16 (-20 ; 51)	695	**70 (15 ; 125)***	268	#	-18 (-65 ; 28)	427	28 (-26 ; 81)	450	5 (-43 ; 53)	245
High	-22 (-92 ; 47)	176	27 (-81 ; 136)	66		-59 (-149 ; 31)	110	-12 (-98 ; 74)	166	-81 (-316 ; 153)	10
Other solvents										
Non-exposed	Ref	9109	Ref	3951		Ref	5158	Ref	3857	Ref	5252
Low	12 (-8 ; 32)	2531	24 (-6 ; 54)	1064		5 (-23 ; 32)	1467	26 (-13 ; 66)	947	7 (-14 ; 29)	1584
High	-19 (-82 ; 44)	211	31 (-69 ; 132)	76		-51 (-132 ; 30)	135	-22 (-150 ; 105)	74	-5 (-69 ; 59)	137
	FEV_1/FVC (%)									
Solvent Exposure[a]	All b (95% CI)	N	Never smokers b (95% CI)	N		Ever smokers b (95% CI)	N	Males b (95% CI)	N	Females b (95% CI)	N
Aromatic solvents										
Non-exposed	Ref	10812	Ref	4668		Ref	6144	Ref	4012	Ref	6800
Low	**-0.5 (-1.0 ; -0.1)***	957	-0.1 (-0.7 ; 0.6)	400		**-0.8 (-1.5 ; -0.2)****	557	-0.5 (-1.0 ; 0)	793	-0.6 (-1.6 ; 0.4)	164
High	0.4 (-1.1 ; 1.8)	82	0.7 (-1.8 ; 3.2)	23		0.1 (-1.6 ; 1.9)	59	0.6 (-1.1 ; 2.2)	73	-1.3 (-5.4 ; 2.8)	9
Chlorinated solvents										
Non-exposed	Ref	10980	Ref	4757		Ref	6223	Ref	4262	Ref	6718
Low	-0.3 (-0.8 ; 0.2)	695	0.4 (-0.3 ; 1.2)	268		**-0.7 (-1.4 ; 0)***	427	-0.1 (-0.8 ; 0.6)	450	-0.5 (-1.3 ; 0.3)	245
High	0 (-1.0 ; 0.9)	176	-0.8 (-2.3 ; 0.7)	66		0.3 (-1.0 ; 1.7)	110	0.1 (-0.9 ; 1.2)	166	-2.6 (-6.5 ; 1.3)	10
Other solvents										
Non-exposed	Ref	9109	Ref	3951		Ref	5158	Ref	3857	Ref	5252
Low	-0.1 (-0.4 ; 0.1)	2531	0.1 (-0.3 ; 0.6)	1064		-0.3 (-0.7 ; 0.1)	1467	-0.2 (-0.7 ; 0.3)	947	-0.1 (-0.4 ; 0.3)	1584
High	-0.3 (-1.2 ; 0.6)	211	-0.3 (-1.7 ; 1.1)	76		-0.4 (-1.5 ; 0.8)	135	0.2 (-1.4 ; 1.9)	74	-0.6 (-1.7 ; 0.4)	137

[a] Occupational exposures (no/low/high) were estimated based on job title and function using the ALOHA+ Job Exposure Matrix. Non-exposed subjects were assigned as reference category (Ref). # Significantly different for never and ever smokers (i.e. p-value for interaction < 0.05).

Supplementary table 5. Associations between occupational exposure to solvents and airway obstruction in the LifeLines and Vlagtwedde-Vlaardingen cohorts.

	LifeLines						Vlagtwedde-Vlaardingen					
Solvent	**Mild obstruction (FEV_1/FVC<70%)**			**Moderate/severe obstruction[b]**			**Mild obstruction (FEV_1/IVC<70%)**			**Moderate/severe obstruction[b]**		
	OR (95% CI)	**N[c]**	**P**	**OR (95% CI)**	**N[c]**	**P**	**OR (95% CI)**	**N[c]**	**P**	**OR (95% CI)**	**N[c]**	**P**
Aromatic solvents												
Non-exposed	Ref	1569		Ref	414		Ref	431		Ref	208	
Low	**1.26 (1.04 ; 1.52)**	173	**0.020**	0.97 (0.67 ; 1.39)	39	0.857	1.13 (0.89 ; 1.44)	199	0.320	1.25 (0.91 ; 1.73)	101	0.165
High	0.92 (0.48 ; 1.75)	12	0.798	1.28 (0.50 ; 3.30)	5	0.604	0.70 (0.30 ; 1.67)	9	0.425	0.75 (0.25 ; 2.23)	5	0.606
Chlorinated solvents												
Non-exposed	Ref	1611		Ref	422		Ref	559		Ref	279	
Low	1.09 (0.87 ; 1.36)	112	0.452	0.93 (0.61 ; 1.41)	27	0.731	0.67 (0.47 ; 0.97)	57	0.032	0.62 (0.38 ; 1.01)	26	0.054
High	1.38 (0.91 ; 2.10)	31	0.130	1.27 (0.62 ; 2.62)	9	0.513	0.53 (0.31 ; 0.89)	23	0.018	**0.40 (0.18 ; 0.87)**	9	**0.020**
Other solvents												
Non-exposed	Ref	1369		Ref	365		Ref	491		Ref	247	
Low	1.04 (0.91 ; 1.18)	357	0.608	0.90 (0.70 ; 1.15)	82	0.392	0.93 (0.73 ; 1.19)	138	0.564	0.85 (0.61 ; 1.19)	62	0.349
High	0.99 (0.65 ; 1.50)	28	0.959	1.43 (0.76 ; 2.70)	11	0.272	0.53 (0.25 ; 1.15)	10	0.107	0.51 (0.18 ; 1.42)	5	0.197

Associations between occupational exposures (no/low/high), mild (pre-bronchodilator FEV_1/FVC<70%) and moderate/severe airway obstruction using logistic regression adjusted for sex, age, height, weight, ever smoking (no/yes) and (log) packyears smoked in the LifeLines and Vlagtwedde-Vlaardingen cohorts.

[a] Occupational exposures (no/low/high) were estimated based on job title and function using the ALOHA+ Job Exposure Matrix. Non-exposed subjects were assigned as reference category (Ref). [b] Moderate/severe airway obstruction = pre-bronchodilator FEV_1/FVC<70% and FEV_1<80%, without obstruction = pre-bronchodilator FEV_1/FVC≥70% and FEV_1≥80%. Subjects with mild obstruction (pre-bronchodilator FEV_1/FVC<70% and FEV_1≥80%predicted) or pre-bronchodilator FEV_1/FVC≥70% and FEV_1<80%predicted were excluded from this analysis (LifeLines n = 1517 (13%) and Vlagtwedde-Vlaardingen n = 436 (18%)). For Vlagtwedde-Vlaardingen: FEV_1/IVC instead of FEV_1/FVC. [c] Number of subjects with obstruction.

Supplementary table 6. Verification of associations between the exposures and level of FEV_1 (ml) adjusted for sex, age, height, weight, current, ex smoking, (log) packyears smoked and and co-exposure to VGDF/pesticides in the Vlagtwedde-Vlaardingen cohort. Stratification according to smoking status (never/ever) and gender is shown.

Vlagtwedde-Vlaardingen	FEV_1 (ml)											
	All		Never smokers			Ever smokers		Males			Females	
Exposure[a]	b (95% CI)	N (%)	b (95% CI)	N		b (95% CI)	N	b (95% CI)	N		b (95% CI)	N
VGDF												
Non-exposed	Ref	895 (38)	Ref	355		Ref	540	Ref	373		Ref	522
Low	**-77 (-123 ; -31)*** **	685 (29)	**-87 (-154 ; -20)***	225		**-73 (-133 ; -13)***	460	**-128 (-211 ; -46)****	251	#	-36 (-83 ; 12)	434
High	**-93 (-149 ; -37)****	784 (33)	**-136 (-245 ; -27)***	180		**-91 (-158 ; -24)****	604	-126 (-163 ; 56)	641	#	-53 (-163 ; 56)	143
Gases/Fumes												
Non-exposed	Ref	1021 (43)	Ref	412		Ref	609	Ref	401		Ref	620
Low	**-54 (-98 ; -11)***	1111 (47)	**-71 (-140 ; -4)***	316		-50 (-106 ; 6)	795	**-94 (-167 ; -21)***	647	#	-18 (-66 ; 30)	464
High	-66 (-134 ; 3)	232 (10)	-103 (-256 ; 51)	32		-61 (-141 ; 19)	200	**-88 (-174 ; -3)***	217		-33 (-223 ; 156)	15
Mineral dust												
Non-exposed	Ref	1491 (63)	Ref	520		Ref	971	Ref	630		Ref	861
Low	-28 (-80 ; 25)	375 (16)	-76 (-159 ; 7)	117		-7 (-73 ; 61)	258	-47 (-125 ; 31)	234		-26 (-93 ; 41)	141
High	-15 (-86 ; 57)	498 (21)	4 (-147 ; 155)	123	#	-21 (-105 ; 62)	375	-20 (-108 ; 67)	401		64 (-107 ; 235)	97
Biological dust												
Non-exposed	Ref	1318 (56)	Ref	423		Ref	895	Ref	726		Ref	592
Low	**-68 (-113 ; -23)****	840 (36)	**-122 (-189 ; -54)*****	292		-42 (-101 ; 17)	548	**-113 (-194 ; -32)****	373		-37 (-84 ; 11)	467
High	-57 (-127 ; 14)	206 (9)	**-152 (-282 ; -21)***	45		-26 (-111 ; 59)	161	-88 (-181 ; 5)	166		-3 (-126 ; 121)	40
All pesticides												
Non-exposed	Ref	1936 (82)	Ref	631		Ref	1305	Ref	945		Ref	991
Low	-39 (-120 ; 41)	153 (6)	54 (-74 ; 181)	59		-78 (-182 ; 26)	94	-44 (-156 ; 67)	96		-42 (-158 ; 74)	57
High	-47 (-113 ; 19)	275 (12)	89 (-33 ; 211)	70	#	**-84 (-165 ; -4)***	205	-53 (-138 ; 31)	224		25 (-96 ; 145)	51
Herbicides												
Non-exposed	Ref	2018 (85)	Ref	654		Ref	1364	Ref	1011		Ref	1007
Low	-78 (-159 ; 5)	145 (6)	34 (-102 ; 170)	46		**-122 (-225 ; -19)***	99	**-124 (-235 ; -12)***	98	#	10 (-112 ; 132)	47
High	-14 (-87 ; 59)	201 (9)	81 (-43 ; 206)	60		-44 (-134 ; 47)	141	-8 (-101 ; 56)	156		-8 (-132 ; 116)	45
Insecticides												
Non-exposed	Ref	1969 (83)	Ref	636		Ref	1333	Ref	977		Ref	992
Low	4 (-87 ; 95)	120 (5)	111 (-21 ; 244)	54		-50 (-173 ; 74)	66	25 (-110 ; 160)	64		-35 (-153 ; 82)	56
High	-38 (-104 ; 29)	275 (12)	107 (-15 ; 228)	70	#	-78 (-158 ; 2)	205	-42 (-126 ; 42)	224		27 (-94 ; 148)	51

[a] Occupational exposures (no/low/high) were estimated based on job title and function using the ALOHA+ Job Exposure Matrix. Non-exposed subjects were assigned as reference category (Ref); VGDF = Vapors, Gases, Dust, Fumes; *p<0.05; **p<0.01; ***p<0.001. # Significantly different for never and ever smokers or males and females (i.e. p-value for interaction < 0.05).

Supplementary table 7. Verification of associations between the exposures and level of FEV_1/IVC (%) adjusted for sex, age, height, weight, current, ex smoking, (log) packyears smoked and co-exposure to VGDF/pesticides in the Vlagtwedde-Vlaardingen cohort. Stratification according to smoking status (never/ever) and gender is shown.

Vlagtwedde-Vlaardingen	FEV$_1$/IVC (%)									
	All		Never smokers		Ever smokers		Males		Females	
Exposure[a]	b (95% CI)	N (%)	b (95% CI)	N	b (95% CI)	N	b (95% CI)	N	b (95% CI)	N
VGDF										
Non-exposed	Ref	895 (38)	Ref	355	Ref	540	Ref	373	Ref	522
Low	**-0.9 (-1.6 ; -0.1)***	685 (29)	-0.7 (-1.9 ; 0.5)	225	**-1.0 (-2.0 ; 0)***	460	-1.1 (-2.4 ; 0.2)	251	-0.7 (-1.7 ; 0.2)	434
High	**-1.5 (-2.5 ; -0.6)***	784 (33)	-1.4 (-3.4 ; 0.5)	180	**-1.7 (-2.8 ; -0.6)****	604	**-1.4 (-2.6 ; -0.2)***	641	**-2.8 (-5.0 ; -0.6)***	143
Gases/Fumes										
Non-exposed	Ref	1021 (43)	Ref	412	Ref	609	Ref	401	Ref	620
Low	-0.7 (-1.4 ; 0)	1111 (47)	-0.7 (-1.9 ; 0.5)	316	-0.8 (-1.7 ; 0.1)	795	-0.9 (-2.1 ; 0.2)	647	-0.6 (-1.6 ; 0.3)	464
High	**-1.2 (-2.4 ; 0)***	232 (10)	-1.1 (-3.8 ; 1.7)	32	-1.3 (-2.6 ; 0)	200	-1.2 (-2.5 ; 0.1)	217	-2.1 (-5.9 ; 1.7)	15
Mineral dust										
Non-exposed	Ref	1491 (63)	Ref	520	Ref	971	Ref	630	Ref	861
Low	-0.5 (-1.4 ; 0.4)	375 (16)	-1.3 (-2.7 ; 0.2)	117	-0.2 (-1.3 ; 1.0)	258	-0.3 (-1.5 ; 0.9)	234	-0.8 (-2.1 ; 0.6)	141
High	-0.2 (-1.4 ; 1.0)	498 (21)	0.8 (-1.9 ; 3.4)	123	-0.4 (-1.8 ; 1.0)	375	-0.3 (-1.7 ; 1.0)	401	1.5 (-1.9 ; 5.0)	97
Biological dust										
Non-exposed	Ref	1318 (56)	Ref	423	Ref	895	Ref	726	Ref	592
Low	**-0.9 (-1.6 ; -0.1)***	840 (36)	-1.1 (-2.3 ; 0.1)	292	-0.8 (-1.8 ; 0.2)	548	-1.0 (-2.2 ; 0.3)	373	-0.9 (-1.8 ; 0.1)	467
High	-0.7 (-1.9 ; 0.5)	206 (9)	-1.1 (-3.4 ; 1.3)	45	-0.6 (-2.0 ; 0.8)	161	-0.4 (-1.8 ; 1.1)	166	-2.2 (-4.6 ; 0.3)	40
All pesticides										
Non-exposed	Ref	1936 (82)	Ref	631	Ref	1305	Ref	945	Ref	991
Low	-0.3 (-1.7 ; 1.1)	153 (6)	0.6 (-1.7 ; 2.8)	59	-0.8 (-2.5 ; 1.0)	94	-0.3 (-2.0 ; 1.5)	96	-0.3 (-2.6 ; 2.0)	57
High	**-1.3 (-2.4 ; -0.2)***	275 (12)	0.1 (-2.1 ; 2.2)	70	**-1.8 (-3.1 ; -0.4)***	205	**-1.8 (-3.1 ; -0.5)****	224	0.6 (-1.8 ; 3.0)	51
Herbicides										
Non-exposed	Ref	2018 (85)	Ref	654	Ref	1364	Ref	1011	Ref	1007
Low	-0.6 (-2.0 ; 0.8)	145 (6)	1.3 (-1.1 ; 3.7)	46	# -1.5 (-3.2 ; 0.2)	99	-1.2 (-3.0 ; 0.5)	98	0.7 (-1.5 ; 3.1)	47
High	**-1.3 (-2.6 ; -0.1)***	201 (9)	-0.7 (-2.9 ; 1.5)	60	**-1.6 (-3.1 ; -0.1)***	141	**-1.8 (-3.2 ; -0.3)***	156	0.1 (-2.4 ; 2.6)	45
Insecticides										
Non-exposed	Ref	1969 (83)	Ref	636	Ref	1333	Ref	977	Ref	992
Low	0.2 (-1.4 ; 1.7)	120 (5)	0.8 (-1.5 ; 3.2)	54	-0.3 (-2.4 ; 1.8)	66	0.6 (-1.5 ; 2.7)	64	-0.8 (-2.5 ; 2.2)	56
High	**-1.2 (-2.3 ; -0.1)***	275 (12)	0.1 (-2.0 ; 2.3)	70	**-1.7 (-3.0 ; -0.3)***	205	**-1.7 (-3.0 ; -0.4)***	224	0.6 (-1.8 ; 3.0)	51

[a] Occupational exposures (no/low/high) were estimated based on job title and function using the ALOHA+ Job Exposure Matrix. Non-exposed subjects were assigned as reference category (Ref); VGDF = Vapors, Gases, Dust, Fumes; *p<0.05; **p<0.01; ***p<0.001. # Significantly different for never and ever smokers (i.e. p-value for interaction < 0.05).

Supplementary table 8. Verification of associations between exposure to various types of solvents and level of FEV_1 and FEV_1/IVC adjusted for sex, age, height, weight, current, ex smoking and (log) packyears smoked in the Vlagtwedde-Vlaardingen cohort. Stratification by smoking status (never/ever) and gender is shown.

Vlagtwedde-Vlaardingen					FEV_1 (ml)					
Solvent Exposure[a]	**All b (95% CI)**	**N**	**Never smokers b (95% CI)**	**N**	**Ever smokers b (95% CI)**	**N**	**Males b (95% CI)**	**N**	**Females b (95% CI)**	**N**
Aromatic solvents										
Non-exposed	Ref	1755	Ref	644	Ref	1111	Ref	731	Ref	1024
Low	**-61 (-108 ; -14)***	577	**-93 (-179 ; -8)***	115	-47 (-104 ; 9)	462	-58 (-117 ; 1)	505	-23 (-111 ; 65)	72
High	-32 (-192 ; 129)	32	217 (-577 ; 1012)	1	-33 (-205 ; 139)	31	-32 (-223 ; 159)	29	-28 (-444 ; 389)	3
Chlorinated solvents										
Non-exposed	Ref	2067	Ref	713	Ref	1354	Ref	1010	Ref	1057
Low	29 (-39 ; 96)	202	-53 (-194 ; 88)	34	48 (-30 ; 126)	168	35 (-50 ; 120)	165	42 (-79 ; 163)	37
High	-13 (-109 ; 83)	95	-213 (-441 ; 15)	13	19 (-89 ; 127)	82	4 (-108 ; 115)	90	-112 (-438 ; 213)	5
Other solvents										
Non-exposed	Ref	1808	Ref	637	Ref	1171	Ref	915	Ref	893
Low	-7 (-53 ; 38)	505	**-85 (-166 ; -5)***	116	17 (-38 ; 73)	389	-6 (-72 ; 60)	318	13 (-46 ; 72)	187
High	6 (-122 ; 133)	51	157 (-143 ; 458)	7	-14 (-159 ; 131)	44	-27 (-209 ; 156)	32	69 (-99 ; 237)	19
					FEV_1/IVC (%)					
Solvent Exposure[a]	**All b (95% CI)**	**N**	**Never smokers b (95% CI)**	**N**	**Ever smokers b (95% CI)**	**N**	**Males b (95% CI)**	**N**	**Females b (95% CI)**	**N**
Aromatic solvents										
Non-exposed	Ref	1755	Ref	644	Ref	1111	Ref	731	Ref	1024
Low	**-0.9 (-1.7 ; -0.1)***	577	-1.0 (-2.6 ; 0.5)	115	-0.8 (-1.8 ; 0.1)	462	-0.9 (-1.8 ; 0)	505	-0.6 (-2.3 ; 1.2)	72
High	-1.3 (-4.0 ; 1.4)	32	3.1 (-10.9 ; 17.1)	1	-1.4 (-4.3 ; 1.5)	31	-1.4 (-4.4 ; 1.6)	29	0.7 (-7.6 ; 9.1)	3
Chlorinated solvents										
Non-exposed	Ref	2067	Ref	713	Ref	1354	Ref	1010	Ref	1057
Low	0.7 (-0.4 ; 1.8)	202	0 (-2.5 ; 2.5)	34	-0.9 (-2.3 ; 0.6)	168	0.9 (-0.4 ; 2.3)	165	-0.1 (-2.5 ; 2.4)	37
High	**1.7 (0.1 ; 3.3)***	95	0.9 (-3.2 ; 4.9)	13	2.5 (-2.8 ; 7.8)	82	**1.9 (0.1 ; 3.6)***	90	1.0 (-5.5 ; 7.5)	5
Other solvents										
Non-exposed	Ref	1808	Ref	637	Ref	1171	Ref	915	Ref	893
Low	0.3 (-0.5 ; 1.1)	505	0.9 (-0.4 ; 2.2)	116	0.7 (-0.3 ; 1.6)	389	0.9 (-0.2 ; 1.9)	318	-0.5 (-1.7 ; 0.6)	187
High	-0.2 (-2.4 ; 1.9)	51	**1.8 (0 ; 3.6)***	7	-0.6 (-3.0 ; 1.9)	44	-0.6 (-3.5 ; 2.2)	32	0.7 (-2.6 ; 4.1)	19

[a] Occupational exposures (no/low/high) were estimated based on job title and function using the ALOHA+ Job Exposure Matrix. Non-exposed subjects were assigned as reference category (Ref). # Significantly different for never and ever smokers (i.e. p-value for interaction < 0.05).

Additional information 1. Pesticide use in the northern Dutch provinces since 1985

Between 1985 and today roughly 90% of agriculture in the northern Dutch provinces consisted of arable crops, on average ~30% potatoes, ~30% cereals, ~15% beets and ~15% maize (for animal feeding). The area of maize has increased since 1985, whereas cereal cultivation has decreased. There is little fruit growing, bulb cultivation or greenhouses in this area. In terms of pesticide use this means that mainly herbicides have been applied (cereals, beets, maize) and substantial fungicide use on potatoes (mainly dithiocarbamate fungicides).

- **Potatoes:** Dithiocarbamate fungicides (maneb, mancozeb), organotin-fungicides (fentin-acetate), quaternary ammonium herbicides (paraquat, diquat) and other fungicides like cymoxanil and fluazinam.
- **Cereals:** Phenoxy herbicides (MCPA, MCPP), urea herbicides (isoproturon), growth regularor chlormequat, conazole fungicides (propiconazole, prochloraz, epoxiconazole etc).
- **Beets:** Carbamate herbicides (phenmedipham, desmedipham), other herbicides like metamitron, chloridazone, ethofumesate and glyphosate.
- **Maize:** triazine herbicides (atrazine, terbutylazin), anilide herbicides (metolachloor, propachloor), other herbicides like bentazone and pyridate.

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2

Pesticides and respiratory health: where do we go from here?

COMMENTARY

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Pesticides and respiratory health: where do we go from here?

For centuries, researchers have focused on exposures to hays, grains and animals as primary contributors to poor respiratory outcomes in farmers and agricultural workers[1,2]. However, growing evidence suggests that other agricultural exposures, namely pesticides, may also adversely impact respiratory health. Recent studies from around the world have suggested that pesticides may be associated with respiratory symptoms and disease, particularly asthma[3-6]. However, these studies have been based on self-reported outcomes and there have been few studies using objective measures of pulmonary function[2,7].

De Jong et al[8] report that occupational pesticide exposure is associated with poorer pulmonary function consistent with airway obstruction as measured by spirometry in two Dutch general population cohorts. These associations with pesticides were seen in both men and women and smokers and non-smokers; some associations were stronger in smokers, but not consistently so. The magnitude of the associations, particularly for herbicides, was of greater or equal magnitude as that observed here for vapours, gases, dusts and fumes, and there was a suggestion of an exposure–response relationship with more intense pesticide exposures. No information on symptoms was reported.

In some ways, the magnitude of the association is surprising as many factors may have contributed to an underestimation of the effect. The first factor was the use of a general population sample. While this was the largest study to date to assess the role of pesticides and pulmonary function, the prevalence of high occupational exposure was 1% in the main cohort (N=11 851) and 12% in the verification cohort (N=2364). Thus, in a sample of over 13 000 individuals, only 387 had high occupational exposure. The second factor is exposure characterization. Occupational pesticide exposure was assigned using a job exposure matrix based on the current or most recent job. Use of a job exposure matrix is an efficient way to classify exposures in a diverse population, but chemical specificity is lost. Exposure intensity was classified as none, low or high, but there was no information on duration of exposure or the specific chemicals used. Pesticides are a diverse group of agents defined based on their ability to kill pests; they differ greatly in their chemical and biological properties. To date, some specific chemicals (eg, paraquat and organophosphate insecticides) have been associated with respiratory outcomes, but other pesticides have not[2, 4-7]. Using a summary measure of pesticides may introduce measurement error as not all pesticides have similar toxicity. However, as all participants came from the same region in The Netherlands, the pesticides used by these individuals may be similar. In addition to a lack of specificity, the authors lack information about the frequency or duration of use of pesticides, so all crop farmers irrespective of commodities raised, farm size or the number of years of farming received the same exposure intensity.

Finally, it is likely that the current study may underestimate the true impact of pesticides on pulmonary function, because exposure was assigned based on current or most recent job. Individuals most affected by respiratory toxicants may change jobs or alter their work environment to reduce their exposure, thus these individuals would not be classified as pesticide exposed. In a meta-analysis on the impact of this loss to follow-up on cohort studies of chronic bronchitis,

Radon et al[9] reported that the prevalence of chronic bronchitis was 25% higher in those who dropped out of occupational cohort studies. Thus by using only the most recent job, de Jong et al would have missed earlier occupational exposures, which may have influenced airway obstruction.

Despite these challenges, de Jong et al[8] provide additional evidence that occupational pesticide use influences pulmonary function and add to the growing body of literature that pesticides may adversely influence respiratory health. Given the ubiquity of pesticide use worldwide, with over 5 billion pounds of pesticides used in 2006,[10] what is now needed is better information on the specific chemicals, exposure–response relationships and mechanisms of action which contribute to these outcomes. In order to protect human health in the presence of ongoing pesticide use, we need to be able to understand the risks associated with specific chemicals to help people make informed choices to protect their respiratory health.

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3

Occupational exposure to vapors, gases, dusts, and fumes is associated with small airways obstruction

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Dear Editor,

Various studies have shown that occupational exposures to vapors, gases, dusts and fumes or their composite measure (VGDF) negatively affect the forced expiratory volume in one second (FEV_1) and the ratio of FEV_1 to forced vital capacity (FEV_1/FVC), indicating obstruction of predominantly the large airways[1-3]. Recently we have shown that occupational exposure to pesticides is associated with substantial losses of large airway function in the general population[3]. The negative effects of occupational exposures on the level of lung function were generally more pronounced in ever than never smokers, suggesting that cigarette smoke-induced damage increases the susceptibility of the airways to other exposures[3]. Next to large airways obstruction there is considerable renewed interest in obstruction of the small airways since small airways obstruction is one of the three main phenotypes of COPD[4,5].

Thus far, only one general population based study in 1,735 individuals has shown associations of occupational exposure to biological dust with forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}), an indicator of small airways obstruction[1]. In addition, some small scale studies in specific populations have shown negative effects of specific occupational exposures on the small airways, like non-asbestos mineral dusts[6], welding fumes[7,8], and pesticide exposure[9,10]. However, these studies included individuals with large airways obstruction, or individuals with reduced FVC that may affect levels of FEF_{25-75} values, and were thus not specifically investigating small airway obstruction.

We used data of 11,851 participants, 9,876 without large airways obstruction, of the LifeLines population for which we have estimated job-specific exposure to the composite measure VGDF (and separately to subcategories biological dust, mineral dust, gases and fumes) and exposure to pesticides in general (and separately to subcategories herbicides and insecticides) as no, low and high (0/1/2) exposure using the ALOHA+ JEM[3]. We assessed associations between occupational exposures and FEF_{25-75} (ml/s) levels using linear regression with adjustment for sex, age, height, weight, current, ex-smoking and (log) packyears. Because of substantial co-exposure between the specific occupational agents, we additionally adjusted the analyses on the composite measure VGDF, biological dust, mineral dust, gases and fumes for co-exposure to pesticides, and conversely the analyses on pesticides, herbicides and insecticides were adjusted for co-exposure to the composite measure VGDF[3].

Of the total of 11,851 subjects, 42% were male, median age being 47 years (range = 18-89), 57% being ever smoker (median number of packyears = 10, range 0-100). Mean FEV_1%predicted was 102%, FEV_1/FVC 76% and FEF_{25-75} 2.9 l/s (78% predicted). Subjects without large airways obstruction (FEV_1/FVC≥70%, FEV_1≥80%; n = 9,876, 83%) had a median age of 46 years (range 18-89), 40% being male and 54% being ever smoker (median number of packyears = 9, range 0-84). In both groups, about 11 percent of the subjects were highly exposed to the composite measure VGDF, whereas high exposure to pesticides in general was less common (1%; table 1).

Table 1. Associations between occupational exposures and level of FEF_{25-75} (ml/s) for the whole sample and for subjects without large airways obstruction ($FEV_1/FVC \geq 70\%$, $FEV_1 \geq 80\%$).

	FEF_{25-75} (ml/s)					
	All (n = 11,851)			Without large airways obstruction (n = 9,876)		
Exposure*	b (95% CI)	p-value	n (%)	b (95% CI)	p-value	n (%)
VGDF						
Non-exposed	Ref		6534 (55)	Ref		5513 (56)
Low	**-47 (-83 ; -10)**	**0.012**	3985 (34)	**-39 (-74 ; -4)**	**0.031**	3325 (34)
High	**-157 (-220 ; -93)**	**<0.001**	1332 (11)	**-102 (-166 ; -39)**	**0.001**	1038 (11)
Biological						
Non-exposed	Ref		8127 (69)	Ref		6787 (69)
Low	-17 (-56 ; 22)	0.389	3256 (28)	-18 (-55 ; 19)	0.343	2707 (27)
High	-84 (-186 ; 17)	0.104	468 (4)	**-143 (-244 ; -43)**	**0.005**	382 (4)
Mineral dust						
Non-exposed	Ref		9389 (79)	Ref		7907 (80)
Low	**-62 (-109 ; -15)**	**0.009**	1924 (16)	-38 (-83 ; 8)	0.104	1551 (16)
High	-69 (-162 ; 24)	0.148	538 (5)	12 (-79 ; 104)	0.790	418 (4)
Gases/Fumes						
Non-exposed	Ref		7007 (59)	Ref		5905 (60)
Low	**-51 (-88 ; -14)**	**0.006**	4159 (35)	**-46 (-18 ; -10)**	**0.011**	3446 (35)
High	**-137 (-212 ; -62)**	**<0.001**	685 (6)	-59 (-134 ; 15)	0.118	525 (5)
All pesticides						
Non-exposed	Ref		11369 (96)	Ref		9494 (96)
Low	-73 (-174 ; 29)	0.162	370 (3)	**-115 (-214 ; -16)**	**0.023**	303 (3)
High	-93 (-270 ; 83)	0.300	112 (0.9)	0 (-184 ; 184)	0.999	79 (0.8)
Herbicides						
Non-exposed	Ref		11680 (99)	Ref		9754 (99)
Low	-96 (-258 ; 65)	0.243	132 (1)	-105 (-267 ; 57)	0.204	101 (1)
High	-193 (-485 ; 99)	0.195	39 (0.3)	218 (-131 ; 567)	0.220	21 (0.2)
Insecticides						
Non-exposed	Ref		11425 (96)	Ref		9540 (97)
Low	-71 (-181 ; 39)	0.206	315 (3)	-114 (-221 ; -7)	0.036	258 (3)
High	-90 (-267 ; 87)	0.320	111 (0.9)	13 (-172 ; 198)	0.890	78 (0.8)

The linear regression model was adjusted for sex, age, height, weight, current, ex smoking and (log) packyears. The analyses on biological dust, mineral dust, gases and fumes, and the composite measure VGDF were additionally adjusted for pesticide exposure, whereas the analyses on pesticides, herbicides and insecticides were additionally adjusted for exposure to the composite measure VGDF. *Occupational exposures (no/low/high) were estimated based on job title and function using the ALOHA+ Job Exposure Matrix. Non-exposed subjects were assigned as reference category (Ref); VGDF = the composite measure of vapors, gases, dusts, and fumes.

Exposure to the composite measure VGDF, and to the subcategories biological dust and gases and fumes, was associated with lower FEF_{25-75} levels (table 1). These associations remained present when we restricted our analysis to subjects without large airways obstruction (table 1). Moreover, findings were similar in ever and never smokers (figure 1) and when adjusted for FVC. Occupational exposure to pesticides in general and to the subcategories herbicides and insecticides tended to be associated with lower FEF_{25-75} in the whole group, yet these associations largely disappeared when the analysis was restricted to subjects without large airways obstruction (table 1).

It is known that occupational exposure to vapors, gases, dusts and fumes affects large airway function and increases the risk for spirometry defined COPD[1-3,11,12]. With the current study we add to this knowledge by showing that the small airways are affected by occupational exposure to the composite measure VGDF, and the subcategories biological dust, gases and fumes as well. Importantly, we find these associations in subjects with normal FEV_1/FVC and FEV_1%predicted values as well, indicating that effects of vapors, gases, dusts and fumes exposure on the small airways are a primary response and independently from effects on the large airways. The observed associations were found to be independent of smoking habits, which is in contrast to our previous findings on large airways obstruction where we found significant differences between ever smokers and never smokers[3]. The lack of effects of smoking on small airways function in interaction with occupational exposure is in line with a previous study investigating biological dust[1].

Although exposure to pesticides was strongly and consistently associated with level of FEV_1 in our previous cross-sectional study[3], the trend for an association with FEF_{25-75} did not reach statistical significance and disappeared when analyses were restricted to subjects without large airways obstruction. In line with our findings, a study from Sri Lanka found no significant reduction in FEF_{25-75} levels of farmers exposed to pesticides, whereas there was a significant effect on FEV_1 and FVC levels[13]. It may be that the aerodynamic diameter of the pesticides aerosols results in deposition mainly in the larger airways. A study assessing different types of pesticides and agricultural application methods showed that aerosols had a median aerodynamic diameter ranging from 4 to 16 μm[14], whereas for example fibrous dust has an aerodynamic diameter <3 μm and the majority of welding aerosols have an aerodynamic diameter <1μm[15].

In conclusion, with the current study we show that occupational exposure to vapors, gases, dusts and fumes induces small airways obstruction independently of large airways obstruction in both ever and never smokers. Loss and narrowing of the small airways is seen in patients with mild COPD even before the onset of emphysematous destruction and becomes increasingly evident in severe COPD[4]. Therefore small airway obstruction should be taken into account when monitoring respiratory health of workers that are exposed to vapors, dust, gases and fumes.

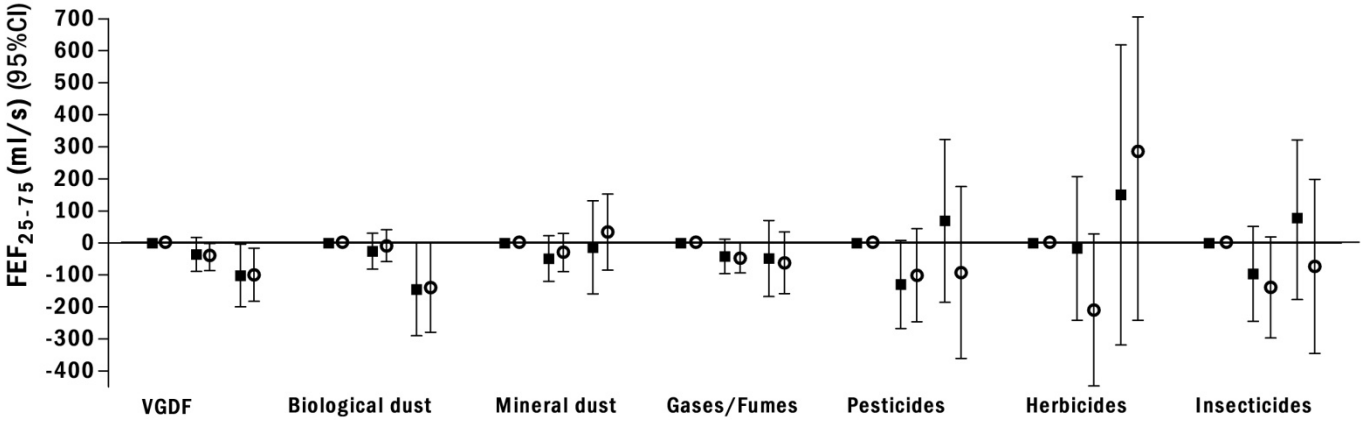

Figure 1. Associations between occupational exposures and level of FEF_{25-75} (ml/s) for subjects without large airways obstruction ($FEV_1/FVC \geq 70\%$, $FEV_1 \geq 80\%$), stratified by smoking status (never/ever). Associations are shown for no (reference: set on 0), low and high exposure to the composite measure VGDF, and the subcategories biological dust, mineral dust, gases and fumes, pesticides in general, and the subcategories herbicides and insecticides.

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4

Association of occupational pesticide exposure with accelerated longitudinal decline in lung function

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ABSTRACT

Background

Cross-sectional studies have shown that occupational exposure to vapors, gases, dusts and fumes (VGDF) and pesticides is associated with a lower level of lung function. These associations seem to be stronger in ever smokers. The current study aimed to assess whether occupational exposure to VGDF and pesticides is associated with the longitudinal decline of lung function.

Methods

We used 12,772 observations from 2,527 subjects participating in the Vlagtwedde-Vlaardingen general population-based cohort that was followed for 25 years from 1965 to the last survey in 1989/1990. Job-specific exposure at the last survey was estimated with the ALOHA+ job exposure matrix. Associations between exposures and annual declines in forced expiratory volume in one second (FEV_1) and the FEV_1 as percentage of the inspiratory vital capacity (FEV_1%VC) were assessed with linear mixed effect models, including sex, age and level of lung function at the first and packyears at the last measurement. We tested for interaction between smoking and occupational exposure, and assessed associations separately for never and ever smokers.

Results

Exposure to VGDF was not associated with accelerated lung function decline after adjustment for co-exposure to pesticides. Exposure to pesticides, both in the last held job and as cumulative measure was associated with accelerated decline of FEV_1 and FEV_1%VC, especially in ever smokers where we found an excess FEV1 of -6.9 mL/year (95% confidence interval: -10.2,-3.7) associated with high pesticides exposure.

Conclusion

Occupational exposure to pesticides is associated with clinically relevant accelerated annual decline of lung function in the general population. This may subsequently increase the risk for development of COPD and thereby contribute to the large burden of morbidity and mortality associated with this disease.

BACKGROUND

A lower level of lung function and accelerated lung function decline is associated with respiratory diseases like chronic obstructive pulmonary disease (COPD). Although cigarette smoking is regarded as the most important environmental risk factor for impaired level of lung function, accelerated lung function decline and development of COPD, there are additional environmental risk factors. About 15-20% of all COPD cases have been attributed to occupational exposures[1]. Cross-sectional studies have shown that occupational exposure to vapors, gases, dusts and fumes (VGDF) is a risk factor for respiratory symptoms, lower level of lung function and risk for COPD in the general population[2-4]. These effects are suggested to be even stronger in ever smokers[5,6]. Moreover, we recently showed that occupational exposure to pesticides was cross-sectionally associated with a lower level of lung function and increased prevalence of COPD in two general populations[5].

Several studies have focused on specific exposures within single industries or companies, such as exposure to dusts and gases in tunnel construction workers, organic dust exposure in pig farmers and cotton versus silk dust exposure in textile workers[7-9]. To date, only one study has assessed the association between occupational exposures, as estimated by means of the ALOHA job exposure matrix (JEM), and longitudinal decline in lung function in a general population. In that study study, using meta-analyzed data from 27 individual European centers, Sunyer et al did not find an association between exposure to dusts, gases and fumes or the composite measure VGDF and decline in lung function between two surveys on average 9 years apart. This null finding may have been due to the relatively young age of the study population (25-45 years) and relatively short follow-up time, hence cumulative exposure may have been too limited[10]. With the current study we extended on the previous studies by using a general population cohort that has been followed for 25 years with surveys performed every 3 years. Moreover, we now have additional estimates of occupational exposure to pesticides from the recently extended ALOHA JEM (ALOHA+). Therefore, the aim of the current study was to assess whether occupational exposure to VGDF and pesticides (comprising herbicides and insecticides) is associated with the longitudinal decline in lung function in a general population, and secondly whether these associations were different for never and ever smokers.

METHODS

Study population and measurements

The study population consisted of subjects participating in the last survey (1989/1990) of the Vlagtwedde-Vlaardingen cohort, a prospective general population based cohort study on the epidemiology of pulmonary diseases. The cohort, including subjects from a rural area in the North-Eastern part of the Netherlands (Vlagtwedde) and subjects from an urban area in the South-West part of the Netherlands (Vlaardingen), started in 1965. Participants were followed for 25

years, with surveys performed every 3 years[11,12]. In Vlaardingen only participants who were included at baseline (1965 or 1969) were approached for follow-up, whereas in Vlagtwedde new subjects aged between 20 and 65 years were invited to participate at every survey. The study protocol was approved by the local university medical hospital ethics committee, University of Groningen, University Medical Center Groningen, The Netherlands. All participants gave their written informed consent. In 1984, the Committee on Human Subjects in Research of the University of Groningen reviewed the study and affirmed the safety of the protocol and study design.

During each survey, information was collected by questionnaires and spirometry was performed using a slow inspiration maneuver (for more information about the inspiratory vital capacity see Web Appendix 1) according to European Respiratory Society criteria (water-sealed spirometer; Lode instruments, Groningen, the Netherlands). Job titles and descriptions reported at the last survey were used to estimate job-specific exposures in the current job (or last held job in case of current unemployment) as no, low and high (0/1/2) exposure using the ALOHA+ JEM[4,5]. Cumulative exposure was calculated as the number of intensity-years in three jobs: the last held job, the previous job and the most important job before the previous job, multiplied by the intensity of exposure (low = 1 and high = 4). All jobs were reported at last survey (the specific survey question can be found in Web Appendix 2). Information on numbers of years in the reported jobs was available for 93% of the subjects.

Statistical analysis

Associations between the exposures and annual declines in forced expiratory volume in one second (FEV_1) and the FEV_1 as percentage of the inspiratory vital capacity (FEV_1%VC) were assessed with linear mixed effect models. For each subject, the linear mixed effect model takes into account every available survey. Only surveys performed at age 30 years or older were included because an individual's maximal level of lung function is assumed to have been reached before that age and thereafter lung function is considered to be in the decline phase[13]. The linear mixed effect models included sex, lung function level at the first measurement (absolute value centered at population mean level), age at the first measurement (centered at age 30), packyears at the last measurement, and their interaction with time since first measurement. Time was defined as years since first measurement. A random factor was assigned to the intercept and time. Statistical analyses were performed in Spotfire S-PLUS version 8.1 (TIBCO Software Inc, Palo Alto, California) and SPSS version 20 (IBM Corporation, Armonk, NY). *P*-values <0.05 were considered statistically significant (tested two-sided).

Because of substantial co-exposure between the specific occupational exposures, the analysis on exposure to VGDF was additionally adjusted for exposure to pesticides, and conversely the analysis on exposure to pesticides was adjusted for exposure to VGDF. A high level of exposure to pesticides was always accompanied by a high level exposure to VGDF in our sample and testing for interaction between the two exposures was therefore not possible. In an attempt to

disentangle the associations with exposure to VGDF and pesticides we created groups based on joint exposure to VGDF and pesticides (unexposed, high exposure to VGDF only, high exposure to both VGDF and pesticides) and assessed associations with annual decline in FEV_1 and FEV_1%VC. Additionally, we assessed whether the associations between occupational exposures and decline in lung function were different for never and ever smokers and tested for interaction.

Table 1. Characteristics of subjects who participated in the last survey (1989/1990) of the Vlagtwedde-Vlaardingen study, the Netherlands, 1989/1990.

	Vlagtwedde-Vlaardingen
No. of Subjects	2,527
No. of Observations	12,772
Number of visits per subject, median (range)	5 (1-8)
Duration of follow-up (years), median (range)	16 (0-25)
Male sex, n (%)	1,343 (53)
Age (years), median (range)	
At first measurement	35 (30-70)
At last measurement	53 (30-80)
Smoking status, n (%)	
Never smoker	877 (35)
Ever smoker	1,650 (65)
Packyears of smoking in ever smokers, median (range)	20 (1-262)
Lung function level at first measurement, mean (sd)	
FEV_1%predicted[a]	91 (13)
FEV_1%VC	77 (8)
Exposure to vapors, gases, dusts and fumes	
High exposure in the last held job, n (%) subjects	837 (33)
Cumulative exposure >0 intensity-years[b] , n (%) subjects	1,626 (69)
Cumulative exposure (intensity-years[c]), median (range)	48 (1-260)
Exposure to pesticides	
High exposure in the last held job, n (%) subjects	298 (12)
Cumulative exposure >0 intensity-years , n (%) subjects	579 (25)
Cumulative exposure (intensity-years[c]), median (range)	56 (1-228)

VGDF : vapors, gases, dusts and fumes; Pesticides comprises herbicides and insecticides.

[a] Based on reference equations by Quanjer et al (24).

[b] Intensity-years estimated as years of exposure weighted by intensity of exposure (low = 1, high = 4).

[c] Within the subjects exposed (>0 intensity-years).

RESULTS

Population characteristics

Population characteristics are shown in table 1. A total of 12,772 observations from 2,527 subjects were available (the median number of observations per subject was 5; range, 1-8). Of all subjects, 53% were male, and the median age at the last visit was 53 years. There were 2 times more ever smokers than never smokers. One-third of the subjects were occupationally exposed to high levels of VGDF (33%), whereas exposure to high levels of pesticides was less common (12%). The median number of intensity-years, as years of exposure weighted by intensity of exposure (low = 1, high = 4),

within the exposed subjects (>0 exposure years) was 48 years (range, 1-260) for VGDF and 56 years (range, 1-228) for pesticides. The mean estimated change in lung function in the whole sample was -18.0 mL/year (95% confidence interval (CI): -19.6,-16.5) for FEV_1 and -0.08%/year (95% CI: -0.11,-0.05) for FEV_1%VC.

VGDF exposure and annual change in lung function

The group that was unexposed to VGDF had an average annual change of -17.2 mL/year (95% CI: -19.0,-15.4) in FEV_1 and -0.07%/year (95% CI: -0.10,-0.03) in FEV1%VC. Compared with no exposure, high occupational exposure to VGDF in the last-held job was significantly associated with an excess change in FEV_1 (-4.0 mL/year, 95% CI:-6.1,-2.0) but was not significantly associated with change in FEV_1%VC (-0.04%/year, 95% CI: -0.08,0.00). When adjusted for pesticide exposure, occupational exposure to VGDF in the last-held job was no longer significantly associated with excess change in FEV_1 (table 2). There was a marginal significant association with cumulative exposure (intensity-years) to VGDF (table 2). There was no significant interaction between smoking and VGDF exposure (table 3).

Table 2. Associations between occupational exposure to pesticides and annual change in FEV_1 and FEV_1%VC, Vlagtwedde-Vlaardingen Study, the Netherlands, 1965-1990[d].

	FEV_1			FEV_1%VC					
Exposure	Excess change (mL/yr)	95% CI	*p*-value	Excess change (%/year)	95% CI	*p*-value	No. subjects	[c]%	No. obs.
VGDF									
[a]no	Ref			Ref			958	38	4643
low	-0.6	-2.6, 1.5	0.591	-0.01	-0.05, 0.03	0.579	732	29	3698
high	-1.8	-4.4, 0.7	0.154	0.00	-0.06, 0.05	0.881	837	33	4431
[b]cumulative	-0.2	-0.4, 0.0	0.047	0.00	-0.01, 0.00	0.243	2359	69	11895
Pesticides									
[a]no	Ref			Ref			2067	82	1025
low	-1.4	-4.9, 2.1	0.435	-0.01	-0.08, 0.06	0.733	162	6	885
high	-5.1	-8.0, -2.1	<0.001	-0.09	-0.15, -0.03	0.004	298	12	1659
[b]cumulative	-0.3	-0.5, -0.1	0.007	-0.01	-0.01, 0.00	0.046	2359	25	11895

VGDF: vapors, gases, dusts and fumes, Pesticides comprises herbicides and insecticides. [a] in the last held job at last survey (1989-1990) (no exposure was assigned as reference category (Ref)). [b] Decline per 10 intensity-years; intensity-years estimated as years of exposure weighted by intensity of exposure (low = 1, high = 4). [c] Percent exposed is the percentage of subjects with no, low, or high exposure or cumulative exposure >0 intensity-years. [d] The Linear mixed effect models were adjusted for sex, lung function level and age at first measurement, packyears smoked at last measurement and co-exposure to VGDF or pesticides.

Pesticide exposure and decline in lung function

The participants who were unexposed to pesticides had an average annual change of -17.6 mL/year (95% CI: -19.1,-16.0) in FEV_1 and an average annual change of -0.07%/year (95% CI: -0.10,-0.04) in FEV_1%VC. Compared with no exposure, occupational exposure to high levels of pesticides in the last-held job was associated with an excess change of -6.2

mL/year (95% CI: -8.6,-3.8) in FEV_1 and a change of -0.09%/year (95% CI: -0.14,-0.04) in FEV_1%VC. This association remained present after adjustment for co-exposure to VGDF (table 2). The negative association between occupational exposure to pesticides and annual change in lung function was confirmed when we used an estimate of cumulative exposure (intensity-years) to pesticides (table 2).

The annual changes in both FEV_1 and FEV_1%VC were significantly larger in ever smokers with high pesticide exposure than in never smokers with high pesticide exposure; the P values for interaction between smoking and high exposure to pesticides in the last-held job were 0.02 and 0.01 for FEV_1 and FEV_1%VC, respectively, after adjustment for coexposure to VGDF and the VGDF-by-smoking interaction. When the associations were assessed for never and ever smokers separately, the associations between occupational exposure to pesticides, both in the last-held job and as a cumulative measure (intensity-years), and change in FEV_1 and FEV_1%VC remained present in ever smokers only (table 3).

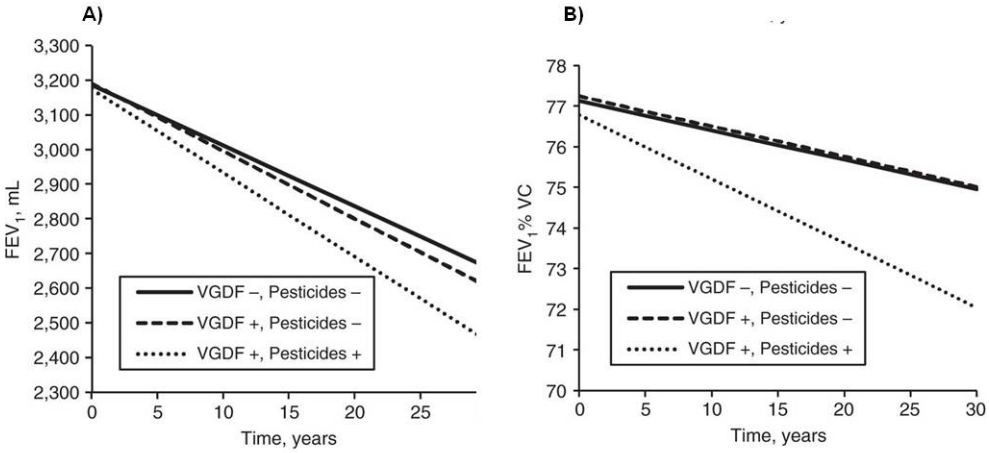

Figure 1. Estimated course of FEV_1 (A) and FEV_1%VC (B) for subjects with high exposure to VGDF only and joint high exposure to VGDF and pesticides compared to the unexposed modeled for time (years) since first survey after the age of 30 years in the Vlagtwedde-Vlaardingen study (1965-1990).

* Significantly accelerated decline for the group with joint high exposure to VGDF and pesticides (VGDF + : Pesticides +) compared to the unexposed (VGDF - : Pesticides -), P-value < 0.001 for both FEV_1 and FEV_1%VC, and for the group with joint high exposure to VGDF and pesticides (VGDF + : Pesticides +) compared to VGDF only (VGDF + : Pesticides -): P-value = 0.001 for FEV_1, P-value = 0.003 for FEV_1%VC. The linear mixed effect models were adjusted for sex, lung function level and age at first measurement, and packyears smoked at last measurement.

Joint exposure to VGDF and pesticides

High exposure to pesticides was always accompanied by high exposure to VGDF in our sample; that is, there was no "high exposure to pesticide only" group. The group with high exposure to both VGDF and pesticides in the last-held job

had a significant excess change of -6.7 mL/year (95% CI: -9.2,-4.1) in FEV_1 and a change of -0.09%/year (95% CI: -0.14,-0.04) in FEV_1%VC, compared with the unexposed group. There was also a significant difference between the group with high exposure to both VGDF and pesticides and the group with high exposure to VGDF only (change in FEV_1 = -4.7 mL/year (95% CI: -7.5,-1.9); change in FEV_1%VC = -0.08%/year (95%CI:-0.14,-0.03)) (Figure 1). There was no difference in lung function change between the group with only high exposure to VGDF and the unexposed group (FEV_1: -2.0 mL/year (95% CI: -4.1,0.1); FEV_1% VC: -0.00%/year (95% CI: -0.05,0.05)).

DISCUSSION

Our current study shows that occupational exposure to pesticides is associated with accelerated annual decline in FEV_1 and FEV_1%VC in this sample from the general population. To our knowledge, no other study to date has investigated the association between occupational exposure to pesticides and decline in lung function in a general population. Cross-sectional studies have shown associations of specific types of pesticides with the presence of chronic bronchitis in U.S. farmers[14] and their spouses[15], and with lower levels of FEV_1 and FVC in occupationally exposed farmers from Sri-Lanka[16] and South Korea[17]. Recently, we have shown that occupational exposure to pesticides, as assessed with the ALOHA+ JEM, was associated with lower levels of FEV_1 and FEV_1%VC, and an increased prevalence of COPD in a cross-sectional analysis of two Dutch general populations, the LifeLines cohort study and the currently used Vlagtwedde-Vlaardingen cohort[5]. With the current study we extended on these findings by showing that high exposure to pesticides had clinically relevant associations, especially in ever smokers where we found an excess annual decline of over 7 ml FEV_1 compared to the unexposed.

Subjects that were highly exposed to pesticides in our sample are field crop and vegetable growers (72%), mixed crop and animal producers (12%), gardeners, horticultural and nursery growers (15%), and tree and shrub crop growers (1%). To exclude the possibility that the associations are driven by exposures related to crop farming that we have not accounted for in the current study we performed an additional analysis in which we excluded the agricultural workers, i.e. the field crop and vegetable growers and the mixed crop and animal producers. Associations between high exposure to pesticides and level of FEV_1 (-13.1 ml/year, 95% CI = -19.1,-7.1) and FEV_1%VC (-0.19%/year, 95% CI = -0.31,-0.06) were even stronger in the remaining group, of which the majority were gardeners, horticultural and nursery growers. In an additional analysis we assessed associations with the pesticide subcategories: insecticides and herbicides (Web tables 1-3). Associations with the subcategory insecticides were similar compared to all pesticides. The strongest association with FEV_1 was seen for low exposure to the subcategory herbicides, an association that may be driven by the gardeners, horticultural and nursery growers.

Table 3. Associations between occupational exposure to pesticides and annual change in FEV_1 and FEV_1%VC among never and ever smokers, Vlagtwedde-Vlaardingen study, the Netherlands, 1965-1990[d].

	FEV_1						FEV_1%VC					
	Never smokers			Ever smokers			Never smokers			Ever smokers		
Exposure	Excess Change (mL/yr)	95% CI	P-value	Excess Change (mL/yr)	95% CI	P-value	Excess change (%/year)	95% CI	P-value	Excess change (%/year)	95% CI	P-value
VGDF												
[a]no	Ref			Ref			Ref			Ref		
low	-1.2	-4.2, 2.0	0.467	-0.1	-2.7, 2.6	0.962	-0.03	-0.10, 0.03	0.305	0.01	-0.05, 0.06	0.773
high	-3.4	-8.8, 2.0	0.218	-1.9	-4.8, 1.1	0.211	-0.06	-0.17, 0.05	0.285	0.00	-0.06, 0.06	0.901
[b]cumulative	-0.3	-0.6, 0.1	0.189	-0.2	-0.4, 0.0	0.080	0.00	-0.01, 0.01	0.833	0.00	-0.01, 0.00	0.117
Pesticides												
[a]no	Ref			Ref			Ref			Ref		
low	2.1	-4.6, 8.8	0.538	-3.2	-7.6, 1.2	0.155	0.07	-0.06, 0.21	0.281	-0.05	-0.14, 0.04	0.241
high	1.9	-4.8, 8.7	0.575	-6.9[c]	-10.2, -3.7	<0.001	0.08	-0.05, 0.22	0.229	-0.13[c]	-0.20, -0.07	<0.001
[b]cumulative years)	0.0	-0.6, 0.6	0.922	-0.4	-0.6, -0.1	0.005	0.00	-0.01, 0.02	0.638	-0.01	-0.01, 0.00	0.030

VGDF: vapors, gases, dusts and fumes, Pesticides comprises herbicides and insecticides. [a] In the last held job at last survey (1989-1990) (no exposure was assigned as reference category (Ref)). [b] Decline per 10 intensity-years; intensity-years estimated as years of exposure weighted by intensity of exposure (low = 1, high = 4). [c] significant interaction between smoking status and occupational exposure P-value <0.05. [d] The linear mixed effect models were adjusted for sex, lung function level and age at first measurement, packyears smoked at last measurement and co-exposure to VGDF or pesticides.

Within our sample, almost all (~99%) subjects with high pesticide exposure originated from the rural area around Vlagtwedde in the North-Eastern part of the Netherlands. About 80 to 90% of the agricultural land around Vlagtwedde was used for cultivation of crops during the study period (1965-1990). During the sixties and seventies the majority of cultivated crops were cereals (~50%) and during the eighties and nineties potatoes (~50%). Within the potatoes sector dinitrophenol herbicides were used until the eighties, whereas the quaternary ammonium herbicides (Diquat and Paraquat) became the most commonly used herbicides from the early eighties on (M. Brouwer, University of Utrecht, personal communication, 2014: for more detailed information Web table 4). Exposure to pesticides in the occupational setting occurs during mixing, loading of equipment, spraying and application of pesticides[18]. An important change in pesticide application during the study period may have been the change from open to closed cabins on tractors. However, the most important changes in application methods occurred after the study period, since the late nineties. We have used a general JEM-based estimate of exposure to pesticides (no/low/high) and the specific intensity of exposure may depend on the prudence and (protective) equipment of the pesticide applier. Moreover, the specific mechanism leading to damage in the lungs is likely different for each pesticide, amongst others it depends on the affected biochemical pathway and on the vapor and aerosol droplet size. For example, the primary mechanism for toxicity due to the herbicide 'paraquat' is related to its cyclic redox reaction and consequently free radical generation resulting in oxidative damage of the lung tissue [19]. It is very well likely that the effect of exposure to such a pesticide is more pronounced when anti-oxidant systems are already depleted by cigarette smoking and the lung tissue is already damaged by the free radicals from tobacco smoke. Occupational exposure to pesticides may then act synergistically with tobacco smoke exposure, as suggested by the interaction between smoking and exposure to pesticides that we found in both the previous cross-sectional and the current longitudinal study[5].

Sunyer et al, using the same JEM to assess occupational exposure, did not detect an accelerated decline in FEV_1 in subjects exposed to dusts, gases and fumes, or the composite measure VGDF in a population of relatively young age (25-40 years)[10]. Our cohort consists of a more heterogeneous sample of older age with lung function measurements every 3 years, yet the association between exposure to VGDF in the last held job and annual decline in FEV_1 in our study disappeared after adjustment for pesticides. There was a marginally significant association between cumulative exposure (intensity-years) to VGDF and annual decline in FEV_1.

Our study has several strengths and limitations. Our general population has been followed for 25 years and we have repeated measurements for the majority of subjects included, on average 5 measurements were available for each subject. Subjects were included from a rural (Vlagtwedde) and urban area (Vlaardingen), yet effect estimates remained similar when we adjusted for area (results not shown). Secondly, we have used two estimates of occupational exposure; exposure in last held job and cumulative exposure (intensity-years) based on years exposed in the last held and two previous jobs reported at last measurement, weighted by intensity of exposure. Duration of the reported jobs was

available for 93% of the subjects. Conclusions remained similar when we assumed exposure of 1, 5 or 10 years in case the duration of the job was missing. Moreover, using estimates of occupational exposure in the last held job or as cumulative exposure (intensity-years) resulted in the same conclusions. Finally, exposures were estimated with the ALOHA+ JEM, that has been specifically designed for general population based studies. In general, an advantage of using JEM-based exposure estimates rather than using self-reported exposures is that they are less likely to be affected by recall bias and differential misclassification of exposure[20, 21].

An important limitation of our study is that we were not able to completely disentangle the associations of VGDF and pesticide exposure, i.e. high pesticide exposure was always accompanied by high VGDF exposure. In the analyses based on joint high levels of exposure to VGDF and pesticides we confirmed that there was no association of only high VGDF exposure. It remains to be elucidated whether the association found with pesticides is driven by an association of only pesticide exposure or of joint exposure to pesticides and VGDF. Moreover, we did not have specific information on the pesticides applied, this information may be required to determine the specific biological pathways by which pesticides affect decline in lung function and consequently to set up interventions. In addition, since the late nineties there have been changes in regulations of application methods and pesticides available on the market, i.e. paraquat has been banned in the European Union since 2007. Also fewer people smoke currently than during the study observation period. Therefore translation to today's workers is uncertain and needs studies in cohorts of historically younger age. Finally, occupational exposure was ascertained at the last visit in 1989/1990. Subjects with respiratory complaints or lung function impairment could have changed occupations or dropped out of the study. This may have resulted in an underestimation of the associations between occupational exposures and the annual decline in lung function.

Notwithstanding, with the current study we show that occupational exposure to pesticides is associated with clinically relevant accelerated decline in both FEV_1 and FEV_1%VC. The agricultural sector employs more than 1.1 billion workers worldwide (about 34% of the global working force)[22], therefore effects associated with occupational exposure to pesticides can have a large public health impact. This is especially true in populations that are highly exposed, such as agricultural workers in developing countries who often apply pesticides with insufficient protective equipment and training[23].

Conclusion

We have shown that occupational exposure to pesticides is associated with clinically relevant accelerated annual decline in lung function in the general population. This may subsequently increase the risk for development of COPD and thereby contribute to the large burden of morbidity and mortality associated with this disease.

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4

SUPPLEMENTARY MATERIAL

Web Appendix 1. FEV_1%VC: FEV_1 as percentage of the inspiratory vital capacity.

Inspiratory vital capacity (IVC) is measured with the slow inspiratory maneuver, in contrast to the forced vital capacity (FVC) that is measured with a full maximal exhalation. In the current study only the IVC was available as a measure of vital capacity. In a statement document by Levy et al[1] the FEV_1%IVC was reported to be a more reliable measurement than the FEV_1%FVC. Moreover, the relaxed slow inspiratory maneuver is more convenient to perform for subjects with airways obstruction and thus increases the chance to fulfill the criteria for a good measurement. A study by Chhabra[2] compared FVC, IVC and SVC (slow vital capacity) among 20 normal and 60 asthmatics with different stages of airways obstruction (mild, moderate, severe). Chhabra found that FVC and IVC did not differ in normal subjects and only marginally in subjects with mild obstruction, but IVC tends to be larger than FVC in subjects with moderate-severe airways obstruction. The authors stated that FEV_1%IVC is a more sensitive indicator of airways obstruction than FEV_1%FVC because FVC, given its forced nature, underestimates the actual vital capacity.

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Web Appendix 2. Survey questions used to estimate occupational exposure.

The categorization of the three jobs (last held/previous/most important job before the previous) is based on a survey question from the last measurement in 1989/1990. The survey question was as follows:

Q. What is your current job? How long did you work in this job? Which jobs did you have before the current job? How long did you work in these jobs?

(fill-in:)
a) Current job and duration .....
b) Job before the current job (for at least 6 months) and duration.....
c) Most important job before b) and duration .....

Job a (or job b in case of current unemployment) is used to estimate exposure in the last held job. Jobs a, b and c are used to estimate cumulative exposure (intensity-years).

Web Table 1. Linear mixed effect model estimating effects of occupational exposure in the last held job and as cumulative exposure (intensity-years), on annual change in FEV_1 and FEV_1%VC in the Vlagtwedde-Vlaardingen cohort.

	FEV_1			FEV_1%VC		
Exposure	**Excess change (mL/yr)**	**95% CI**	***P*-value**	**Excess change (%/year)**	**95% CI**	***P*-value**
VGDF						
[b]no	Reference			Reference		
low	-0.55	-2.57, 1.46	0.591	-0.011	-0.052, 0.029	0.579
high	-1.84	-4.37, 0.69	0.154	-0.004	-0.055, 0.047	0.881
[c]cumulative	**-0.20**	**-0.40, 0.00**	**0.047**	-0.002	-0.006, 0.002	0.243
Biological dust						
[b]no	Reference			Reference		
low	0.23	-1.76, 2.21	0.824	-0.008	-0.048, 0.032	0.688
high	-1.50	-4.74, 1.74	0.365	-0.011	-0.076, 0.055	0.745
[c]cumulative	-0.20	-0.44, 0.03	0.091	-0.002	-0.006, 0.003	0.480
Mineral dust						
[b]no	Reference			Reference		
low	-0.18	-2.48, 2.13	0.882	0.036	-0.010, 0.082	0.129
high	-0.16	-3.23, 2.90	0.917	0.045	-0.016, 0.107	0.150
[c]cumulative	-0.13	-0.39, 0.13	0.338	0.000	-0.005, 0.006	0.904
Gases/Fumes						
[b]no	Reference			Reference		
low	-1.13	-3.06, 0.80	0.251	-0.022	-0.061, 0.017	0.260
high	-2.58	-5.66, 0.50	0.101	-0.002	-0.065, 0.060	0.957
[c]cumulative	-0.20	-0.45, 0.05	0.112	-0.002	-0.007, 0.003	0.450
Pesticides						
[b]no	Reference			Reference		
low	-1.41	-4.94, 2.12	0.435	-0.012	-0.084, 0.059	0.733
high	**-5.07**	**-8.00, -2.13**	**<0.001**	**-0.087**	**-0.147, -0.028**	**0.004**
[c]cumulative	**-0.31**	**-0.54, -0.08**	**0.007**	**-0.005**	**-0.009, 0.000**	**0.046**
Herbicides						
[b]no	Reference			Reference		
low	**-6.82**	**-10.39, -3.24**	**<0.001**	**-0.076**	**-0.148, -0.003**	**0.041**
high	-2.56	-5.73, 0.60	0.112	**-0.074**	**-0.138, -0.010**	**0.024**
[c]cumulative	-0.17	-0.41, 0.07	0.153	-0.004	-0.009, 0.001	0.126
Insecticides						
[b]no	Reference			Reference		
low	-1.13	-5.15, 2.89	0.582	-0.004	-0.085, 0.077	0.927
high	**-4.99**	**-7.94, -2.03**	**<0.001**	**-0.085**	**-0.145, -0.025**	**0.005**
[c]cumulative	**-0.31**	**-0.54, -0.08**	**0.007**	**-0.005**	**-0.009, 0.000**	**0.046**

The models were adjusted for sex, level of lung function and age at first measurement, packyears and co-exposure. The analyses with VGDF, biological dust, mineral dust, and gases and fumes were adjusted for pesticide exposure, whereas the analyses with pesticides, herbicides and insecticides were additionally adjusted for VGDF exposure. [b] In last held job at last survey (1989-1990). [c] Decline /10 intensity-years. Intensity-years estimated as years of exposure weighted by intensity of exposure (low = 1, high = 4).

Web Table 2. Linear mixed effect model estimating effects of occupational exposure in the last held job, and as cumulative exposure (intensity-years) on annual decline of FEV_1 in the Vlagtwedde-Vlaardingen cohort, for never and ever smokers from the model including the interaction between ever smoking and occupational exposure.

	FEV_1						
	Never smokers				Ever smokers		
Exposure	Excess change (mL/yr)	95% CI	*P*-value	interaction	Excess change (mL/yr)	95% CI	*P*-value
VGDF							
[b]no	Reference				Reference		
low	-1.15	-4.24, 1.95	0.467		-0.07	-2.71, 2.58	0.962
high	-3.39	-8.77, 2.00	0.218		-1.86	-4.77, 1.05	0.211
[c]cumulative	-0.25	-0.63, 0.12	0.189		-0.20	-0.42, 0.02	0.080
Biological dust							
[b]no	Reference				Reference		
low	-2.72	-5.83, 0.40	0.172	*	1.82	-0.73, 4.37	0.162
high	-3.88	-10.49, 2.73	0.250		-0.19	-3.92, 3.55	0.922
[c]cumulative	-0.27	-0.84, 0.31	0.360		-0.19	-0.45, 0.07	0.148
Mineral dust							
[b]no	Reference				Reference		
low	-2.15	-5.98, 1.69	0.273		0.87	-2.01, 3.74	0.554
high	1.43	-5.88, 8.75	0.701		-0.84	-4.24, 2.56	0.629
[c]cumulative	-0.04	-0.57, 0.49	0.891		-0.17	-0.46, 0.13	0.268
Gases/Fumes							
[b]no	Reference				Reference		
low	-2.05	-5.23, 1.13	0.206		-0.75	-3.21, 1.70	0.548
high	-4.92	-12.00, 2.17	0.174		-2.25	-5.68, 1.19	0.201
[c]cumulative	-0.43	-0.96, 0.10	0.111		-0.15	-0.42, 0.11	0.260
Pesticides							
[b]no	Reference				Reference		
low	2.10	-4.58, 8.78	0.538		-3.19	-7.59, 1.21	0.155
high	1.93	-4.82, 8.68	0.575	*	**-6.94**	**-10.22, -3.66**	**<0.001**
[c]cumulative	-0.03	-0.61, 0.56	0.922		**-0.35**	**-0.60, -0.11**	**0.005**
Herbicides							
[b]no	Reference				Reference		
low	2.04	-4.76, 8.84	0.557	*	**-10.67**	**-14.91, -6.42**	**<0.001**
high	2.05	-4.52, 8.61	0.541		**-4.10**	**-7.71, -0.49**	**0.026**
[c]cumulative	-0.03	-0.62, 0.56	0.912		-0.20	-0.46, 0.06	0.139
Insecticides							
[b]no	Reference				Reference		
low	1.86	-5.21, 8.94	0.606		-2.96	-8.21, 2.29	0.270
high	1.76	-5.05, 8.57	0.613	*	**-6.84**	**-10.13, -3.55**	**<0.001**
[c]cumulative	-0.06	-0.64, 0.53	0.852		**-0.35**	**-0.59, -0.10**	**0.005**

The models were adjusted for sex, level of lung function and age at first measurement, packyears and co-exposure. The analyses with VGDF, biological dust, mineral dust, and gases and fumes were adjusted for pesticide exposure, whereas the analyses with pesticides, herbicides and insecticides were additionally adjusted for VGDF exposure. [b] in last held job at last survey (1989-1990). [c] Decline /10 intensity-years. Intensity-years estimated as years of exposure weighted by intensity of exposure (low = 1, high = 4). * Interaction p-value < 0.05.

Web Table 3. Linear mixed effect model estimating effects of occupational exposure in the last held job, and as cumulative exposure (intensity-years) on annual decline of FEV_1%VC in the Vlagtwedde-Vlaardingen cohort, for never and ever smokers from the model including the interaction between ever smoking and occupational exposure.

	FEV$_1$%VC						
	Never smokers				Ever smokers		
Exposure	Excess change (%/year)	95% CI	*P*-value	interaction	Excess change (%/year)	95% CI	*P*-value
VGDF							
[b]no	Reference				Reference		
low	-0.033	-0.095, 0.030	0.305		0.008	-0.046, 0.061	0.773
high	-0.060	-0.168, 0.049	0.285		0.004	-0.055, 0.063	0.901
[c]cumulative	-0.001	-0.008, 0.007	0.833		-0.004	-0.008, 0.001	0.117
Biological dust							
[b]no	Reference				Reference		
low	-0.048	-0.111, 0.014	0.129	#	0.021	-0.031, 0.072	0.430
high	-0.070	-0.203, 0.063	0.302		0.022	-0.054, 0.097	0.576
[c]cumulative	-0.004	-0.015, 0.008	0.515		-0.001	-0.006, 0.004	0.601
Mineral dust							
[b]no	Reference				Reference		
low	-0.018	-0.095, 0.059	0.640		**0.063**	**0.005, 0.121**	**0.034**
high	0.036	-0.112, 0.185	0.630		0.041	-0.027, 0.110	0.236
[c]cumulative	0.003	-0.008, 0.014	0.574		-0.001	-0.007, 0.005	0.726
Gases/Fumes							
[b]no	Reference				Reference		
low	-0.046	-0.110, 0.018	0.162		-0.011	-0.060, 0.039	0.671
high	-0.028	-0.172, 0.116	0.699		0.002	-0.068, 0.071	0.957
[c]cumulative	0.000	-0.011, 0.010	0.958		-0.003	-0.008, 0.003	0.347
Pesticides							
[b]no	Reference				Reference		
low	0.074	-0.061, 0.209	0.281		-0.053	-0.142, 0.036	0.241
high	0.084	-0.053, 0.221	0.229	*	**-0.131**	**-0.198, -0.065**	**<0.001**
[c]cumulative	0.003	-0.009, 0.015	0.638		**-0.005**	**-0.010, -0.001**	**0.030**
Herbicides							
[b]no	Reference				Reference		
low	0.095	-0.042, 0.232	0.175	*	**-0.148**	**-0.234, -0.062**	**<0.001**
high	0.055	-0.078, 0.189	0.415	*	**-0.115**	**-0.188, -0.042**	**0.002**
[c]cumulative	0.002	-0.010, 0.013	0.800		-0.004	-0.010, 0.001	0.100
Insecticides							
[b]no	Reference				Reference		
low	0.065	-0.078, 0.208	0.375		-0.040	-0.146, 0.066	0.458
high	0.078	-0.060, 0.216	0.268	*	**-0.128**	**-0.195, -0.062**	**<0.001**
[c]cumulative	0.003	-0.009, 0.014	0.675		**-0.005**	**-0.010, 0.000**	**0.032**

The models were adjusted for sex, level of lung function and age at first measurement, packyears and co-exposure. The analyses with VGDF, biological dust, mineral dust, and gases and fumes were adjusted for pesticide exposure, whereas the analyses with pesticides, herbicides and insecticides were additionally adjusted for VGDF exposure. [b] in last held job at last survey (1989-1990). [c] Decline /10 intensity-years. Intensity-years estimated as years of exposure weighted by intensity of exposure (low = 1, high = 4). * Interaction p-value < 0.05:
Interaction p-value < 0.10

Web Table 4. Additional information on pesticide use in Vlagtwedde. Between 1965 and 1990 about 80 to 90% of the agricultural area around Vlagtwedde in the North-Eastern part of the Netherlands was used for cultivation of crops, the majority for potatoes.

		Indication of pesticides/chemicals applied
Crop	**percent**	**1960-1980**
Potatoes	35	Herbicides: Dinitrophenoles (DNOC, Dinoseb) Fungicides: Dithiocarbamates (Maneb), Organotin fungicides (Fentin-acetate)
Cereals	50	Growth regulators: Chlormequat Herbicides: Dinitrophenoles (DNOC, Dinoterp)
Beets/other	15	Herbicides: Fenmedifam, Ethofumesaat, Desmedifam, Chloridazon Fungicides: Dithiocarbamates (Thiram)
		1980-1990
Potatoes	50	Herbicides: Quaternary ammonium herbicides (Diquat, Paraquat) Fungicides: Dithiocarbamates (Maneb), Organotin fungicides (Fentin-acetate)
Cereals	30	Growth regulators: Chlormequat Herbicides: Phenoxy herbicides (MCPA, MCPP), Herbicides: Dinitrophenoles (DNOC, Dinoterp), Herbicides: Urea herbicides (Chlortoluron, Isoproturon) Fungicides: Dithiocarbamates (Thiram), Benzimidazoles (Carbendazim)
Beets/other	20	Herbicides: Fenmedifam, Ethofumesaat, Desmedifam, Chloridazon Fungicides: Dithiocarbamates (Thiram)

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5

Risk factors for chronic mucus hypersecretion in individuals with and without COPD: influence of smoking and job exposure on CMH

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ABSTRACT

Background

Chronic mucus hypersecretion (CMH) is highly prevalent in smokers and associated with an accelerated lung function decline and chronic obstructive pulmonary disease (COPD). Several risk factors contribute to CMH and to COPD. It is, however, unknown if risk factors for CMH are similar in subjects with and without COPD.

Methods

1,479 subjects with and 8,529 without COPD, participating in the general population based LifeLines cohort, completed questionnaires and underwent spirometry. Occupational exposure was assessed using the ALOHA+ job exposure matrix. Analyses were performed using multiple logistic regression models.

Results

In COPD, a significantly higher risk for CMH was associated with higher pack-years smoking (per 10 pack-years) (OR=1.28; 1.12-1.46) and environmental tobacco smoke (ETS) (OR=2.06; 1.33-3.19). In non-COPD; male gender (OR=1.91; 1.51-2.41), higher body mass index (OR=1.04; 1.01-1.06), higher pack-years smoking (OR=1.28; 1.14-1.44), current smoking (OR=1.50; 1.04-2.18), low and high exposure to mineral dust (OR=1.39; 1.04-1.87 and OR=1.60; 1.02-2.52), high exposure to gases & fumes (OR=2.19; 1.49-3.22). Significant interactions were found between COPD and exposure to gases & fumes (p=0.018) and aromatic solvents (p=0.038).

Conclusions

A higher risk for CMH was associated with higher pack-years smoking regardless of COPD status. However, a higher risk for CMH was associated with high occupational exposure to gases & fumes in individuals without COPD only.

INTRODUCTION

The secretion of mucus is a normal response of epithelial cells in order to protect the airways and lung tissue against inhaled pathogens, particles and noxious chemicals. In contrast, chronic mucus hypersecretion (CMH) is abnormal. CMH is a condition of mucus overproduction defined by mucus production for at least 3 months during the last 2 years, when specific causes have been excluded[1]. The prevalence of CMH in the general population varies from 3.5% to 12.7% depending on the study population and the CMH definitions used[2,3]. In the general population, CMH is associated with an increased duration and frequency of respiratory infections, excess decline of the forced expiratory volume in 1 second (FEV_1), and increased hospitalization and mortality rates[2,4-6].

The best studied and most important risk factor for CMH is cigarette smoking[2,7]. Other risk factors for CMH are higher age and male gender[8,9]. Of interest, the presence of respiratory infections in childhood is a risk factor for CMH and also for development of Chronic Obstructive Pulmonary Disease (COPD), as is smoking[7,10]. Next to active smoking there is evidence that exposure to maternal smoking during pregnancy (passive smoking in utero) and environmental tobacco smoke exposure (ETS) in childhood are additional risk factors for the presence of CMH in adulthood[11-15]. Occupational exposures have been mentioned as risk factors for CMH in many general population based studies, and have also been reported as risk factors for COPD in different studies[3,16,17]. In addition, CMH is present in about 30% of COPD patients where it constitutes a risk factor for increased duration and frequency of respiratory infections, hospitalization and mortality and higher risk for exacerbations[18,19].

Above studies show that CMH can be present, both in subjects with and without COPD and some risk factors for COPD overlap with those for CMH, like smoking and bacterial infections. However, not all patients with COPD have CMH and conversely not all individuals with CMH have COPD. We therefore investigated whether risk factors for CMH differ between subjects with and without COPD. To this aim we used data of the LifeLines cohort, a general population based study in the northern part of The Netherlands, and determined risk factors for CMH in subjects with and without COPD taking into account well-known clinical, demographic and environmental factors contributing to CMH (active smoking, exposure to environmental tobacco smoke, and occupational exposures).

METHODS

Study Population and Methods

To investigate risk factors for CMH we included subjects participating in the Dutch LifeLines cohort study. The LifeLines study is a multidisciplinary prospective general population-based study among residents of the three northern provinces of The Netherlands, investigating the origins and the development of chronic diseases and multimorbidity[20]. Subjects

were recruited via general practitioners. In the current study, we included 13,301 Caucasian adults, aged between 18 and 90 years, from the second data release of the LifeLines cohort All participants gave written informed consent, completed questionnaires and underwent a medical examination and standardized spirometry, according to the ERS guidelines[21]. In this population-based study we did not administer a bronchodilator.

The exact question used to define CMH was "do you usually expectorate sputum during day or night in winter on the majority of days ≥ 3 months a year? (yes/no)". Since it is known that the presence of asthma can cause symptoms of CMH, subjects with asthma (ever having asthma confirmed by a physician) were excluded from the analyses (n=953).

Environmental tobacco smoke (ETS) exposure and smoking habits

Exposure to smoke during childhood was determined by the question: "did your mother/father smoke regularly during your childhood? (yes/no)". Furthermore, current ETS exposure was determined by questions about regularly exposure to smoke from others during the last year for at least 1 hour per day (yes/no), and in case of a paid job, whether smoking was present in the workplace (yes/no). Smoking habits were defined as never smoking, ex-smoking and current smoking and the lifetime number of pack-years smoked.

An individual was defined as being a current smoker if he/she answered 'yes' to the question "do you smoke now or have you been smoking in the last month?". A never smoker when answered 'no' to the question "have you ever smoked for as long as a year?", and an ex-smoker answered 'yes' to the question "have you ever smoked for as long as a year?" and 'no' to the question "do you smoke now or have you been smoking in the last month?" and 'yes' to the question "did you currently quit smoking?". Pack-years of smoking were calculated as the number of packs of cigarettes (1 pack = 20 cigarettes) smoked per day times the number of years of smoking.

Occupational exposure

Information on employment status, job title and description of work tasks of the current job (or last held job in case of retirement) was obtained by questionnaire and coded according to the International Standard Classification of Occupations version 1988 (ISCO-88)[22]. Employed and unemployed subjects were included in this study. The ALOHA+ Job Exposure Matrix (JEM) was used to classify the reported jobs into no, low or high exposure to various agents (coded respectively 0, 1 or 2)[16]. If someone had two or more jobs (n = 232, 2.3%), the average occupational exposure was determined by rounding the average to the nearest integer (0.5 = 1 and 1.5 = 2).

Statistical analyses

Analyses were stratified for COPD defined as $FEV_1/FVC < 70\%$. Body mass index (BMI) was defined as weight/height^2 (kg/m^2). Differences in characteristics and occupational exposures between subjects with and without CMH stratified by COPD were analyzed using chi-square tests and 2-tailed unpaired Student's t-tests.

Characteristics significantly associated with CMH (except for the occupational exposures and lung function), were included in a multivariate logistic regression model. Subsequently, each occupational exposure was included in this model one-by-one without taking into account other occupational exposures. The interaction effect between COPD and the other possible risk factors was tested by using a multivariate regression model including COPD x risk factor as an extra variable in the model.

Since the prevalence of exposures to herbicides and insecticides was very low in our population (1.3% vs. 3.5%), we analyzed all pesticides as one variable (prevalence 4.0%). Differential effects of the possible risk factors between subjects with and without COPD were tested in unstratified multivariate models by including the appropriate interaction terms. In an additional analysis, retired and unemployed subjects were excluded (n=1,996) to assess the effect of current occupational exposure only. Finally, analyses were stratified by age, gender, and smoking habits to investigate possible effect modification by these variables. Analyses were conducted using SPSS version 19.0. A two-sided p-value < 0.05 was considered statistically significant.

RESULTS

From the initial LifeLines sample of 13,301 subjects a total of 2,340 was excluded because of incomplete data on CMH (n = 356), lacking information on smoking habits and ETS (n = 1,568) and incomplete data on lung function (n = 416). After exclusion of asthmatics (n = 953) 10,008 subjects (75.8% of all subjects) remained, including 1,479 (14.8%) with and 8,529 without COPD.

Characteristics, ETS and smoking habits related to CMH

Table 1 presents the demographics of subjects with and without CMH, stratified by COPD status. The overall prevalence of CMH was 4.2% and was significantly higher in subjects with COPD (8.7%) than in subjects without COPD (3.4%, $p < 0.001$). In subjects with and without COPD, the prevalence of CMH was significantly higher in males, in ever smokers and current smokers and in subjects with ETS exposure; the number of pack-years smoked was also significantly higher in subjects with CMH. COPD subjects with CMH had significantly worse lung function than those without CMH.

Table 1. Demographic characteristics of subjects with and without chronic mucus hypersecretion, and association of these characteristics with chronic mucus hypersecretion (expressed as p-value), stratified by COPD (n = 10,008).

	Non-COPD n = 8,529 (85.2%)			COPD n = 1,479 (14.8%)		
Variable	**No CMH**	**CMH**	**P**	**No CMH**	**CMH**	**p**
N (%)	**8,236 (96.6)**	**293 (3.4)**		**1,350 (91.3)**	**129 (8.7)**	
Males, n (%)	**1,907 (40.0)**	**164 (56.0)**	**<0.001**	**619 (45.9)**	**73 (56.6)**	**0.020**
Age (years), median (range)	46.4 (18-89)	47.0 (26-79)	0.257	52.3 (26-86)	51.0 (34-86)	0.933
BMI (kg/height2), mean (SD)	**26.2 (4.2)**	**27.0 (4.7)**	**0.001**	26.0 (3.7)	26.3 (4.6)	0.549
Lung function						
FEV_1 (liter), mean (SD)	3.5 (0.8)	3.6 (0.8)	0.142	**2.8 (0.8)**	**2.7 (0.8)**	**0.006**
FEV_1/FVC (%), median (range)	**78.8 (70-100)**	**78.0 (70-96)**	**0.016**	**66.0 (39-70)**	**65.0 (33-70)**	**<0.001**
# FEV_1, % predicted, mean (SD)	105.2 (12.6)	104.0 (12.6)	0.097	**91.7 (14.3)**	**84.6 (17.1)**	**<0.001**
ETS						
By mother during childhood, n (%)	2,820 (34.3)	92 (31.5)	0.316	492 (31.8)	48 (33.8)	0.400
By father during childhood, n (%)	6,288 (76.6)	229 (78.2)	0.545	1,283 (83.4)	124 (87.3)	0.284
*By others, n (%)	**1,756 (21.3)**	**108 (36.9)**	**<0.001**	**323 (23.9)**	**56 (43.4)**	**<0.001**
At work, n (%)	**472 (5.7)**	**36 (12.3)**	**<0.001**	97 (7.2)	15 (11.6)	0.071
Smoking habits						
Never smoking, n (%)	**3,711 (45.1)**	**97 (33.1)**	**<0.001**	**357 (26.4)**	**23 (17.8)**	**<0.001**
Ex-smoking, n (%)	**3,006 (36.5)**	**86 (29.4)**	**0.013**	580 (43.0)	47 (36.4)	0.153
Pack-years, median (range)	7.0 (0.05-7.5)	7.9 (0.1-47.0)	0.525	**10.9 (0.05-100)**	**16.3 (0.05-67.5)**	**0.005**
Current smoking, n (%)	**1,519 (18.4)**	**110 (37.5)**	**<0.001**	**413 (30.6)**	**59 (45.7)**	**0.001**
Pack-years, median (range)	**13.5 (0.25-70.5)**	**21.0 (1.05-84.0)**	**<0.001**	**21.4 (0.45-100)**	**26.3 (2.75-69.0)**	**0.003**

CMH = chronic mucus hypersecretion; BMI = body mass index; ETS = environmental tobacco smoke ; * at least one hour per day during the last year; bold = p-value < 0.05; # Lung function reference equations according to Quanjer et al.[21]

Table 2 and figure 1 present the results of the multivariate logistic regression analysis on the associations between risk factors and CMH, stratified by COPD, and the results of interaction analysis between risk factors and COPD. In subjects with COPD, a higher number of pack-years and current ETS exposure were significantly associated with a higher risk for CMH. In subjects without COPD, next to a higher number of pack-years also male gender, higher BMI and current smoking were associated with a significant higher risk for CMH. None of the investigated interactions between the risk factors and COPD was statistically significant.

Table 2. Interaction analysis between COPD and characteristics, ETS (by others and at work), ex- and current smoking and pack-years and multivariate logistic regression on association between chronic mucus hypersecretion and gender, BMI, ETS (by others and at work), ex- and current smoking and pack-years, stratified by COPD.

Variables	Non-COPD		COPD		Interaction with COPD
	OR (95% CI)	p	OR (95% CI)	p	p
Gender (male)	**1.63 (1.29-2.10)**	**< 0.001**	1.33 (0.91-1.94)	0.142	0.276
BMI	**1.04 (1.01-1.06)**	**0.010**	1.00 (0.96-1.05)	0.860	0.345
ETS					
By others*	1.29 (0.96-1.74)	0.088	**2.06 (1.33-3.19)**	**0.001**	0.475
At work	1.37 (0.91-2.06)	0.128	0.99 (0.53-1.85)	0.975	0.561
Smoking habits					
Ex-smoking	0.80 (0.58-1.12)	0.191	0.78 (0.44-1.40)	0.408	0.853
Current smoking	**1.50 (1.04-2.18)**	**0.032**	0.85 (0.45-1.63)	0.629	0.180
Pack-years per 10	**1.28 (1.14-1.44)**	**< 0.001**	**1.28 (1.12-1.46)**	**< 0.001**	0.362

BMI = body mass index; ETS = environmental tobacco smoke; * at least one hour per day during the last year; bold = p-value < 0.05; Pack-years per 10: the unit in the analysis is 10 pack-years so the OR is the estimate of 10 pack-years; #Interaction between variable and COPD status (non-COPD/COPD).

Occupational exposure and risk for CMH

Table 3 presents the proportion of subjects without, with low or high exposure to occupational agents according to the ALOHA+ JEM, in subjects with and without chronic mucus hypersecretion, stratified by COPD. Almost 45% of the population had some occupational exposure, either low or high. Exposure to gases & fumes was the most frequent occupational exposure (40.1%). An overview of the most prevalent occupations within those exposed is given in table 1 in the supplementary file.

In subjects with COPD, there was no significant difference in occupational exposures between subjects with and without CMH. In contrast, in subjects without COPD, the prevalence of 5 out of the 8 investigated occupational exposures was significantly different between subjects with and without CMH.

Statistically significant interactions were found between COPD and high exposure to gases & fumes and between COPD and low exposure to aromatic solvents (Supplementary Table 2). In the stratified analyses, significant associations were found particularly between low and high exposure to mineral dust and CMH and between high exposure to gases & fumes, chlorinated solvents or heavy metals and CMH (adjusted for gender, BMI, ETS and smoking habits) in subjects without COPD. Figure 2 shows the odds ratios and 95% confidence intervals of occupational risk factors studied with respect to the presence of CMH, stratified by COPD. In subjects with COPD there were no significant associations between occupational exposures and CMH (Supplementary Table 2).

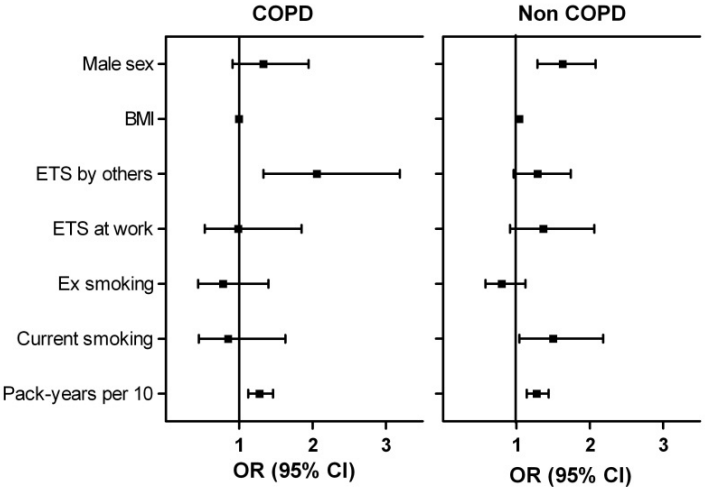

Figure 1. Odds ratios and 95% CI for multivariate analysis showing association between chronic mucus hypersecretion and gender, BMI, ETS and smoking habits, stratified by COPD.

Pack-years per 10: the unit in the analysis is 10 pack-years so the OR is the estimate of 10 pack-years.

Exclusion of retired and unemployed subjects to assess the effect of current occupational exposures did not change the results (results not shown). Stratification by age, gender or smoking habits (never-smoker, ex-smoker and current smoking) did not consistently indicate effect modification by these variables of the associations between occupational exposures and CMH (Supplementary Tables 3, 4 and 5).

DISCUSSION

We report results from a large cross-sectional general population based study, relating demographic characteristics, environmental smoke exposure, smoking habits and occupational exposures to CMH in subjects with and without COPD. Subjects with COPD had a higher prevalence of CMH (defined by expectoration of sputum on most days ≥3 months during the last year) (8.7%) than to those without COPD (3.4%). The risk for CMH in subjects with COPD increased with higher pack-years and ETS exposure only, without any effect of occupational exposures. In contrast, risk factors for CMH

in subjects without COPD were male gender, higher BMI, current smoking, higher pack-years and several occupational exposures. Interestingly, the association between CMH and high occupational exposure to gases & fumes differed significantly between subjects with and without COPD. Although the differences in the associations of the other occupational risk factors with CMH between subjects with and without COPD failed to reach statistical significance, the observed differences in effect sizes may be important.

Table 3. Prevalence of occupational exposures, according to the ALOHA+ JEM, in subjects with and without chronic mucus hypersecretion, stratified by COPD.

		Non-COPD (n = 8,529)			COPD (n = 1,479)		
		No CMH	CMH		No CMH	CMH	
Exposure		n (%)	n (%)	p*	n (%)	n (%)	p*
Biological	No	5,674 (68.9)	196 (66.9)		935 (69.3)	88 (68.2)	
	Low	2,240 (27.2)	84 (28.7)	0.541	359 (26.6)	36 (27.9)	0.747
	High	322 (4.2)	13 (4.4)	0.715	56 (4.1)	5 (3.9)	0.882
Mineral dust	No	6,673 (81.0)	201 (68.6)		1,032 (76.4)	89 (69.0)	
	Low	**1,234 (15.0)**	**66 (22.5)**	**<0.001**	240 (17.8)	31 (24.0)	0.079
	High	**329 (4.0)**	**26 (8.9)**	**<0.001**	78 (5.8)	9 (7.0)	0.580
Gases &	No	5,008 (60.8)	146 (49.8)		775 (57.4)	68 (52.7)	
	Low	2,812 (34.1)	105 (35.8)	0.423	482 (35.7)	48 (37.2)	0.733
	High	**416 (5.1)**	**42 (14.3)**	**<0.001**	93 (6.9)	13 (10.1)	0.180
All pesticides	No	7,992 (96.2)	279 (95.2)		1,285 (95.2)	122 (94.6)	
	Low	251 (3.0)	11 (3.8)	0.607	47 (3.5)	4 (3.1)	0.821
	High	63 (0.8)	3 (1.0)	0.212	18 (1.3)	3 (2.3)	0.363
Aromatic	No	7,559 (91.8)	250 (85.3)		1,212 (89.8)	116 (89.9)	
	Low	**618 (7.5)**	**38 (13.0)**	**0.003**	131 (9.7)	12 (9.3)	0.883
	High	59 (0.7)	5 (1.7)	0.081	7 (0.5)	1 (0.8)	0.704
Chlorinated	No	7,665 (93.1)	250 (85.3)		1,247 (92.4)	118 (91.5)	
	Low	**464 (5.6)**	**27 (9.2)**	**0.013**	78 (5.8)	9 (7.0)	0.580
	High	**107 (1.3)**	**10 (3.4)**	**0.013**	25 (1.9)	2 (1.6)	0.807
Other	No	6,301 (76.8)	221 (75.4)		1,055 (78.1)	103 (79.8)	
	Low	1,788 (21.7)	64 (21.8)	0.729	279 (20.7)	24 (18.6)	0.579
	High	147 (1.8)	8 (2.7)	0.302	16 (1.2)	2 (1.6)	0.718
Heavy metals	No	7,729 (93.8)	258 (88.1)		1,244 (92.1)	115 (89.1)	
	Low	**366 (4.4)**	**19 (6.5)**	**0.046**	70 (5.2)	9 (7.0)	0.387
	High	**141 (1.7)**	**16 (5.5)**	**<0.001**	36 (2.7)	5 (3.9)	0.424

CMH = chronic mucus hypersecretion; bold = p-value < 0.05; *p-value: unadjusted logistic regression, reference is not exposed (to the current investigated agent).

The commonly reported prevalence of CMH in the general population ranges from 3.5% to 12.7%[2,9]. The prevalence of CMH was 4.2% in our study, which is in the lower range of reported prevalences. When asthmatics also were included, the prevalence was 4.8%. The prevalence of CMH in our study was comparable with the prevalence of CMH (defined in the same way), in another general population based cohort from the northern part of The Netherlands (Vlagtwedde), also when stratified for gender, smoking habits or COPD.

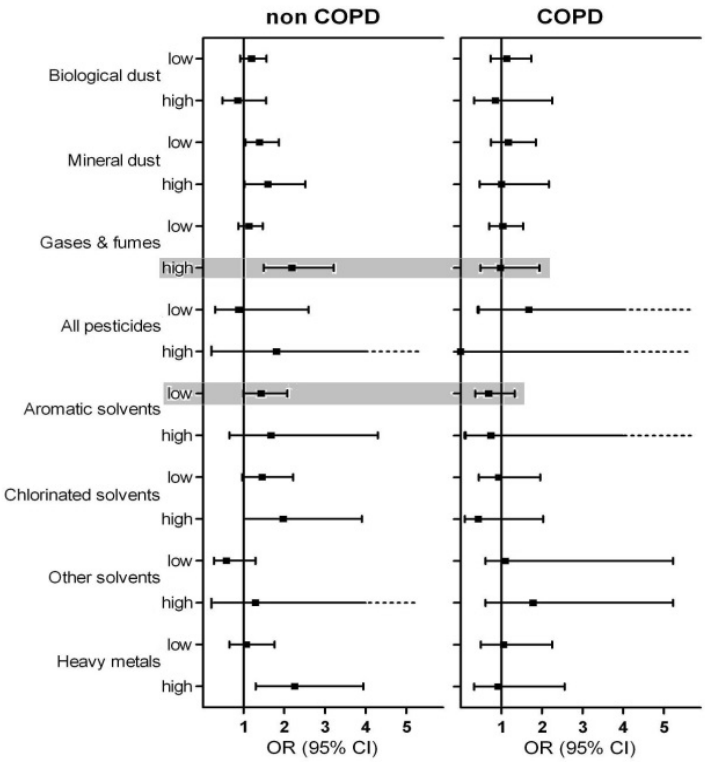

Figure 2. Odds ratios and 95% CI for multivariate analysis showing associations between chronic mucus hypersecretion and occupational exposures, stratified by COPD.

Reference is not exposed; analysis corrected for gender, BMI, ETS, ex- and current smoking and pack-years. Occupational exposures were added one by one. Gray frame: significant interaction ($p < 0.05$) between occupational exposure and COPD.

It has been well established that the presence of CMH increases with the severity of airflow limitation[18,23]. Since our population encompassed subjects with relatively mild COPD according to GOLD the guidelines (80% stage 1, 20% stage 2), the relatively low prevalence of CMH in subjects with COPD of 8.7% is in line with the association of CMH with the lung function level[24].

We had only prebronchodilator lung function available in this population-based study, which may have affected our prevalence of COPD and especially very mild COPD. For this same reason it is also possible that few undiagnosed asthmatics may incorrectly have been included in the COPD group. In a sensitivity analysis we used the lower limit of normal (LLN) to define COPD[25]. The results of this analysis showed that the prevalence of CMH and the directions and magnitudes of the associations remained similar (Supplementary Table 6).

In accordance with many other general population-based studies we found that CMH is significantly more prevalent in males than in females[2,3,17]. A potential reason for this difference is a tendency for women to report more dyspnea and cough, but less phlegm symptoms than men[26].

The association between pack-years smoking and CMH is in accordance with the literature but was rarely examined separately for subjects with and without COPD in the general population[27]. We found this association to be present in both groups. This could mean that the cigarette smoke-induced chronic inflammatory process and its associated remodeling of the airway walls, are the most important risk factors for CMH, thereby reducing the effects of other potential risk factors.

In addition to pack-years, current smoking was significantly associated with an increased CMH-risk in subjects without but not in subjects with COPD. Since some individuals would have quitted for only a short time, this may have affected the results. Even when we excluded individuals who quitted smoking for only a short period (smoking cessation < 1 year, n=31) or added these 31 subjects to the analysis in current smokers with COPD, current smoking was still not a significant risk factor for CMH. It is possible that the extensive and longstanding smoking history in subjects with COPD has resulted in irreversible airway damage which constitutes an overwhelming important contributor to CMH, more so than the current smoking status.

Occupational exposures

The ALOHA+ JEM assigns exposures to gases & fumes as well as exposures to mineral and biological dusts. Exposure to gases & fumes includes exposures to; aromatic, chlorinated and other solvents, to heavy metals and to all pesticides, which were also additionally separately allocated. Exposure to heavy metals contributes also to exposure to mineral dust. In our study occupational exposures like mineral dust, gases & fumes, chlorinated solvents and heavy metals are significantly contributing to CMH in subjects without COPD, but not at all in subjects with COPD.

Supplementary Table 7 shows how this is related to findings in the literature published since 2000, reporting risk factors for CMH including occupational exposures, demographic characteristics and smoking habits in the general population. Of importance we have not found any study in general populations that performed stratified analyses for COPD status combined with detailed information on occupational exposures (JEM-based), and our findings are new in this respect. Given the low numbers of subjects with COPD in the general population, results of the above mentioned studies will be driven primarily by subjects without COPD. This makes the results of these population-based studies comparable to our results in subjects without COPD. However, a considerable variation in the definitions used for CMH or chronic bronchitis (CB), and in definitions for (extent of) occupational exposures complicates comparisons. Comparison of studies is further

complicated by differences in age between populations, differences in habits (exposure in home caused by cooking) belonging to a continent, the registration of exposure (lifetime versus last job, self-reported versus a JEM).

Notwithstanding this, some studies have found an association between CMH and exposure to gases & fumes, and most studies have not found an association between CMH and biological dust, similar to our results. The significant associations between CMH and low or high exposure to mineral dust, and between CMH and high exposure to heavy metals (separately) we found, were not found in other studies.

Since there are differential effects of occupational exposures on CMH in subjects with and without COPD, the question arises whether the pathophysiology of CMH is different as well. This clearly needs further study into differences given the composition, tenacity, viscosity and produced volume of sputum, as well as the type and level of inflammation, the involved genes and epigenetic phenomena. Furthermore, cigarette smoke causes damage from the central to the peripheral airways. This is a slow process which is accompanied by metaplasia of goblet cells and mucus hypersecretion that is located in the larger airways and also in the small airways in a later stage, accompanied by closure of the small airways and subsequently airway obstruction. It remains to be established whether occupational exposures mainly affect the larger airways in subjects without COPD, yet with similar symptoms of CMH as occurring in smoking-related COPD.

The strength of this study is that we had access to a large population, with a very wide age range and a considerable number of subjects with airflow limitation, which allowed us to study risk factors for CMH in subjects with and without COPD separately. A limitation is the lack of information about life-time occupational exposure since we had information about occupational exposures during the current or last job only. Symptomatic subjects might have left jobs with exposures to occupational exposures before (early) retirement. However, an additional analysis in which unemployed and retired subjects were excluded contradicts the possibility of selective avoidance of hazardous occupational exposures; subjects with COPD had a similar or even higher prevalence of occupational exposures in their current job than subjects without (19% had exposure to mineral dust in non-COPD versus 24.4% in COPD, for gases and fumes being 39.9% and 44.9% respectively (results not shown)). Comparison of provided reasons for unemployment in non-COPD and COPD revealed that the mean age in the COPD-group was considerably higher explaining the higher number of subjects who were retired or pre-retired in this group. The percentage of subjects who were incapable to work was comparable in both groups.

We believe that through legislation and awareness of the danger of these exposures, people are nowadays less exposed. We hypothesize that with using current or last held job we rather have under- than over-estimated the association between occupational exposures or ETS and risk for CMH. Clearly, studies including information on lifetime (cumulative) exposure are desirable to confirm the effects found.

Conclusions

We conclude that occupational exposures contribute differentially to CMH in subjects with and without COPD. In subjects with established COPD only the number of pack-years smoked is associated with an increased risk for CMH and occupational exposures do not contribute. In contrast, high occupational exposure to gases & fumes (among which solvents, all pesticides and heavy metals) is an important driver of CMH in subjects without airflow limitation, next to pack-years smoking.

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5

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Most prevalent occupations in subjects exposed.

Exposure		Jobs
Biological dust	Low	Institution- and home-based personal care workers, Domestic and office cleaners, Nursing and midwifery professionals, Nursing associate professionals
	High	Dairy and livestock producers, Carpenters, Freight handlers, Bakers
Mineral dust	Low	Cleaners, Dairy and livestock producers, Machine operators, Heavy truck and lorry drivers
	High	Welders and flame-cutters , Freight handlers, Agricultural and industrial mechanics, Gardeners, (Building) Construction workers, Field crop and vegetable growers
Gases & fumes	Low	Institution- and home-based personal care workers, Cleaners, Nursing and midwifery professionals
	High	Heavy truck and lorry drivers, Motor vehicle mechanics, Welders and flame-cutters, Agricultural and industrial mechanics, Plumbers and pipe fitters, Painters
Aromatic solvents	Low	Carpenters and joiners, Motor vehicle mechanics, Agricultural and industrial mechanics, Life science technicians, Gardeners and horticultural growers, Plumbers and pipe-fitters
	High	Painters, Printing machine operators, Varneshers and related painters
Chlorinated solvents	Low	Hairdressers and beauticians, Plumbers and pipe fitters, Painters, Mechanical engineers, Decorators and commercial designers, Electronics mechanics and servicers
	High	Motor vehicle mechanics, Agricultural and industrial mechanics sheet metal workers
Heavy metals	Low	Plumbers and pipe fitters, Painters, Machine tool operators, Mechanical engineers, Electronics mechanics and servicers, Building construction laborers, Mechanical engineering technicians, Electrical mechanics,
	High	Welders and flame-cutters, Motor vehicle mechanics, Agricultural and industrial mechanics, Sheet metal workers

Supplementary Table 2. Multivariate logistic regression on association between chronic mucus hypersecretion and occupational exposures, stratified by COPD, and p-values for the interaction between the occupational exposures and COPD.

Exposure		Non-COPD			COPD			Interaction with COPD
		OR (95% CI)	p*	p**	OR (95% CI)	p*	p**	p_{int}
Biological dust	Low	1.19 (0.91-1.56)	0.203	0.622	1.14 (0.74-1.74)	0.560	0.827	0.902
	High	0.86 (0.48-1.55)	0.626		0.86 (0.33-2.25)	0.766		0.801
Mineral dust	Low	**1.39 (1.04-1.87)**	**0.028**	**0.007**	1.18 (0.75-1.85)	0.479	0.684	0.420
	High	**1.60 (1.02-2.52)**	**0.041**		1.01 (0.47-2.17)	0.980		0.190
Gases & fumes	Low	1.13 (0.87-1.47)	0.357	0.001	1.04 (0.70-1.54)	0.858	0.959	0.666
	High	**2.19 (1.49-3.22)**	**< 0.001**		0.98 (0.49-1.94)	0.948		**0.018**
All pesticides	Low	0.88 (0.30-2.60)	0.817	0.835	1.68 (0-44-6.44)	0.451	0.720	0.532
	High	1.81 (0.22-14.9)	0.584		0.00 (0.00-)	0.999		0.784
Aromatic solvents	Low	1.43 (0.99-2.07)	0.057	**0.036**	0.69 (0.36-1.33)	0.269	0.285	**0.038**
	High	1.68 (0.65-4.30)	0.282		0.75 (0.09-6.47)	0.794		0.496
Chlorinated solvents	Low	1.46 (0.96-2.22)	0.075	**0.010**	0.94 (0.45-1.96)	0.862	0.356	0.269
	High	**1.98 (1.00-3.91)**	**0.049**		0.44 (0.10-2.03)	0.292		0.066
Other solvents	Low	0.58 (0.27-1.27)	0.172	0.438	1.10 (0.61-5.23)	0.713	0.454	0.464
	High	1.29 (0.26-6.51)	0.757		1.78 (0.61-5.23)	0.295		0.597
Heavy metals	Low	1.07 (0.65-1.76)	0.795	**0.015**	1.06 (0.50-2.25)	0.881	0.956	0.843
	High	**2.26 (1.30-3.94)**	**0.004**		0.92 (0.33-2.56)	0.876		0.093

Reference is not exposed; Analysis corrected for gender, BMI, ETS, ex- and current smoking and pack-years; Occupational exposures were added one-by-one; bold = p-value < 0.05; * p = p-value for separated (low and high) exposure (no exposure = reference); ** p = p-value for linear trend of intensity of exposure; p_{int} = p-value for interaction analysis.

Supplementary Table 3. Age-stratified (<50 and ≥50 years) multivariate logistic regression on association between chronic mucus hypersecretion and gender, BMI, ETS (by others and at work), ex- and current smoking, pack-years, and occupational exposures (added one by one), stratified by COPD.

		< 50 years				≥ 50 years			
		non COPD		COPD		non COPD		COPD	
N		5,621		566		2,908		913	
CMH %		3.3		9.5		7.8		8.2	
		OR (95% CI)	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Gender (male)		**1.54 (1.14-2.08)**	**0.005**	1.16 (0.64-2.12)	0.630	**1.77 (1.18-2.64)**	**0.005**	1.55 (0.93-2.56)	0.091
BMI, kg/m2		1.03 (1.00-1.06)	0.089	1.04 (0.97-1.11)	0.304	1.05 (1.00-1.10)	0.053	0.98 (0.91-1.04)	0.492
Smoking									
ETS by others*		1.21 (0.84-1.75)	0.302	**2.56 (1.30-5.05)**	**0.006**	1.53 (0.92-2.57)	0.103	1.58 (0.85-2.92)	0.148
ETS at work		**1.66 (1.05-2.65)**	**0.031**	0.65 (0.27-1.55)	0.328	0.84 (0.35-1.99)	0.685	1.56 (0.62-3.92)	0.348
Ex-smoking		**0.61 (0.38-0.96)**	**0.033**	0.41 (0.14-1.20)	0.105	1.02 (0.60-1.72)	0.952	0.90 (0.43-1.89)	0.779
Current smoking		1.44 (0.91-2.27)	0.116	0.55 (0.19-1.55)	0.256	1.58 (0.78-3.22)	0.204	0.79 (0.32-1.96)	0.605
Pack-years, per 10**		**1.44 (1.14-1.61)**	**0.001**	**1.66 (1.24-2.21)**	**0.001**	1.19 (1.00-1.42)	0.056	**1.21 (1.03-1.42)**	**0.017**
Occupational exposure									
Biological dust	Low	**1.42 (1.02-1.98)**	**0.040**	1.05 (0.54-2.06)	0.885	0.86 (0.54-1.37)	0.525	1.18 (0.67-2.07)	0.560
	High	0.91 (0.43-1.92)	0.812	0.51 (0.06-4.07)	0.527	0.83 (0.32-2.13)	0.694	1.02 (0.34-3.10)	0.969
Mineral dust	Low	**1.64 (1.14-2.36)**	**0.008**	1.30 (0.65-2.60)	0.453	1.05 (0.63-1.76)	0.839	0.93 (0.50-1.76)	0.834
	High	**2.12 (1.25-3.60)**	**0.006**	0.42 (0.09-2.03)	0.278	0.84 (0.32-2.17)	0.714	1.31 (0.54-3.17)	0.548
Gases & fumes	Low	**1.43 (1.03-1.98)**	**0.032**	1.11 (0.58-2.12)	0.746	0.75 (0.48-1.17)	0.207	0.94 (0.56-1.57)	0.809
	High	**2.54 (1.57-4.11)**	**<0.001**	1.98 (0.74-5.34)	0.175	1.83 (0.94-3.55)	0.073	0.55 (0.20-1.53)	0.252
All pesticides	Low	1.05 (0.48-2.32)	0.902	0.00 (0.00-)	0.998	0.96 (0.34-2.71)	0.942	1.36 (0.45-4.12)	0.583
	High	2.33 (0.68-7.96)	0.176	0.00 (0.00-)	0.999	0.00 (0.00-)	0.998	2.13 (0.58-7.78)	0.255
Aromatic solvents	Low	1.25 (0.78-2.00)	0.355	0.62 (0.22-1.79)	0.378	1.85 (1.02-3.37)	0.043	0.66 (0.28-1.54)	0.341
	High	1.42 (0.42-4.80)	0.569	0.70 (0.06-8.61)	0.784	2.40 (0.54-10.79)	0.252	0.00 (0.00-)	0.999
Chlorinated solvents	Low	1.26 (0.74-2.15)	0.400	0.93 (0.29-3.01)	0.905	**2.01 (1.03-3.94)**	**0.040**	0.87 (0.33-2.33)	0.785
	High	1.85 (0.81-4.18)	0.142	0.76 (0.15-3.93)	0.740	2.55 (0.75-8.74)	0.136	0.00 (0.00-)	0.999
Other solvents	Low	1.00 (0.70-1.44)	0.981	0.89 (0.44-1.82)	0.755	1.15 (0.71-1.86)	0.557	0.77 (0.40-1.48)	0.437
	High	1.25 (0.49-3.16)	0.635	0.56 (0.06-5.70)	0.626	2.13 (0.63-7.17)	0.221	1.13 (0.13-9.54)	0.909
Heavy metals	Low	0.94 (0.50-1.76)	0.848	0.90 (0.27-3.02)	0.871	1.33 (0.59-3.03)	0.491	1.01 (0.37-2.73)	0.981
	High	**2.19 (1.11-4.32)**	**0.023**	1.33 (0.37-4.83)	0.661	2.61 (0.97-7.01)	0.057	0.37 (0.05-2.94)	0.348

BMI = body mass index; ETS = environmental tobacco smoke; bold = p-value < 0.05; * at least one hour per day during the last year; ** Pack-years, per 10: the unit in the analysis is 10 pack-years so the OR is the estimate of 10 pack-years.

Supplementary Table 4. Gender stratified multivariate logistic regression on association between chronic mucus hypersecretion and BMI, ETS (by others and at work), ex- and current smoking, pack-years, and occupational exposures (added one by one), stratified by COPD.

		Males				Females			
		non COPD		COPD		non COPD		COPD	
N		3,294		692		5,071		787	
CMH %		4.7		10.5		2.5		7.1	
		OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
BMI, kg/m^2		1.04 (0.99-1.08)	0.101	1.00 (0.93-1.07)	0.897	**1.04 (1.00-1.07)**	**0.038**	1.02 (0.95-1.09)	0.592
Smoking									
ETS by others*		1.35 (0.91-2.01)	0.134	1.43 (0.78-2.60)	0.246	1.20 (0.76-1.88	0.432	3.09 (1.60-6.00)	0.001
ETS at work		1.51 (0.93-2.47)	0.099	1.08 (0.48-2.45)	0.854	1.12 (0.53-2.38)	0.762	1.08 (0.40-2.89)	0.879
Ex-smoking		0.82 (0.52-1.29)	0.396	1.00 (0.46-2.17)	0.998	0.76 (0.46-1.26)	0.287	0.46 (0.18-1.16)	0.099
Current smoking		1.25 (0.76-2.06)	0.388	0.95 (0.39-2.29)	0.906	1.84 (1.05-3.24)	**0.033**	0.58 (0.21-1.60)	0.290
Pack-years, per 10**		**1.25 (1.08-1.45)**	**0.003**	**1.18 (1.00-1.38)**	**0.049**	**1.35 (1.10-1.65)**	**0.004**	**1.56 (1.22-2.00)**	**<0.001**
Occupational exposure									
Biological dust	Low	**1.49 (1.01-2.19)**	**0.045**	0.55 (0.26-1.20)	0.135	0.99 (0.69-1.44)	0.970	1.74 (0.98-3.08)	0.057
	High	0.88 (0.46-1.66)	0.686	0.85 (0.32-2.23)	0.736	1.02 (0.24-4.28)	0.976	0.00 (0.00-)	0.999
Mineral dust	Low	1.32 (0.90-1.92)	0.155	1.08 (0.60-1.94)	0.794	1.50 (0.94-2.38)	0.087	1.35 (0.66-2.77)	0.417
	High	1.57 (0.97-2.54)	0.069	0.92 (0.41-2.08)	0.850	1.54 (0.36-6.62)	0.563	2.84 (0.32-25.01)	0.347
Gases & fumes	Low	1.09 (0.75-1.59)	0.639	0.58 (0.32-1.04)	0.068	1.17 (0.81-1.68)	0.405	**1.84 (1.03-3.28)**	**0.041**
	High	**2.25 (1.48-3.42)**	**<0.001**	0.85 (0.41-1.75)	0.657	1.38 (0.32-5.95)	0.667	1.88 (0.21-16.43)	0.570
All pesticides	Low	0.89 (0.43-1.86)	0.757	0.95 (0.32-2.78)	0.924	1.69 (0.52-5.52)	0.383	0.00 (0.00-)	0.999
	High	1.31 (0.40-4.31)	0.652	1.75 (0.48-6.31)	0.394	0.00 (0.00-)	0.394	0.00 (0.00-)	0.999
Aromatic solvents	Low	1.50 (1.02-2.23)	0.042	0.74 (0.38-1.47)	0.396	0.91 (0.28-2.96)	0.880	0.37 (0.04-3.401)	0.383
	High	1.85 (0.72-4.76)	0.204	1.04 (0.12-9.04)	0.970	0.00 (0.00-)	0.999	0.00 (0.00-)	1.000
Chlorinated solvents	Low	1.55 (0.96-2.50)	0.076	1.03 (0.44-2.38)	0.952	1.25 (0.54-2.93)	0.602	0.62 (0.13-3.01)	0.551
	High	**1.99 (1.00-3.94)**	**0.048**	0.60 (0.13-2.72)	0.511	0.00 (0.00-)	0.999	0.00 (0.00-)	0.999
Other solvents	Low	1.38 (0.95-2.00)	0.094	0.65 (0.33-1.28)	0.210	0.75 (0.47-1.17)	0.205	1.07 (0.54-2.11)	0.854
	High	1.86 (0.72-4.81)	0.199	0.90 (0.11-0.759)	0.920	1.12 (0.34-3.61)	0.855	1.10 (0.13-9.45)	0.928
Heavy metals	Low	1.03 (0.61-1.74)	0.919	1.09 (0.50-2.41)	0.825	1.35 (0.31-5.94)	0.691	0.57 (0.05-5.99)	0.638
	High	**2.24 (1.28-3.93)**	**0.005**	1.11 (0.40-3.03)	0.843	0.00 (0.00-)	0.999	0.00 (0.00-)	0.999

BMI = body mass index; ETS = environmental tobacco smoke; bold = p-value < 0.05; * at least one hour per day during the last year; ** Pack-years, per 10: the unit in the analysis is 10 pack-years so the OR is the estimate of 10 pack-years.

Supplementary Table 5. Smoking habits stratified (never, ex and current smokers) multivariate logistic regression on association between chronic mucus hypersecretion and gender, BMI, ETS (by others and at work), pack-years, and occupational exposures (added one by one), stratified by COPD.

		Never smokers				Ex-smokers				Current smokers			
		non COPD		COPD		non COPD		COPD		non COPD		COPD	
N		3,808		380		3,092		627		1,629		472	
CMH %		2.5		6.1		2.8		7.5		6.8		12.5	
		OR (95% CI)	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Gender(male)		**1.90 (1.26-2.87)**	**0.002**	1.68 (0.72-3.95)	0.231	**2.03 (1.05-3.89)**	**0.034**	1.18 (0.79-1.77)	0.414	0.85 (0.57-1.27)	0.424	0.85 (0.47-1.52)	0.577
BMI, kg/m^2		1.04 (1.00-1.09)	0.079	0.93 (0.81-1.06)	0.276	1.03 (0.98-1.09)	0.211	1.00 (0.92-1.08)	0.992	1.04 (0.99-1.08)	0.117	1.04 (0.97-1.12)	0.268
Smoking													
ETS by others*		1.43 (0.81-2.53)	0.223	1.37 (0.36-5.26)	0.648	**1.71 (0.94-3.12)**	**0.07**	1.37 (0.57-3.33)	0.483	0.99 (0.65-1.50)	0.962	**2.77 (1.49-5.15)**	**0.001**
ETS at work		1.41 (0.60-3.27)	0.430	1.68 (0.27-10.45)	0.579	1.37 (0.56-3.33)	0.488	1.37 (0.32-5.85)	0.668	1.26 (0.72-2.19)	0.422	1.02 (0.47-2.22)	0.956
Packyears, per 10**						0.94 (0.75-1.20)	0.633	**1.23 (1.02-1.49)**	**0.027**	**1.54 (1.33-1.80)**	**<0.001**	**1.31 (1.08-1.60)**	**0.007**
Occupational exposure													
Biological dust	Low	1.08 (0.67-1.74)	0.752	2.11 (0.83-5.35)	0.116	1.01 (0.60-1.68)	0.981	1.24 (0.62-2.49)	0.547	1.39 (0.90-2.15)	0.139	0.78 (0.40-1.52)	0.468
	High	0.55 (0.17-1.76)	0.311	0.00 (0.00-)	0.999	1.04 (0.37-2.96)	0.937	1.47 (0.41-5.32)	0.557	1.02 (0.40-2.56)	0.972	0.78 (0.17-3.61)	0.747
Mineral dust	Low	1.65 (1.00-2.74)	0.051	1.16 (0.37-3.65)	0.799	1.04 (0.58-1.86)	0.895	0.90 (0.41-1.98)	0.796	1.39 (0.87-2.23)	0.174	1.45 (0.76-2.76)	0.262
	High	2.00 (0.94-4.28)	0.074	0.00 (0.00-)	0.999	0.99 (0.37-2.59)	0.977	0.82 (0.26-2.58)	0.736	1.85 (0.91-3.76)	0.088	1.82 (0.60-5.56)	0.290
Gases & fumes	Low	1.02 (0.64-1.62)	0.942	1.31 (0.53-3.25)	0.557	0.86 (0.53-1.40)	0.547	0.84 (0.44-1.60)	0.598	1.48 (0.95-2.28)	0.080	1.07 (0.58-1.97)	0.825
	High	**3.19 (1.69-6.02)**	**<0.001**	0.95 (0.10-8.67)	0.965	1.31 (0.60-2.83)	0.497	0.54 (0.15-1.97)	0.350	**2.41 (1.26-4.59)**	**0.008**	1.51 (0.60-3.81)	0.380
All pesticides	Low	1.20 (0.47-3.03)	0.703	0.00 (0.00-)	0.999	0.82 (0.20-3.43)	0.785	0.50 (0.06-3.86)	0.503	0.88 (0.30-2.60)	0.817	1.68 (0.44-6.43)	0.451
	High	0.86 (0.12-6.44)	0.887	0.00 (0.00-)	0.999	1.27 (0.17-9.65)	0.819	2.53 (0.65-9.82)	0.179	1.81 (0.22-14.98)	0.584	0.00 (0.00-)	0.999
Aromatic solvents	Low	1.47 (0.79-2.75)	0.224	1.23 (0.26-5.85)	0.796	1.23 (0.61-2.50)	0.560	0.96 (0.40-2.34)	0.936	1.54 (0.83-2.84)	0.171	0.31 (0.09-1.11)	0.073
	High	3.38 (0.75-15.21)	0.113	0.00 (0.00-)	1.000	2.09 (0.46-9.44)	0.338	0.00 (0.00-)	0.999	0.67 (0.09-5.15)	0.697	1.08 (0.10-11.68)	0.947
Chlorinated solvents	Low	1.23 (0.56-2.72)	0.608	1.30 (0.16-10.79)	0.807	1.00 (0.43-2.37)	0.993	1.11 (0.36-3.43)	0.850	**2.03 (1.09-3.77)**	**0.025**	0.75 (0.25-2.28)	0.612
	High	**4.40 (1.74-11.14)**	**0.002**	4.00 (0.36-44.77)	0.260	0.89 (0.21-3.83)	0.876	0.00 (0.00-)	0.999	1.29 (0.29-5.71)	0.733	0.25 (0.03-2.55)	0.244
Other solvents	Low	1.09 (0.67-1.77)	0.731	1.95 (0.73-5.23)	0.186	0.95 (0.55-1.62)	0.845	0.79 (0.37-1.69)	0.543	1.09 (0.68-1.77)	0.713	0.58 (0.27-1.27)	0.172
	High	1.38 (0.33-5.82)	0.658	0.00 (0.00-)	0.999	1.09 (0.25-4.65)	0.911	0.00 (0.00-)	0.999	1.78 (0.60-5.23)	0.295	1.29 (0.26-6.51)	0.757
Heavy metals	Low	0.93 (0.36-2.38)	0.874	1.17 (0.13-10.41)	0.887	0.76 (0.27-2.15)	0.599	1.13 (0.36-3.51)	0.831	1.40 (0.68-2.89)	0.361	1.04 (0.33-3.26)	0.950
	High	**3.41 (1.44-8.06)**	**0.005**	2.03 (0.21-19.62)	0.540	1.48 (0.50-4.36)	0.473	0.61 (0.08-5.00)	0.648	2.02 (0.74-5.52)	0.170	0.88 (0.21-3.63)	0.855

BMI = body mass index; ETS = environmental tobacco smoke; bold = p-value < 0.05; * at least one hour per day during the last year; ** Pack-years, per 10: the unit in the analysis is 10 pack-years so the OR is the estimate of 10 pack-years.

Supplementary Table 6. Multivariate logistic regression on the association between chronic mucus hypersecretion and gender, BMI, ETS (by others and at work), ex- and current smoking, pack-years, and occupational exposures (added one by one), stratified by COPD based on the lower limit of normal (LLN).

		Non COPD based on LLN		COPD based on LLN	
N		9,060		948	
CMH %		3.7		9.3	
		OR (95% CI)	**p**	**OR (95% CI)**	**p**
Gender (male)		**1.63 (1.29-2.08)**	**< 0.001**	1.33 (0.91-1.94)	0.142
BMI, kg/m2		**1.04 (1.01-1.06)**	**0.010**	1.00 (0.96-1.05)	0.860
Smoking					
ETS, by others		1.29 (0.96-1.74)	0.088	**2.06 (1.33-3.19)**	**0.001**
ETS, at work		1.37 (0.91-2.06)	0.128	0.99 (0.53-1.85)	0.975
Ex-smoking		0.80 (0.57-1.12)	0.191	0.78 (0.44-1.40)	0.408
Current smoking		1.50 (1.04-2.18)	0.032	0.85 (0.44-1.63)	0.629
Pack-years per 10**		**1.28 (1.14-1.44)**	**< 0.001**	**1.28 (1.12-1.46)**	**<0.001**
Occupational exposure					
Biological dust	Low	1.18 (0.90-1.55)	0.219	1.14 (0.74-1.74)	0.555
	High	0.87 (0.48-1.56)	0.629	0.87 (0.33-2.26)	0.770
Mineral dust	Low	**1.39 (1.04-1.87)**	**0.028**	1.18 (0.75-1.85)	0.479
	High	**1.60 (1.02-2.52)**	**0.041**	1.01 (0.47-2.17)	0.980
Gases & fumes	Low	1.13 (0.87-1.47)	0.357	1.04 (0.70-1.54)	0.858
	High	**2.19 (1.49-3.22)**	**< 0.001**	0.98 (0.49-1.94)	0.948
All pesticides	Low	1.02 (0.54-1.91)	0.953	0.80 (0.28-2.30)	0.677
	High	1.15 (0.36-3.74)	0.812	1.64 (0.46-5.83)	0.446
Aromatic solvents	Low	1.43 (0.99-2.07)	0.057	0.69 (0.36-1.33)	0.269
	High	1.68 (0.65-4.30)	0.282	0.75 (0.09-6.47)	0.794
Chlorinated solvents	Low	1.46 (0.96-2.21)	0.075	0.94 (0.45-1.96)	0.862
	High	**1.98 (1.00-3.91)**	**0.049**	0.44 (0.09-2.03)	0.292
Other solvents	Low	1.05 (0.78-1.39)	0.757	0.82 (0.51-1.32)	0.414
	High	1.46 (0.70-3.04)	0.313	0.91 (0.20-4.15)	0.904
Heavy Metals	Low	1.07 (0.65-1.76)	0.795	1.06 (0.50-2.25)	0.881
	High	**2.26 (1.29-3.94)**	**0.004**	0.92 (0.33-2.56)	0.876

LLN = lower limit of normal; BMI = body mass index; ETS = environmental tobacco smoke; bold = p-value < 0.05; * at least one hour per day during the last year; ** Pack-years, per 10: the unit in the analysis is 10 pack-years so the OR is the estimate of 10 pack-years.

Supplementary Table 7. Studies on occupational risk for CMH or CB in the general population, published since 2000.

Study	N	Age (year)	Male (%)	Exposure	Definition	Exposed to	Exposure effect compared to		CMH OR (95% CI)	CB OR (95% CI)	Adjusted for
Suadicani, 2001, Denmark[1]	3,331	53-75	100	Self reported	CB: cough & phlegm ≥3 months/year ≥2 successive yrs	Dust	≥ 5 years exp/ ≤ 5 years exp			1.5 (1.1-2.1)	smoking, age, alcohol, blood pressure, MNS phenotype
						Organic solvents	≥ 5 years exp/ ≤ 5 years exp			1.5 (1.1-2.1)	
Lange, 2003, Denmark[2]	3.736	≥ 65	40	Self reported, ever exposed	CMH: phlegm ≥3 months/year ≥2 successive years	Industrial dust and fumes	Exp/non exp		2.2 (1.7-2.7)		smoking, gender, childhood resp. infections, alcohol.
De Meer, 2004, Netherlands[3]	1,906	45-70	52	JEM	CB: morning phlegm or cough ≥3 months , ≥1 yr	Organic dust	Exp/non exp			0.89 (0.56-1.42)	living area, age, gender, smoking, working years
						Mineral dust				2.22 (1.16-4.23)	
						Gases/Fumes				0.67 (0.36-1.26)	
Sunyer, 2005, Spain[4]	1,735	20-44	48	JEM (Current job/ Job quitted because of respiratory symptoms)	CMH: phlegm ≥3 months, last year	Biological dust	Low/non exp		1.1 (0.7-1.5)		age, gender, centre, other exposure, smoking, (asthma excluded)
							High/non exp		2.0 (1.1-3.8)		
						Mineral dust	Low/non exp		1.1 (0.6-1.8)		
							High/non exp		0.9 (0.6-1.8)		
						Gases/Fumes	Low/non exp		1.0(0.7-1.4)		
							High/non exp		1.5 (0.9-2.5)		
Matheson, 2005, Australia[5]	1,213	45-70	52	ALOHA JEM (Lifetime)	CMH: phlegm ≥3 months/year ≥2 successive yrs *CMH + COPD: phlegm ≥3 months/year ≥2 successive years + $FEV_1/FVC<0.70$	Biological dust	Exp/non exp		1.74 (0.97-2.06)		gender, age, smoking
						Mineral dust	Exp/non exp		1.32 (0.71-2.44)		
						Gases/Fumes	Exp/non exp		1.31 (0.72-2.40)		
						Biological dust	Exp/non exp		3.19 (1.27-7.97)		
							Exp/non exp	Men	2.02 (0.17-2.08)		
							Exp/non exp	Women	5.83 (1.24-27.4)		
						Mineral dust	Exp/non exp		1.40 (0.56-3.51)		
							Exp/non exp	Men	0.59 (0.17-2.08)		
							Exp/non exp	Women	3.60 (1.06-12.3)		
						Gases/Fumes	Exp/non exp		2.81 (1.01-7.79)		
							Exp/non exp	Men	1.62 (0.42-6.26)		
							Exp/non exp	Women	4.85 (1.03-22.9)		

Continuation Supplementary Table 7. Studies on occupational risk for CMH or CB in the general population, published since 2000.

Jaen, 2006, Spain[6]	576	20-70	49	Jobs self reported exposure >1 year (Lifetime)	CMH: phlegm ≥3 months/year ≥2 successive years	Dust, gases or fumes	Exp/non exp		2.0 (1.1-3.7)		gender, age, smoking
							Exp/non exp	Never smok	3.7 (0.9-15)		
							Exp/non exp	Ex-smok	1.9 (0.5-7.2)		
							Exp/non exp	Current smok	1.6 (0.7-3.6)		
Zock, 2006, Netherlands[7]	13,253	20-44	48	JEM (Current job or quitted because of respiratory symptoms)	CMH: phlegm ≥3 months/ last year CB: cough + phlegm ≥3 months/last year	VGDF	Low/non exp	Never smok	1.0 (0.7-1.4)	0.8 (0.4-1.5)	age, gender, centre, height, current nr. of cigarettes (asthma excluded)
							High/non exp		1.4 (0.9-2.1)	1.0 (0.4-2.1)	
							Low/non exp	Ex-smok	1.1 (0.6-1.8)	0.7 (0.3-1.8)	
							High/non exp		1.1 (0.6-2.2)	1.0 (0.4-2.9)	
							Low/non exp	Current smok	1.2 (1.0-1.5)	1.3 (0.9-1.8)	
							High/non exp		1.6 (1.2-2.1)	1.7 (1.2-2.4)	
LeVan, 2006, Singapore[8]	52,325	45-74	43	Industry or occupation > 1 year been working	CMH: phlegm ≥3 months/year ≥2 successive yrs	Dust from mineral, cotton, wood, metal or asbestos	Exp/non exp		1.08 (0.95-1.22)	1.26 (1.01-1.57)	
						Vapors from chemical solvents	Exp/non exp		1.03 (0.89-1.18)	0.93 (0.71-1.22)	
						Mineral dust	Exp/non exp		2.22 (1.16-4.23)		
							Exp/non exp	Current smok		3.97 (1.08-14.5)	
						Gases/Fumes	Exp/non exp		0.67 (0.36-1.26)		
Krstev, 2008, China[9]	14,500	40-70	0	Every job > 1year, categorized	CB: ever diagnosed with bronchitis by a physician	#Textile/sewing	Ever/never employed		1.11 (1.03-1.19)		
						Postal/ communication			1.57 (1.14-2.17)		
						Education/culture			1.14 (1.05-1.25)		
Skorge, 2009, Norway[10]	2,312	15-65	49	ALOHA JEM (last 11 yrs)	CMH: phlegm when coughing	Biological dust	Low/non exp	Men	1.37 (0.9-2.1)		
							High/non exp		0.89 (0.4-2.0)		
							Low/non exp	Woman	0.87 (0.6-1.3)		
							High/non exp		1.57 (0.6-4.2)		
						Mineral dust	Low/non exp	Men	0.64 (0.4-1.0)		
							High/non exp		0.55 (0.3-1.0)		
							Low/non exp	Woman	0.53 (0.3-0.9)		
							High/non exp		1.68 (0.7-4.1)		
						Gases & fumes	Low/non exp	Men	1.01 (0.6-1.6)		
							High/non exp		0.67 (0.4-1.1)		
							Low/non exp	Woman	1.42 (1.0-2.1)		
							High/non exp		2.03 (0.9-4.8)		

CMH = chronic mucus hypersecretion; CB = chronic bronchitis; JEM = job exposure matrix; VGDF = vapors, gases, dusts and fumes

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6

GST-omega genes interact with environmental tobacco smoke on adult level of lung function

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ABSTRACT

Background

Lung growth *in utero* and lung function loss during adulthood can be affected by exposure to environmental tobacco smoke (ETS). The underlying mechanisms have not been fully elucidated. Both ETS exposure and single nucleotide polymorphisms (SNPs) in *Glutathione S-Transferase* (*GST*) *Omega* genes have been associated with the level of lung function. This study aimed to assess if *GSTO* SNPs interact with ETS exposure *in utero* and during adulthood on the level of lung function during adulthood.

Methods

We used cross-sectional data of 8,128 genotyped participants from the LifeLines cohort study. Linear regression models (adjusted for age, sex, height, weight, current smoking, ex-smoking and packyears smoked) were used to analyze the associations between *in utero*, daily and workplace ETS exposure, *GSTO* SNPs, the interaction between ETS and *GSTOs*, and level of lung function (FEV_1, FEV_1/FVC). Since the interactions between ETS and *GSTOs* may be modified by active tobacco smoking we additionally assessed associations in never and ever smokers separately. A second sample of 5,308 genotyped LifeLines participants was used to verify our initial findings.

Results

Daily and workplace ETS exposure was associated with significantly lower FEV_1 levels. *GSTO* SNPs (recessive model) interacted with *in utero* ETS and were associated with higher levels of FEV_1, whereas the interactions with daily and workplace ETS exposure were associated with lower levels of FEV_1, effects being more pronounced in never smokers. The interaction of *GSTO2* SNP rs156697 with *in utero* ETS associated with a higher level of FEV_1 was significantly replicated in the second sample. Overall, the directions of the interactions of *in utero* and workplace ETS exposure with the SNPs found in the second (verification) sample were in line with the first sample.

Conclusions

GSTO genotypes interact with *in utero* and adulthood ETS exposure on adult lung function level, but in opposite directions.

BACKGROUND

Lung function loss is common in chronic respiratory diseases like chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and interstitial lung fibrosis, and associates with all-cause and other specific mortality[1,2]. Both environmental and genetic factors contribute to lung function loss. Active cigarette smoking is regarded as the most important environmental risk factor, yet other factors exist. Like active smoking, passive cigarette smoking or environmental tobacco smoke (ETS) exposure induces inflammation and oxidative stress in the lungs[3]. ETS exposure has been associated with reduced level of lung function at birth[4,5] and in adulthood[6,7], as well as with respiratory symptoms[8,9] and increased COPD risk[10,11]. In other words, ETS exposure can affect *in utero* lung development, lung growth during childhood and lung function loss during adulthood. However, the underlying mechanisms have not been elucidated. Furthermore, these underlying mechanism are not necessarily similar for ETS exposure *in utero* and during adulthood given the fact that both the mode of exposure and the period of exposure within the life-span are different.

Although it has been very well established that genetic factors contribute to lung function level[12], less is known about how genetic factors modify effects of ETS exposure on the level of lung function during the life-span. Glutathione S-Transferases (GSTs) are a family of enzymes involved in the detoxification of xenobiotic substances such as tobacco smoke, and play an essential role in oxidative stress reactions[13,14]. Polymorphisms in the *GST-mu*, *-pi*, and *-theta* genes have been described to interact with tobacco smoke exposure with respect to asthma development and atopy in asthmatic children[15,16] and lower childhood level of lung function[17]. The *GST-omega* (*GSTO*) class has been less well studied. Of interest, GSTO enzymes have thioltransferase activity and can catalyze specific reduction reactions with compounds that are not substrates for other GSTs, suggesting an important role for GSTO in oxidative stress reactions[18,19] and in biotransformation of inorganic arsenic[20], a component present in tobacco smoke. GSTO1 has also been reported to activate IL-1β[21], a cytokine that is important for tobacco smoke induced inflammation and fibrosis[22,23]. Harju et al. showed that GSTO1 is abundantly expressed in alveolar macrophages[24]. Furthermore, a genome wide association analysis in the Framingham Heart Study found a *GSTO2* SNP (rs156697) to be associated with both lower level of FEV_1 and FVC[12]. Another study could not replicate this association between rs156697 and FEV_1, but found an association with COPD, defined by lower lung function[25]. It is unknown whether *GSTO1* and *GSTO2* SNPs modify effects of ETS exposure on the level of lung function.

This study aimed to assess if *GSTO* SNPs interact with *in utero* and/or adulthood ETS exposure on lung function level in a general population.

METHODS

Study sample and measurements

We included 8,128 genetically unrelated individuals from the LifeLines cohort study. The LifeLines cohort is designed to investigate universal risk factors and their modifiers for multifactorial chronic diseases and comorbidities[26]. All subjects received a questionnaire and underwent a medical examination including collection of a blood sample for DNA extraction. The questionnaire included questions regarding personal characteristics, smoking habits and ETS exposure. We used self-reported *in utero* ETS exposure (coded as: no/yes/do not know), daily ETS exposure based on self-reported hours of exposure to other person's tobacco smoke per day (coded as: <1/≥1 hour per day), and ETS exposure at work (answer categories: no/yes/not applicable). The medical examination included spirometry (FEV_1 and FEV_1/FVC) performed in a standardized setting following ATS guidelines using a Welch Allyn Version 1.6.0.489, PC-based SpiroPerfect with CardioPerfect Workstation software. A second sample, including 5,308 individuals from the LifeLines cohort study genotyped at a later stage, was used to verify our initial findings. Questionnaires, medical examinations and genotyping at baseline were performed according to the same standardized protocol in sample 1 and sample 2.

Genotyping

Genotyping was performed using IlluminaCytoSNP-12 arrays. Beagle (version 3.3) and the HapMap3-database were used to impute additional SNPs. Three Haplotype-tagging SNPs in the *GSTO1-2* cluster with minor allele frequency (MAF) > 0.1, HW-equilibrium p-value > 0.05, and R^2 < 0.8 were selected with Haploview (version 4.2). We additionally included SNP rs156697 that was associated with lower FEV_1 and FVC in the Framingham Heart Study[12]. The four selected SNPs were rs4925, rs1147611, rs156697 and rs156699. LD-plot (supplementary figure 1) and genotype frequencies (supplementary table 1) are presented in the online supplement.

Statistical analysis

Linear regression models adjusted for age, sex, height, weight, current smoking, ex-smoking, and packyears smoked, were used to analyze the associations between ETS exposure, *GSTO* SNPs, the interaction between ETS and *GSTOs* and level of lung function (FEV_1, FEV_1/FVC). Since the interactions between ETS and *GSTOs* may be modified by active tobacco smoking we additionally assessed associations in never and ever smokers separately. All analyses were performed using SPSS version 20.0 (IBM Corporation, USA). P-values < 0.05 (tested 2-sided) were considered statistically significant. To examine the robustness of our findings we used False Discovery Rate (FDR) correction for multiple testing[27], taking into account the number of tests performed for each of the exposures (4 SNPs * 3 separate analyses (all/never/ever) * 2 outcomes (FEV_1, FEV_1/FVC)).

Ethical approval

The study was approved by the Medical Ethics Committee of the University Medical Center Groningen, Groningen, The Netherlands (ref. METc 2007/152).

RESULTS

Population characteristics

Characteristics of both samples 1 and 2 are shown in table 1. Briefly, both samples included more females than males and more ever than never smokers. 15% of the participants did not know whether their mother smoked during pregnancy, and this group was excluded in the analyses on the effect of *in utero* exposure on the level of lung function. Of the remaining participants, 13% reported *in utero* ETS exposure. Almost 25% of the participants reported daily ETS exposure (≥1 hours), and 7% reported ETS exposure at the workplace (in 12% of the participants this was not applicable because of unemployment; this group was coded as a separate category in the analyses). The median level of self-reported exposure was 2 hours per day within the group with daily ETS exposure (25th percentile = 1 hour, 75th percentile = 4 hours).

Table 1. Characteristics participants included in sample 1 and sample 2.

	Sample 1	Sample 2 (verification)
n	8128	5308
Males, n (%)	3483 (43)	2133 (40)
Age, median (min-max)	47 (18–89)	48 (21–90)
Smoking status, n (%)		
Never, n (%)	3277 (40)	2154 (41)
Ex, n (%) [median packyears (min-max)]	2882 (36) [8 (0–86)]	2014 (39) [7 (0–100)]
Current, n (%) [median packyears (min-max)]	1936 (24) [15 (0–100)]	1065 (20) [16 (0–81)]
ETS exposure, n (%)		
In utero	867 (13)	559 (13)
≥ 1 hour/day	1788 (24)	1029 (21)
At the workplace	565 (7)	303 (6)
Lung function, mean (sd)		
FEV_1 (ml)	3412 (831)	3331 (840)
FEV_1pp (%)[1]	102 (14)	102 (14)
FEV_1/FVC (%)	77 (7)	76 (7)
Spirometry available, n	7635	5070

The second sample (sample 2) was used to verify the initial findings from sample 1, both samples are part of the LifeLines population-based cohort study. [1] FEV_1pp = FEV_1 as percentage predicted based on reference equations constructed by Quanjer et al[33].

ETS exposure and level of lung function

Complete data on all covariates was available for n = 6003, 6822 and 7149 subjects for *in utero* ETS exposure (excluding 'do not know'), daily and workplace ETS exposure respectively. *In utero* ETS exposure was not associated with FEV_1 and was negatively associated with FEV_1/FVC [b = -0.6 % (95% CI = -1.1 ; -0.1)]. The association with FEV_1/FVC was similar for never and ever smokers (supplementary table 2). Daily ETS exposure (≥1 hour) was significantly associated with lower FEV_1 [-37 ml (-65 ; -8)], and not with FEV_1/FVC. Workplace ETS exposure was significantly associated with lower FEV_1 [-43 ml (-86 ; 0)], and FEV_1/FVC [-0.6 % (-1.2 ; 0)]. Stratification by smoking status resulted in significant associations of ETS exposure with FEV_1 in never smokers only, effect estimates being -45 ml (-91 ; 0) and -82 ml (-153 ; -11) for daily and workplace ETS respectively (figure 1). Daily and workplace ETS exposure were not significantly associated with FEV_1/FVC in never or ever smokers (for all effect estimates see supplementary table 2).

SNPs and level of lung function

Subjects heterozygous for SNP rs4925 had a significantly higher FEV_1 [23 ml (0 ; 45)] and subjects heterozygous for rs156699 a significantly higher FEV_1/FVC [0.3 % (0 ; 0.7)] than wild types. There were no other significant associations between genotype and lung function (supplementary table 3).

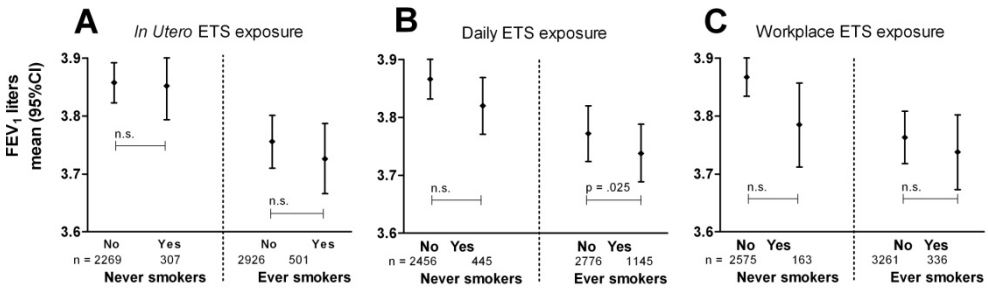

Figure 1. Mean FEV_1 (liters) for non-exposed and exposed subjects stratified by smoking status (never/ever smoker). The analysis was adjusted for sex, current smoking, packyears smoked, and centered for group specific (never/ever smokers) means for age, height and weight. A: In utero ETS (no/yes). B: daily ETS exposure (</≥1hr). C: ETS exposure at the workplace (no/yes).

Effect of interaction between *GSTO* SNPs and *in utero* ETS exposure on level of lung function

Mean FEV_1 levels were significantly different (i.e. higher with *in utero* ETS, and lower with daily and workplace ETS exposure) in subjects carrying both minor alleles for all four *GSTO* SNPs compared to wild type and heterozygote genotypes (figure 2). Therefore we used a recessive genetic model in subsequent analyses. *In utero* ETS exposure interacted with all four *GSTO* SNPs and these interactions were associated with higher level of FEV_1 level (table 2), i.e. being homozygote for the minor alleles was associated with a higher FEV_1 only in subjects that were exposed to ETS *in*

utero. There was no association with FEV_1/FVC (supplementary table 4). Associations were more pronounced in never smokers, except for SNP rs156697 (table 2). Most of these interactions remained significant after FDR correction for multiple testing.

Effect of interaction between *GSTO* SNPs and adulthood ETS exposure on level of lung function

Daily ETS exposure (≥1 hour) interacted significantly with SNPs rs4925, rs1147611 and rs156699 and these interactions were associated with lower FEV_1 level (table 3). Workplace ETS interacted significantly with all four SNPs and these interactions were associated with lower FEV_1 (table 4). In other words, being homozygote for the minor alleles of the *GSTO* SNPs was associated with lower level of lung function only in subjects that were exposed to daily and workplace ETS exposure. Stratification by smoking status showed that the negative interaction effects between ETS exposure and the SNPs on FEV_1 level were consistently more pronounced in never smokers (tables 3 and 4). No significant interactions were found between daily and workplace ETS exposure and the *GSTO* SNPs on FEV_1/FVC in the whole group, or when stratified by smoking status (never/ever) (supplementary tables 5 and 6). All significant interactions of the *GSTO* SNPs with workplace ETS on level of FEV_1 remained significant after FDR correction for multiple testing, the interactions with daily ETS exposure did not remain significant.

Table 2. Effects for *in utero* ETS exposure (no/yes), the SNPs, and the interaction of *GSTO* SNPs (recessive model) with *in utero* ETS exposure on FEV_1.

Gene	Variable	FEV_1 (ml) b (95% CI)		
	N, in analysis	All 6003	Never smokers 2576	Ever smokers 3427
GSTO1	*In utero* ETS	−33 (−69 ; 4)	−25 (−81 ; 32)	−43 (91 ; 5)
	rs4925	−33 (−77 ; 10)	−1 (65 ; 63)	−58 (−117 ; 1)
	ETS*rs4925	$^{\$}$177 (50 ; 305)**	196 (15 ; 376)*	161 (−18 ; 340)
GSTO1	*In utero* ETS	−40 (−77 ; -2)*	−32 (−89 ; 26)	−51 (−100 ; -2)*
	rs1147611	−22 (−58 ; 14)	6 (−46 ; 58)	−46 (−96 ; 3)
	ETS*rs1147611	$^{\$}$177 (72 ; 283)***	$^{\$}$206 (47 ; 365)*	166 (25 ; 307)*
GSTO2	*In utero* ETS	−41 (−78 ; -3)*	−28 (−85 ; 30)	−55 (−104 ; -6)*
	rs156697	−28 (−64 ; 9)	9 (−44 ; 62)	−59 (−110 ; -8)*
	ETS*rs156697	$^{\$}$198 (90 ; 307)***	181 (20 ; 342)*	$^{\$}$219 (72 ; 366)**
GSTO2	*In utero* ETS	−33 (−70 ; 3)	−24 (−81 ; 32)	−45 (−93 ; 4)
	rs156699	−30 (−70 ; 10)	−9 (−66 ; 49)	−48 (−104 ; 7)
	ETS*rs156699	$^{\$}$160 (41 ; 278)**	193 (12 ; 374)*	143 (−15 ; 300)

The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders. *p-value<0.05 **p-value<0.01 *** p-value<0.001. $^{\$}$significant after FDR correction for multiple testing.

Table 3. Effects for daily ETS exposure (</≥1hr), the SNPs, and the interaction of *GSTO* SNPs (recessive model) with daily ETS exposure on FEV_1.

Gene	Variable	FEV_1 (ml) b (95% CI)		
	N, in analysis	All 6822	Never smokers 2901	Ever smokers 3921
GSTO1	Daily ETS	−27 (−56 ; 3)	−31 (−79 ; 16)	−28 (−66 ; 10)
	rs4925	61 (−37 ; 49)	67 (6 ; 128)*	−46 (−106 ; 15)
	ETS*rs4925	−115 (−209 ; -22)*	−153 (−306 ; 0)	−72 (−192 ; 47)
GSTO1	Daily ETS	−25 (−55 ; 5)	−28 (−77 ; 22)	−28 (−67 ; 11)
	rs1147611	15 (−22 ; 51)	56 (5 ; 107)*	−24 (−75 ; 28)
	ETS*rs1147611	−83 (−159 ; -7)*	−122 (−247 ; 3)	−45 (−147 ; 52)
GSTO2	Daily ETS	−29 (−59 ; 1)	−28 (−77 ; 22)	−34 (−73 ; 5)
	rs156697	6 (−31 ; 43)	57 (5 ; 109)*	−40 (−93 ; 12)
	ETS*rs156697	−59 (−137 ; 19)	−127 (−252 ; -1)*	−2 (−104 ; 99)
GSTO2	Daily ETS	−26 (−56 ; 3)	−29 (−78 ; 19)	−28 (−66 ; 10)
	rs156699	6 (−35 ; 46)	44 (−12 ; 100)	−29 (−86 ; 28)
	ETS*rs156699	−94 (−178 ; -10)*	−133 (−268 ; 2)	−56 (−165 ; 54)

The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders. *p-value<0.05. There were no significant effects after FDR correction for multiple testing.

Verification of initial findings in the second sample

Population characteristics (table 1) and genotype frequencies (supplementary table 1) were similar in the second (verification) and the first sample. Complete data was available for n = 3914, 4527 and 4702 subjects for *in utero*, daily and workplace ETS respectively. Estimates for the negative associations of *in utero*, daily and workplace ETS exposure on FEV_1 and FEV_1/FVC in the verification sample, were in line with associations found in sample 1, yet did not all reach statistical significance (supplementary table 7). Associations between the heterozygote genotypes for rs4925 and rs156699 with FEV_1 and FEV_1/FVC respectively, found in sample 1, could not be replicated in the second sample (supplementary table 3).

Interaction GSTO SNPs with in utero ETS

Similar to sample 1, *GSTO2* SNP rs156697 significantly interacted with *in utero* ETS exposure and was associated with a higher level of FEV_1 in sample 2 (table 5). The other *GSTO* SNPs consistently had effects in the similar direction, yet without reaching statistical significance.

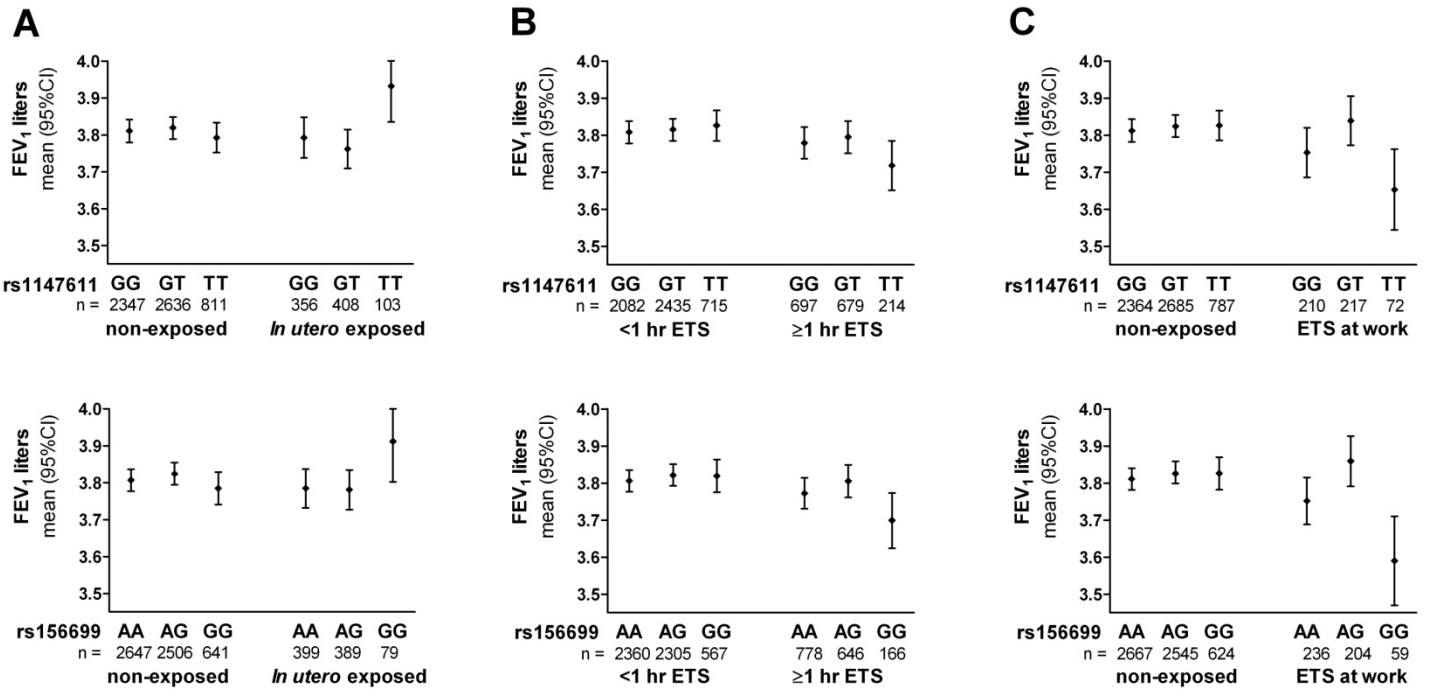

Figure 2. Mean FEV_1 (liters) for non-exposed and ETS exposed subjects stratified by genotype for the two genotyped SNPs rs1147611 and rs156699. *GSTO1*SNP rs1147611 (upper) and *GSTO2* SNP rs156699 (lower row). The analysis was adjusted for sex, current, ex-smoking, packyears smoked, and centered for mean age, height and weight. A: In utero ETS (no/yes). B: daily ETS exposure (</≥1hr). C: ETS exposure at the workplace (no/yes).

Table 4. Effects for workplace ETS exposure (n/y), the SNPs, and the interaction of *GSTO* SNPs (recessive model) with workplace ETS exposure on FEV_1.

Gene	Variable	FEV_1 (ml) b (95% CI)		
	N, in analysis	All	Never smokers	Ever smokers
		7149	3051	4098
GSTO1	Workplace ETS	−26 (−71 ; 20)	−51 (−126 ; 25)	−17 (−73 ; 40)
	rs4925	8 (−34 ; 50)	78 (17 ; 138)*	−48 (−105 ; 9)
	ETS*rs4925	$-173 (−313 ; -33)*	$-281 (−498 ; -64)*	−84 (−268 ; 100)
GSTO1	Workplace ETS	−22 (−68 ; 25)	−41 (−117 ; 36)	−15 (−73 ; 43)
	rs1147611	8 (−27 ; 42)	49 (0 ; 99)#	−29 (−78 ; 19)
	ETS*rs1147611	$-151 (−272 ; -31)*	$-286 (−486 ; -87)**	−65 (−218 ; 87)
GSTO2	Workplace ETS	−21 (−67 ; 25)	−41 (−118 ; 36)	−14 (−72 ; 44)
	rs156697	8 (−28 ; 44)	50 (−1 ; 100)	−29 (−79 ; 20)
	ETS*rs156697	$-170 (−295 ; -46)**	$-287 (−486 ; -88)**	−84 (−245 ; 77)
GSTO2	Workplace ETS	−17 (−63 ; 28)	−41 (−117 ; 35)	−9 (−66 ; 48)
	rs156699	6 (−32 ; 45)	43 (−12 ; 98)	−26 (−79 ; 28)
	ETS*rs156699	$-218 (−349 ; -87)**	$-318 (−525 ; -111)**	−140 (−311 ; 30)

The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders. *p-value<0.05 **p-value<0.01. $significant after FDR correction for multiple testing.

Table 5. Verification of the interaction of *GSTO* SNPs (recessive model) with different types of ETS exposure on FEV_1 in sample 2.

Gene	Variable	FEV_1 (ml) b (95% CI)		
	N, in analysis	*In utero* ETS	Daily ETS	Workplace ETS
		3914	4527	4702
GSTO1	ETS	−45 (−91 ; 1)	−41 (−78 ; -4)*	−40 (−98 ; 18)
	rs4925	11 (−43 ; 65)	15 (−36 ; 66)	33 (−17 ; 83)
	ETS*rs4925	111 (−32 ; 253)	25 (−93 ; 142)	−163 (−390 ; 65)
GSTO1	ETS	−49 (−96 ; -1)*	−40 (−78 ; -2)*	−40 (−100 ; 20)
	rs1147611	4 (−40 ; 47)	21 (−21 ; 63)	26 (−15 ; 67)
	ETS*rs1147611	94 (−22 ; 211)	5 (−89 ; 98)	−87 (−254 ; 81)
GSTO2	ETS	−52 (−99 ; -5)*	−41 (−79 ; -4)*	−38 (−98 ; 21)
	rs156697	2 (−42 ; 46)	17 (−25 ; 59)	28 (−13 ; 69)
	ETS*rs156697	119 (0 ; 237)*	17 (−79 ; 113)	−99 (−269 ; 70)
GSTO2	ETS	−46 (−92 ; 1)	−40 (−78 ; -3)*	−42 (−101 ; 17)
	rs156699	−9 (−58 ; 39)	0 (−46 ; 47)	10 (−35 ; 56)
	ETS*rs156699	106 (−29 ; 241)	9 (−97 ; 115)	−101 (−292 ; 91)

Effects for in utero ETS exposure (no/yes), daily ETS exposure (</≥1hr), workplace ETS exposure (no/yes), the SNPs, and the interaction of GSTO SNPs (recessive model) with different types of ETS exposure on FEV_1 in sample 2 (verification). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. *p-value<0.05.

Interaction GSTO SNPs with daily and workplace ETS exposure

Analyses in the verification sample did not show any significant interaction or trend for interaction between the *GSTO* SNPs and daily (≥1 hour) ETS exposure (table 5). In line with findings in sample 1, there were clear interactions of the *GSTO* SNPs with workplace ETS exposure that were associated with lower level of FEV_1, but these interactions were non-significant in sample 2 (table 5). Full results of the (stratified) analyses in sample 2 can be found in supplementary tables 8-10.

Overall, the directions of the interactions of *in utero* and workplace ETS exposure with the SNPs found in the second (verification) sample were in line with the first sample, but effect estimates were somewhat smaller and not always significant.

DISCUSSION

Main finding

This study is the first to show that *GSTO* SNPs interact with ETS exposure on FEV_1, findings that were significant after FDR correction for multiple testing and replicated in the second (verification) sample or showed similar directions of effects. Interestingly, interactions were in opposite directions for ETS exposure *in utero* and during adulthood.

Results in relation to other studies

Smoking during pregnancy has been shown to reduce tidal flow-volume ratios in healthy newborn babies[4,5] and to reduce small airway flows in school age children[28]. We found no significant effect of *in utero* ETS exposure on level of FEV_1 in adulthood in both our study samples, which does not exclude that effects might be present when studying more specifically small airway dimensions as derived in school age children[28]. In line with other studies investigating effects of ETS exposure during adulthood[6,7], we found daily and workplace ETS to be associated with lower levels of FEV_1, and these effects were more pronounced in never smokers. Our effect estimate of a 45 ml lower FEV_1 level with daily ETS exposure in never smokers was comparable with the 35 ml (-66 ; -4) reduced FEV_1 level with daily ETS exposure of 1 to 4 hours in never smokers from the European Community Respiratory Health Survey (ECRHS)[7].

The homozygote mutant genotype for SNP rs156697 was not associated with the level of FEV_1 in our sample, but there was a significant interaction with *in utero* ETS exposure that was associated with higher level of FEV_1. This was a robust finding that remained significant after FDR correction for multiple testing and was moreover significantly replicated in the verification sample. Interactions between the other three SNPs and *in utero* ETS exposure showed clear trends for an association with higher level of FEV_1 in both samples but only reached significance in the first sample. Interestingly, the homozygote mutant genotypes for SNPs rs4925, rs1147611, rs156699 significantly interacted with daily ETS exposure, and all SNPs (rs4925, rs1147611, rs156697, and rs156699) significantly interacted with workplace ETS exposure and were

associated with lower level of FEV_1 in the first sample. The interactions with workplace ETS exposure remained significant after FDR correction for multiple testing and showed a clear trend for interaction in the similar direction in the verification sample. These latter results support previous findings that *GST02* is a risk gene for lower levels of FEV_1 and FVC[12].

How we can reconcile that exposure to (harmful) ETS *in utero* does not result in lower but higher adult level of lung function in subjects who are homozygote mutant for the *GST0* "risk" alleles, whereas adult ETS exposure in these individuals associates with lower lung function? First, exposure to ETS *in utero* likely leads to exposure to different substances and concentrations of substances than 'direct' inhalation of ETS. It is conceivable that substances of ETS will be 'filtered' by the maternal lung and circulation, the placenta and fetal circulation. In addition, it is also conceivable that chronic ETS during pregnancy induces maternal changes that are important for lung growth. For example, it is well-known that nicotine inhaled with cigarette smoking stimulates secretion of growth hormone in humans[29]. This is particularly interesting because growth hormone has been shown to stimulate lung growth as well as lung development during the period of alveolarization[30].

Another explanation relates to exposure to ETS taking place in completely different periods of the life-span. Different biochemical and biological processes are involved in lung development *in utero*, lung growth in childhood and early adulthood, and lung function decline in adulthood. ETS may therefore cause differential and even contradictory effects in different periods of life. For example, oxidative stress *in utero* possibly does not only damage, but is additionally necessary for cell apoptosis during lung morphogenesis. A recent study showed that risk genotypes for the non-synonymous SNPs rs4925 (Ala140Asp) in *GST01* and rs156697 (Asn142Asp) in *GST02* reduce *GST02* expression levels, leading to accumulation of oxidative damage[31]. Increased oxidant levels may contribute to cell apoptosis and subsequently better airway branching *in utero*, with positive effects on FEV_1 levels. This may contrast to adult life where airway branching has stopped and oxidative stress has predominantly negative effects, i.e. induced epithelial and endothelial cell damage and apoptosis that may contribute to airway wall and/or lung tissue fibrosis and subsequently a lower level of FEV_1. Obviously, different biological processes and pathways underlie the differential effects of ETS *in utero* versus later in life. However, all given explanations are speculative and merit further research.

Generally we found that the interactions between daily and workplace ETS exposure and the *GST0* SNPs were more pronounced in the never smokers. For *in utero* ETS exposure this difference was less evident. These findings might suggest that among ever smokers the effects are somewhat overruled by the effects of personal smoking, that may damage the lung by similar mechanisms yet with higher doses. We were not able to test if the interaction between *GST0* SNPs and ETS was significantly different between the never and ever smokers since we did not have enough study power for testing this three-way interaction between smoking status and *GST0* SNPs and ETS.

We did not find consistent significant interaction effects of the *GSTO* SNPs and ETS exposure on FEV_1/FVC. Since the interactions were negatively associated with FEV_1 but not with FEV_1/FVC, in an additional analysis we investigated effects on FVC. In line with effects on FEV_1, all four *GSTO* SNPs interacted positively with *in utero* ETS exposure and negatively with workplace ETS exposure on FVC level. Daily ETS exposure interacted negatively with the *GSTO* SNPs on FVC, but these associations were not significant. These findings suggest restrictive rather than obstructive effects on lung function.

Strengths and limitations

The extensive standardized characterization of the LifeLines population and the large sample size provided the unique opportunity to investigate gene-by-environment interactions. A major strength was the inclusion of a large verification sample that is very similar to the discovery sample. Since the verification sample was somewhat smaller than the identification sample, its power might have been too low to replicate the significant associations, but we observed clear trends in similar directions. Haplotype analysis did not provide additional information and was therefore not shown. In the current study we have adjusted for traditional covariates related to level of lung function. Additional adjustment for highest obtained level of education, as proxy for socio-economic status, ever having had a cardiovascular event or bronchodilator use did not change our results.

A limitation of our study might be the cross-sectional design with rather crude assessment of ETS exposure, without data on lifetime exposure and quantitative measurement of workplace exposure. Objective measures of exposure to environmental tobacco smoking such as cotinine levels in serum or urine were unfortunately not available. However, the exact questions as defined in the ECRHS surveys were used in our study, and these questions were validated in an Italian subsample of the ECRHS. The question about the number of hours that a person is exposed to other people's tobacco smoke showed a modest correlation with serum cotinine levels, with a clear dose-response effect between the number of hours and cotinine levels[32]. Notwithstanding this, it should be acknowledged that using self-reports may lead to recall bias, i.e. people experiencing respiratory illness are more likely to recall and report ETS exposure.

Conclusions

Our data show that polymorphisms in *GSTO* genes, involved in oxidative stress pathways and detoxification of xenobiotic substances interact with ETS exposure both *in utero* and in adulthood and significantly affect the level of FEV_1.

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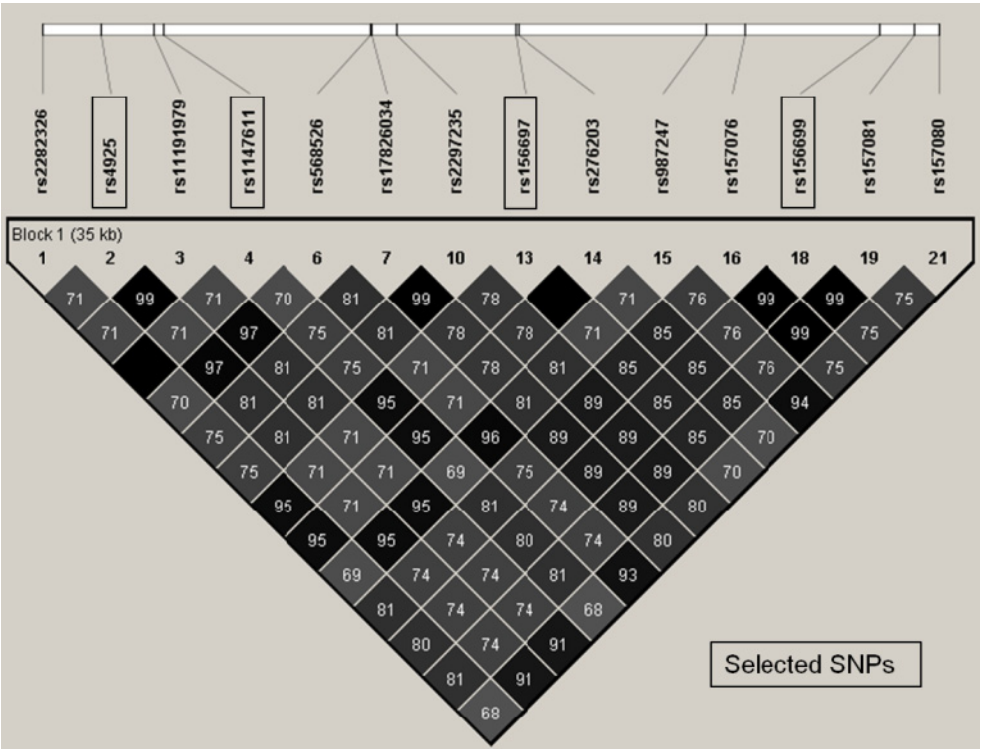
6

SUPPLEMENTARY MATERIAL

Supplementary figure 1. LD plot showing R^2 between genotyped (rs1147611 and rs156699) and imputed (rs4925 and rs156697) *GST01* and *GST02* SNPs with MAF ≥ 0.1 and HW-equilibrium p-value > 0.05, in n = 8,128 subjects included in sample 1.



Supplementary table 1. Genotype frequencies and minor allele frequency (MAF) for the four tagging SNPs in the *GST01-2* cluster in n = 8,128 subjects included in sample 1 and n = 5,308 subjects in sample 2 (verification).

			Sample 1				
Gene	**SNP**	**Wild type**	**Heterozygote**	**Homozygote**	**MAF**	**Major**	**Minor**
GST01	rs4925	4060 (50%)	3345 (41%)	723 (9%)	0.30	C	A
GST01	rs1147611	3329 (41%)	3688 (45%)	1111 (14%)	0.36	G	T
GST02	rs156697	3419 (42%)	3663 (45%)	1046 (13%)	0.35	A	G
GST02	rs156699	3744 (46%)	3508 (43%)	876 (11%)	0.32	A	G
			Sample 2				
Gene	**SNP**	**Wild type**	**Heterozygote**	**Homozygote**	**MAF**	**Major**	**Minor**
GST01	rs4925	2642 (50%)	2202 (42%)	464 (9%)	0.29	C	A
GST01	rs1147611	2122 (40%)	2442 (46%)	744 (14%)	0.37	G	T
GST02	rs156697	2157 (41%)	2435 (46%)	716 (14%)	0.36	A	G
GST02	rs156699	2394 (45%)	2336 (44%)	578 (11%)	0.33	A	G

[#] = Major (wild type) and minor allele (modeled as risk allele) respectively.

Supplementary table 2. Associations between ETS exposure and lung function level in the n = 8,128 subjects included in sample 1. Associations were assessed by linear regression models, all adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Additionally the model was stratified by smoking status (never/ever), and adjusted for the other confounders.

		FEV_1 (ml)	
		b (95% CI)	
ETS exposure	**All**	**Never smokers**	**Ever smokers**
In utero	-19 (-54 ; 17)	-6 (61 ; 48)	-31 (-78 ; 15)
Daily	**-37 (-65 ; -8)***	-45 (-91 ; 0)#	-34 (-70 ; 3)
At work	**-43 (-86 ; 0)***	**-82 (-153 ; -11)***	-25 (-79 ; 29)
		FEV_1/FVC	
		b (95% CI)	
ETS exposure	**All**	**Never smokers**	**Ever smokers**
In utero	**-0.6 (-1.1 ; -0.1)***	-0.6 (-1.4 ; 0.1)	-0.6 (-1.3 ; 0.1)
Daily	-0.3 (-0.7 ; 0.1)	0 (-0.7 ; 0.6)	-0.4 (-1.0 ; 0.1)
At work	**-0.6 (-1.2 ; 0)***	-0.4 (-1.4 ; 0.5)	-0.7 (-1.5 ; 0.1)

#p = 0.051; *p<0.05

Supplementary table 3. Associations between genotypes and lung function, adjusted for sex, age, height, weight, current smoking, ex-smoking and packyears smoked in sample 1 and sample 2 (verification). The wild type genotype was used as reference category.

		Sample 1 (n=8,128)		Sample 2 (n=5,308)	
SNPs	**Alleles**	**FEV_1 (ml)**	**FEV_1/FVC**	**FEV_1 (ml)**	**FEV_1/FVC**
		b (95% CI)	**b (95% CI)**	**b (95% CI)**	**b (95% CI)**
rs4925	CA	**23 (0 ; 45)***	0.3 (-0.8 ; 0.6)	-7 (-34 ; 20)	0.2 (-0.2 ; 0.5)
	AA	-7 (-45 ; 32)	-0.1 (-0.7 ; 0.4)	24 (-23 ; 70)	0.2 (-0.2 ; 1.1)
rs1147611	GT	11 (-12 ; 34)	0.2 (-0.1 ; 0.5)	-1 (-29 ; 26)	-0.1 (-0.5 ; 0.3)
	TT	-2 (-36 ; 31)	-0.2 (-0.7 ; 0.3)	22 (-18 ; 62)	0.5 (-0.1 ; 1.0)
rs156697	AG	11 (-12 ; 34)	0.2 (-0.9 ; 0.6)	1 (-27 ; 28)	0 (0 ; 0.4)
	GG	-5 (-39 ; 29)	-0.2 (-0.7 ; 0.3)	23 (-17 ; 63)	0.5 (-0.1 ; 1.0)
rs156699	AG	21 (-2 ; 43)	**0.3 (0 ; 0.7)***	-8 (-36 ; 19)	0 (-0.4 ; 0.4)
	GG	-8 (-44 ; 29)	-0.4 (-0.9 ; 0.2)	2 (-41 ; 45)	0.3 (-0.3 ; 1.0)

*p-value<0.05

Supplementary table 4. Effects for *in utero* ETS exposure (no/yes), the SNPs, and the interaction of *GST0* SNPs (recessive model) with *in utero* ETS exposure on FEV_1/FVC (%). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders.

			FEV_1/FVC b (95% CI)	
Gene	**SNPs**	**All**	**Never smokers**	**Ever smokers**
	N, in analysis	6003	2576	3427
GST01	*In utero* ETS	**-0.6 (-1.0 ; -0.1)***	-0.7 (-1.5 ; 0.1)	-0.5 (-1.2 ; 0.2)
	rs4925	-0.3 (-0.9 ; 0.3)	-0.3 (-1.2 ; 0.6)	-0.3 (-1.2 ; 0.5)
	ETS*rs4925	-0.4 (-2.2 ; 1.4)	0.1 (-2.0 ; 3.0)	-1.2 (-3.8 ; 1.4)
GST01	*In utero* ETS	**-0.7 (-1.2 ; -0.1)***	-0.7 (-1.5 ; 0.1)	-0.6 (-1.3 ; 0.1)
	rs1147611	-0.4 (0.9 ; 0.1)	-0.3 (-1.0 ; 0.5)	-0.6 (-1.3 ; 0.2)
	ETS*rs1147611	0.3 (-1.2 ; 1.8)	0.6 (-1.6 ; 2.8)	0.2 (-1.9 ; 2.3)
GST02	*In utero* ETS	**-0.7 (-1.2 ; -0.1)***	-0.7 (-1.5 ; 0.1)	-0.6 (-1.3 ; 0.1)
	rs156697	-0.5 (-1.0 ; 0)	-0.4 (-1.1 ; 0.4)	-0.6 (-1.4 ; 0.1)
	ETS*rs156697	0.4 (-1.2 ; 1.9)	0.5 (-1.8 ; 2.7)	0.3 (-1.9 ; 2.4)
GST02	*In utero* ETS	**-0.6 (-1.1 ; 0)***	-0.7 (-1.5 ; 0.1)	-0.5 (-1.2 ; 0.2)
	rs156699	**-0.6 (-1.1 ; 0)***	-0.5 (-1.3 ; 0.3)	-0.6 (-1.4 ; 0.2)
	ETS*rs156699	-0.5 (-2.2 ; 1.2)	0.5 (-2.0 ; 2.9)	-1.1 (-3.4 ; 1.2)

*p-value<0.05

Supplementary table 5. Effects for daily ETS exposure (</≥1hr), the SNPs, and the interaction of *GST0* SNPs (recessive model) with daily ETS exposure on FEV_1/FVC (%). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders.

			FEV_1/FVC	
			b (95% CI)	
Gene	**SNPs**	**All**	**Never smokers**	**Ever smokers**
	N, in analysis	6822	2901	3921
GST01	Daily ETS	-0.2 (-0.6 ; 0.2)	0.1 (-0.5 ; 0.8)	-0.4 (-0.9 ; 0.2)
	rs4925	0 (-0.6 ; 0.6)	0.3 (-0.6 ; 1.1)	-0.2 (-1.1 ; 0.7)
	ETS*rs4925	-1.1 (-2.4 ; 0.2)	-1.7 (-3.9 ; 0.4)	-0.7 (-2.4 ; 1.0)
GST01	Daily ETS	-0.2 (-0.6 ; 0.2)	0.1 (-0.6 ; 0.8)	-0.3 (-0.9 ; 0.2)
	rs1147611	-0.1 (-0.6 ; 0.5)	0.1 (-0.6 ; 0.8)	-0.1 (-0.9 ; 0.6)
	ETS*rs1147611	-0.7 (-1.8 ; 0.4)	-0.7 (-2.4 ; 1.1)	-0.7 (-2.1 ; 0.7)
GST02	Daily ETS	-0.2 (-0.7 ; 0.2)	0.1 (-0.6 ; 0.8)	0.1 (-0.5 ; 0.8)
	rs156697	-0.1 (-0.7 ; 0.4)	0 (-0.7 ; 0.7)	0.1 (-0.7 ; 0.9)
	ETS*rs156697	-0.6 (-1.7 ; 0.5)	-0.7 (-2.4 ; 1.1)	-1.3 (-0.3 ; 0.6)
GST02	Daily ETS	-0.2 (-0.6 ; 0.2)	-0.4 (-0.9 ; 0.2)	-0.3 (-0.9 ; 0.2)
	rs156699	-0.2 (-0.7 ; 0.4)	-0.2 (-1.0 ; 0.5)	-0.4 (-1.2 ; 0.4)
	ETS*156699	-1.0 (-2.2 ; 0.1)	-0.5 (-2.0 ; 0.9)	-0.8 (-2.4 ; 0.8)

Supplementary table 6. Effects for workplace ETS exposure (n/y), the SNPs, and the interaction of *GST0* SNPs (recessive model) with workplace ETS exposure on FEV_1/FVC (%). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders.

			FEV_1/FVC b (95% CI)	
Gene	**SNPs N, in analysis**	**All** 7149	**Never smokers** 3051	**Ever smokers** 4098
GST01	Workplace ETS	-0.6 (-1.2 ; 0.1)	-0.2 (-1.3 ; 0.8)	-0.7 (-1.5 ; 0.1)
	rs4925	-0.3 (-0.9 ; 0.3)	0.1 (-0.7 ; 0.9)	-0.6 (-1.4 ; 0.3)
	ETS*rs4925	-0.5 (-2.5 ; 1.5)	-1.8 (-4.8 ; 1.2)	0.4 (-2.3 ; 3.0)
GST01	Workplace ETS	-0.5 (-1.1 ; 0.2)	-0.1 (-1.1 ; 1.0)	-0.7 (-1.6 ; 0.1)
	rs1147611	-0.2 (-0.7 ; 0.3)	0.1 (-0.6 ; 0.8)	-0.5 (-1.2 ; 0.2)
	ETS*rs1147611	-0.8 (-2.5 ; 0.9)	-2.6 (-5.3 ; 0.1)	0.2 (-2.0 ; 2.4)
GST02	Workplace ETS	-0.5 (-1.1 ; 0.2)	-0.1 (-1.1 ; 1.0)	-0.7 (-1.5 ; 0.2)
	rs156697	-0.3 (-0.8 ; 0.2)	0 (-0.7 ; 0.7)	-0.5 (-1.2 ; 0.2)
	ETS*rs156697	-1.0 (-2.7 ; 0.8)	-2.5 (-5.3 ; 0.2)	0 (-2.3 ; 2.4)
GST02	Workplace ETS	-0.5 (-1.1 ; 0.2)	-0.1 (-1.2 ; 0.9)	-0.7 (-1.5 ; 0.2)
	rs156699	-0.4 (-0.9 ; 0.2)	0 (-0.7 ; 0.8)	-0.7 (-1.5 ; 0.1)
	ETS*rs156699	-0.9 (-2.8 ; 0.9)	-2.3 (-5.1 ; 0.6)	0 (-2.5 ; 2.5)

Supplementary table 7. Verification: Associations between ETS exposure and lung function level (FEV_1 and FEV_1/FVC (%)) in the n = 5,308 subjects included in sample 2. Associations were assessed by linear regression models, all adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Additionally the model was stratified by smoking status (never/ever) and adjusted for the other confounders.

		FEV_1 (ml) b (95% CI)	
ETS exposure	**All**	**Never smokers**	**Ever smokers**
In utero	-34 (-78 ; 10)	-35 (-103 ; 33)	-39 (-97 ; 19)
Daily	**-39 (-75 ; -4)***	-25 (-83 ; 34)	**-51 (-11 ; -7)***
At work	-51 (-107 ; 5)	-87 (-181 ; 6)	-35 (-106 ; 36)
		FEV_1/FVC (%) b (95% CI)	
ETS exposure	**All**	**Never smokers**	**Ever smokers**
In utero	**-0.7 (-1.4 ; -0.1)***	-0.5 (-1.4 ; 0.5)	**-0.9 (-1.7 ; 0)***
Daily	-0.5 (-1.0 ; 0.1)	0.2 (-0.6 ; 1.0)	**-0.8 (-1.4 ; -0.1)***
At work	-0.7 (-1.5 ; 0.1)	-0.9 (-2.2 ; 0.4)	-0.4 (-1.5 ; 0.6)

*p-value<0.05

Supplementary table 8. Verification: Effects for *in utero* ETS exposure (no/yes), the SNPs, and the interaction of *GSTO* SNPs (recessive model) with *in utero* ETS exposure on FEV_1 in sample 2 (verification). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders.

			FEV_1 (ml) b (95% CI)	
Gene	**SNPs N, in analysis**	**All** 3914	**Never smokers** 1713	**Ever smokers** 2201
GSTO1	*In utero* ETS	-45 (-91 ; 1)	-47 (-117 ; 24)	-49 (-110 ; 12)
	rs4925	11 (-43 ; 65)	1 (-79 ; 81)	18 (-55 ; 91)
	ETS*rs4925	111 (-32 ; 253)	123 (-102 ; 347)	106 (-80 ; 291)
GSTO1	*In utero* ETS	**-49 (-96 ; -1)***	-69 (-142 ; 4)	-43 (-105 ; 20)
	rs1147611	4 (-40 ; 47)	5 (-59 ; 70)	1 (-58 ; 60)
	ETS*rs1147611	94 (-22 ; 211)	**214 (33 ; 394)***	28 (-125 ; 181)
GSTO2	*In utero* ETS	**-52 (-99 ; -5)***	-67 (-140 ; 6)	-49 (-111 ; 14)
	rs156697	2 (-42 ; 46)	5 (-60 ; 71)	-1 (-61 ; 59)
	ETS*rs156697	**119 (0 ; 237)***	**208 (25 ; 391)***	69 (-86 ; 224)
GSTO2	*In utero* ETS	-46 (-92 ; 1)	-52 (-123 ; 19)	-47 (-108 ; 14)
	rs156699	-9 (-58 ; 39)	1 (-71 ; 74)	-17 (-83 ; 48)
	ETS*rs156699	106 (-29 ; 241)	180 (-42 ; 403)	73 (-99 ; 244)

*p-value<0.05

Supplementary table 9. Verification: Effects for daily ETS exposure (</≥1hr), the SNPs, and the interaction of *GST0* SNPs (recessive model) with daily ETS exposure on FEV_1 in sample 2 (verification). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders.

			FEV_1 (ml) b (95% CI)	
Gene	**SNPs**	**All**	**Never smokers**	**Ever smokers**
	N, in analysis	4527	1898	2629
GST01	Daily ETS	-41 (-78 ; -4)	-35 (-97 ; 26)	-50 (-97 ; -3)
	rs4925	15 (-36 ; 66)	1 (-75 ; 77)	24 (-45 ; 93)
	ETS*rs4925	25 (-93 ; 142)	123 (-83 ; 328)	-15 (-161 ; 131)
GST01	Daily ETS	-40 (-78 ; -2)	-36 (-98 ; 27)	-49 (-97 ; -1)
	rs1147611	21 (-21 ; 63)	25 (-36 ; 85)	15 (-43 ; 72)
	ETS*rs1147611	5 (-89 ; 98)	92 (-82 ; 266)	-15 (-130 ; 100)
GST02	Daily ETS	**-41 (-79 ; -4)***	-32 (-94 ; 30)	-52 (-101 ; -4)
	rs156697	17 (-25 ; 59)	23 (-38 ; 84)	11 (-47 ; 69)
	ETS*rs156697	17 (-79 ; 113)	64 (-116 ; 243)	10 (-107 ; 127)
GST02	Daily ETS	**-40 (-78 ; -3)***	-35 (-97 ; 26)	-49 (-96 ; -1)
	rs156699	0 (-46 ; 47)	-10 (-79 ; 59)	5 (-58 ; 68)
	ETS*156699	9 (-97 ; 115)	111 (-85 ; 307)	-24 (-154 ; 106)

*p-value<0.05

Supplementary table 10. Verification: Effects for workplace ETS exposure (n/y), the SNPs, and the interaction of *GSTO* SNPs (recessive model) with workplace ETS exposure on FEV_1 in sample 2 (verification). The linear regression model for the whole group was adjusted for sex, age, height, weight, current, ex-smoking and packyears smoked. Consequently we stratified by smoking status (never/ever) and adjusted for the other possible confounders.

			FEV_1 (ml) b (95% CI)	
Gene	**SNPs N, in analysis**	**All** 4702	**Never smokers** 2003	**Ever smokers** 2699
GSTO1	Workplace ETS	-40 (-98 ; 18)	-90 (-186 ; 6)	-17 (-91 ; 56)
	rs4925	33 (-17 ; 83)	21 (-54 ; 96)	42 (-24 ; 109)
	ETS*rs4925	-163 (-390 ; 65)	57 (-339 ; 111)	-258 (-538 ; 23)
GSTO1	Workplace ETS	-40 (-100 ; 20)	-87 (-184 ; 11)	-17 (-94 ; 59)
	rs1147611	26 (-15 ; 67)	32 (-29 ; 92)	21 (-34 ; 76)
	ETS*rs1147611	-87 (-254 ; 81)	-26 (-191 ; 139)	-118 (-316 ; 80)
GSTO2	Workplace ETS	-38 (-98 ; 21)	-83 (-180 ; 14)	-17 (-94 ; 60)
	rs156697	28 (-13 ; 69)	31 (-30 ; 93)	25 (-31 ; 80)
	ETS*rs156697	-99 (-269 ; 70)	-39 (-402 ; 324)	-122 (-320 ; 76)
GSTO2	Workplace ETS	-42 (-101 ; 17)	-88 (-183 ; 8)	-19 (-94 ; 56)
	rs156699	10 (-35 ; 56)	3 (-66 ; 72)	16 (-44 ; 76)
	ETS*rs156699	-101 (-292 ; 91)	7 (-432 ; 445)	-139 (-359 ; 81)

7

Genes and pathways underlying susceptibility to environmental tobacco smoke exposure in relation to the level of FEV_1

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ABSTRACT

Background

Polymorphisms in several genes such as *glutathione S-transferases* and *ADAM33* have been associated with lung function in interaction with environmental tobacco smoke exposure (ETS). Thus far, most studies assessing interactions between genes and ETS exposure focused on candidate-genes in biologically plausible pathways. In the current study, we used a genome-wide hypothesis free approach to assess SNP-by-ETS exposure interactions in relation to the level of FEV_1, and additionally explored biological pathways potentially underlying ETS susceptibility.

Methods

SNP-by-ETS exposure interactions in relation to the level of FEV_1 were investigated in 10,817 subjects from the Dutch LifeLines study and verified in 1,276 subjects from the Swiss SAPALDIA study. ETS exposure was based on the self-reported hours per day of ETS exposure and classified as non-exposed (0 hour/day) and exposed (≥1 hour/day). SNP-by-ETS exposure p-values obtained from the identification analysis in LifeLines were used to perform a pathway analysis.

Results

45 SNP-by-ETS exposure interactions with p-values $<10^{-4}$ were identified in the LifeLines study, two being replicated with nominally significant p-values (<0.05) in the SAPALDIA study, i.e. SNPs located in *actin, beta-like 2* (*ACTBL2*) and *zinc finger homeobox 4 (ZFHX4)*. The latter SNP-by-ETS association was replicated in never-smokers but not in smokers. Three pathways were significantly or suggestively enriched in the pathway-level analysis, i.e. the apoptosis, p38 MAPK and TNF pathways.

Conclusion

Our hypothesis-free genome wide gene-by-ETS interaction study on the level of FEV_1 showed that pathways previously implicated in COPD pathology may underlie susceptibility to ETS exposure.

BACKGROUND

Chronic obstructive pulmonary disease (COPD) is a complex disease with a worldwide high burden of morbidity and mortality[1]. COPD is caused by an abnormal inflammatory response to noxious particles and gases leading to airflow obstruction and an accelerated decline in lung function. Detrimental effects of environmental tobacco smoke (ETS) exposure on the level of lung function has been shown in various studies[2-4]. Moreover, polymorphisms in several genes such as *glutathione S-transferases* (*GSTs*)[5, 6] and *ADAM33*[7], have been reported to interact with ETS exposure on the level and decline of lung function. In other words, ETS exposure has differential effects in subjects carrying mutant alleles compared to wild type alleles. Thus far, studies assessing interactions between genetic variants and ETS exposure have mostly relied on a-priori knowledge of potentially involved biological pathways, for example the detoxification of noxious particles and gases by proteins such as the *glutathione S-transferases*.

Genome wide interaction (GWI) studies are hypothesis free approaches testing hundreds of thousands genetic markers across the entire genome in interaction with an exposure of interest. Studying genome-wide gene-by-environment interactions may yield new loci in addition to those already known to be associated with the development of COPD directly[8]. This may eventually provide us with insights in molecular pathways important in disease development. In general, genome-wide studies focus on the most significant SNPs and ignore the effect of weaker SNPs that may be involved in a shared pathway. Therefore, pathway analysis may add knowledge to better understanding of the etiology of complex diseases[9] such as COPD, a disease that likely results from interactions between multiple genes and multiple environmental exposures[8].

In the current study we performed a GWI study assessing SNP-by-ETS exposure interactions in relation to the level of FEV_1. Additionally we used the i-GSEA-4-GWAS tool to explore biological pathways underlying susceptibility to ETS exposure in relation to the level of lung function.

METHODS

Sample and measurements

We included subjects from the baseline investigation of the LifeLines study (2006-2011), a multi-disciplinary prospective population-based cohort study examining health and health-related behaviour of persons living in the North East region of the Netherlands[10]. Participants received a general health questionnaire and a basic medical examination. The medical examination included pre-bronchodilator spirometry carried out in a standardized setting following ATS guidelines using a Welch Allyn Version 1.6.0.489, PC-based SpiroPerfect with Ca Workstation software. All subjects provided written informed consent.

Exposure assessment

We used self-reported ETS exposure as hours per day as determined by the response to the question "how many hours per day are you exposed to other person's tobacco smoke". Subjects were classified as non-exposed when self-reported ETS exposure was 0 hour/day, whereas subjects were classified as ETS exposed when self-reported exposure was at least 1 hour/day (≥1 hour/day). All subjects with self-reported ETS exposure between 0 and 1 hour/day were excluded in order to have a clear exposure contrast.

Table 1. Characteristics of the LifeLines and SAPALDIA study samples.

	LifeLines (identification)	SAPALDIA (replication)
N	1,0817	1,276
ETS exposed (≥1 hour/day), n (%)	2,473 (23)	296 (23)
FEV_1 (ml), mean (sd)	3,401 (825)	3,153 (864)
FEV_1 percentage of predicted, mean (sd)	102 (14)	96 (16)
FEV_1/FVC (%), mean (sd)	76 (7)	74 (8)
Males, n (%)	4,473 (41)	611 (48)
Age, median (range)	47 (18-88)	52 (29-72)
Ever smokers, n (%)	6,266 (58)	703 (55)
Packyears*, median (range)	10 (0.05-100)	12 (0-130)

* Packyears in ever smokers.

Genotyping

Blood samples of subjects included in the second LifeLines release were genotyped using IlluminaCytoSNP-12 arrays. SNPs with a genotype call-rate ≥95%, minor allele frequency ≥1% and Hardy-Weinberg p-value ≥10^{-4} were included. Non-Caucasian samples and first-degree relatives were excluded. A total of 227,981 genotyped SNPs were included in the identification analysis.

SNP-by-ETS exposure interactions

Interactions of SNPs with daily ETS exposure and their association with FEV_1 were tested in an additive genetic model adjusted for sex, age, height, ever smoking and packyears smoked using linear regression using the software package PLINK, version 1.07[11]. SNPs with p-values <10^{-4} were taken for replication in a second independent cohort. SNP annotation was performed using HaploReg version 2 (Broad Institute). SNP-by-exposure interactions were additionally assessed for never and ever smokers separately.

Replication of SNP-by-ETS exposure interactions

SNP-by-ETS exposure interactions identified in the LifeLines study (p-values <10^{-4}) were verified in a second independent cohort, the SAPALDIA study[12]. The phenotype data from the SAPALDIA study was derived from the first follow-up survey

collected in 2002. The study was enriched with asthmatics (40%). ETS exposure within the SAPALDIA study was ascertained using the same question and coded the same as in the LifeLines study. Pre-bronchodilator spirometry was performed according to a standardized protocol equivalent to that of the European Community Respiratory Health Survey (ECRHS), using a Sensormedics model 2200 (Yorba Linda, California, USA) and following American Thoracic Society criteria.

Blood samples were used to genotype 567,589 SNPs using the Illumina 610K quad array. Consequently this sample was imputed using MACH v1.00 software 13 and the HapMap2 Release 22 CEU reference sample. To account for population stratification, ancestry-informative principal components were inferred using software package EIGENSTRAT2.0 using HapMap data (CEU, YRI, JPT and CHB) and additional European reference samples. Non-European and related samples were excluded.

SNP-by-ETS exposure interactions and their association with the level of FEV_1 were tested in an additive genetic model adjusted for sex, age, height, ever smoking, packyears smoked, study area and principle components ev3 and ev4. Additional smoking-stratified analyses were performed analogous to the identification analysis. Software used for statistical analyses were STATA MP12 and plink version 10.7[11].

Pathway analysis

We performed pathway analysis using the online improved gene set enrichment analysis tool i-GSEA-4-GWAS[9]. I-GSEA-4-GWAS is an online tool for identification of pathways based on p-values obtained from genome-wide association studies. This tool has previously been used in a genome-wide gene-environment interaction analysis for asbestos exposure in relation to lung cancer susceptibility[13].

P-values for SNP-by-ETS interactions in the identification sample were used in the pathway analysis. All log transformed p-values for SNPs 100 kb upstream and downstream of each gene were used to represent that specific gene. Each gene was represented by the lowest SNP p-value annotated to that gene. These SNP p-values were used to rank the genes, and the proportion of significant genes as a number of the total amount of genes (gene set) belonging to a pathway was calculated. Based on the rank, p-values were calculated for the association between the total gene set/pathway and the outcome. Additionally FDR corrected p-values were calculated. Gene sets/pathways with FDR corrected p-values <0.25 are regarded as suggestively associated with the outcome, whereas FDR p-values <0.05 are regarded as highly confident for an association with the outcome[9].

RESULTS

Descriptive statistics

In the LifeLines study, complete data on all covariates was available for 11,187 subjects. 370 subjects (3%) were excluded because they had self-reported ETS exposure between 0 and 1 hour per day. Finally, the analysis included 10,817 subjects, of which 2473 (23%) subjects reported at least one hour of ETS exposure per day (table 1). In the SAPALDIA study (replication sample) complete data was available for 1,276 subjects, of which 296 (23%) subjects reported at least one hour of ETS exposure per day (table 1).

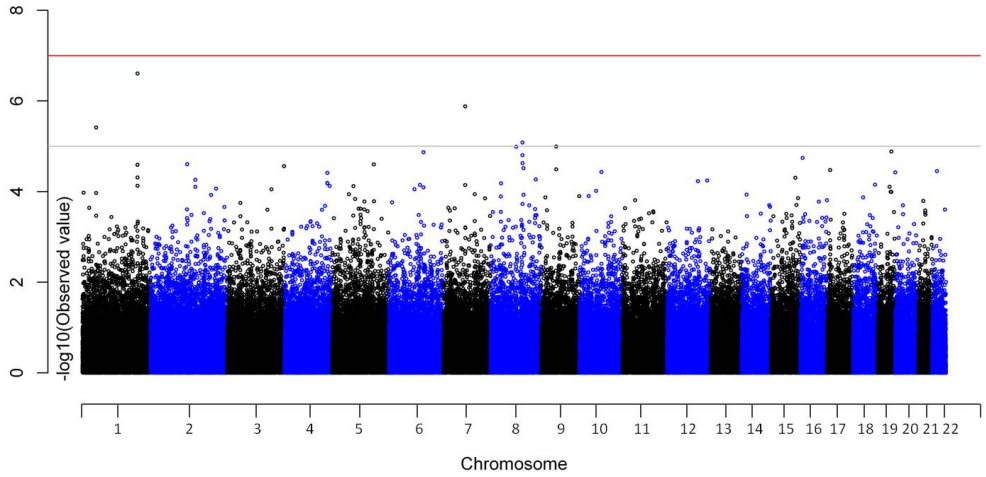

Figure 1. Manhattan plot showing SNP-by-ETS exposure interactions on the level of FEV_1 in the identification sample (LifeLines).

SNP-by-ETS exposure interactions

227,964 genotyped SNPs were included in the identification analysis. P-values for each SNP-by-ETS exposure interaction on the level of FEV_1 are shown in a Manhattan plot (figure 1). The top-interacting SNP rs924568 was associated with a higher level of FEV_1 in subjects with ETS exposure in the identification analysis (figure 2a). This SNP was located in *potassium voltage-gated channel, subfamily H member 1* (*KCNH1*) on chromosome 1 (p-value = $2.49*10^{-7}$). A total of five SNPs in *KCNH1* interacted with ETS exposure on the level of FEV_1 with p-values $<10^{-4}$ (table 2); these SNPs were in moderate/strong LD (supplementary figure 1). Associations in never and ever smokers separately are shown in the supplementary table 1. None of these interactions with SNPs in *KCNH1* were replicated in the SAPALDIA study.

Table 2. Interactions between SNPs (additive effect for minor allele A1) and ETS exposure on the level of FEV_1 (ml) with p-values < 10^{-4} in the identification analysis (LifeLines). MAF is given for minor allele A1.

				95% CI					Functional	LD
CHR	SNP	A1	B	Lower	Upper	P	MAF	Gene	annotation	R2
1	rs2859741	T	69	40	98	3.86E-06	0.45	9.5kb 5' of GRIK3		
1	rs1846946	T	63	32	94	7.41E-05	0.33	KCNH1	intronic	LD block
1	rs7526579	C	-75	-111	-39	4.88E-05	0.20	KCNH1	intronic	
1	rs924568	G	79	49	109	2.49E-07	0.38	KCNH1	intronic	
1	rs4951491	C	67	36	98	2.58E-05	0.32	KCNH1	intronic	
1	rs6540647	G	63	34	92	2.57E-05	0.44	KCNH1	intronic	
2	rs17031275	A	120	64	176	2.49E-05	0.08	31kb 3' of LOC285000		
2	rs4954603	G	93	48	138	5.51E-05	0.12	185kb 5' of CXCR4		0.54
2	rs7570134	G	75	38	112	7.84E-05	0.20	210kb 5' of CXCR4		
2	rs10497902	C	78	39	117	8.62E-05	0.17	77kb 3' of PTH2R		
3	rs528581	C	60	30	90	8.88E-05	0.37	75kb 5' of RAP2B		
3	rs2084386	C	95	51	140	2.74E-05	0.12	PAK2	intronic	
4	rs17062990	G	-102	-150	-53	3.87E-05	0.10	118kb 3' of SPCS3		LD block
4	rs4146433	T	-89	-133	-45	6.44E-05	0.13	147kb 3' of SPCS3		
4	rs11133161	A	-92	-138	-47	6.58E-05	0.11	138kb 3' of VEGFC		
4	rs4861505	G	-68	-102	-34	7.53E-05	0.25	ODZ3	intronic	
5	rs11950494	G	-110	-164	-55	7.60E-05	0.08	ACTBL2	3'-UTR	
5	rs1393082	A	68	36	99	2.51E-05	0.30	H2AFY	intronic	
6	rs4421160	T	-90	-135	-45	8.89E-05	0.11	144kb 3' of CD109		
6	rs982124	A	-294	-439	-149	7.12E-05	0.01	KLHL32	intronic	
6	rs9386622	T	65	33	97	8.08E-05	0.29	PDSS2	intronic	0.60
6	rs11153056	C	72	39	104	1.36E-05	0.29	PDSS2	intronic	
7	rs1533956	G	74	44	104	1.31E-06	0.34	12kb 5' of MIR3147		0.74
7	rs11135646	T	64	32	96	7.15E-05	0.28	18kb 5' of ZNF716		
8	rs2090789	A	-60	-89	-30	6.54E-05	0.46	LOC100128993	intronic	
8	rs2733727	A	144	80	208	1.02E-05	0.05	ZFHX4	intronic	
8	rs16894633	C	118	66	171	8.31E-06	0.08	6.2kb 5' of SDC2		0.92
8	rs16894649	A	111	59	162	2.37E-05	0.08	SDC2	intronic	
8	rs12056723	G	-131	-191	-72	1.58E-05	0.06	SDC2	intronic	
8	rs7831729	C	-80	-118	-43	3.03E-05	0.18	15kb 5' of SNX31		
8	rs13282467	A	-195	-290	-100	5.37E-05	0.02	LRRC6	intronic	
9	rs7030493	T	-112	-165	-59	3.24E-05	0.08	TMEM2	intronic	0.93
9	rs1552708	G	-117	-168	-65	1.02E-05	0.08	TMEM2	intronic	
10	rs2174257	G	-59	-89	-29	9.69E-05	0.39	PRKG1	intronic	
10	rs2593163	G	103	54	152	3.71E-05	0.09	PSAP	intronic	
12	rs12581724	C	-111	-165	-57	5.88E-05	0.08	APAF1	intronic	
12	rs225574	T	-78	-116	-40	5.70E-05	0.18	LOC400084	intronic	
15	rs6496799	G	-80	-118	-41	4.95E-05	0.18	124kb 3' of SV2B		
16	rs8052564	A	120	65	175	1.81E-05	0.08	RBFOX1	intronic	
17	rs8067644	T	-65	-95	-34	3.34E-05	0.35	PIK3R6	intronic	
18	rs7233554	C	-108	-161	-55	7.08E-05	0.09	20kb 3' of CYB5A		
19	rs7976	T	71	36	106	7.88E-05	0.22	KRTDAP	3'-UTR	
19	rs311384	G	70	39	102	1.31E-05	0.31	ARHGAP35	intronic	
20	rs753320	A	-89	-132	-47	3.76E-05	0.13	27kb 5' of SCRT2		
22	rs743262	A	274	144	404	3.53E-05	0.01	206kb 3' of MN1		

Linear regression models were adjusted for sex, age, height, ever smoking and packyears smoked.

One SNP (rs4421160) identified in the LifeLines study with an interaction p-value $<10^{-4}$ was not available in the SAPALDIA study. A total of 44 SNPs was taken for replication in the SAPALDIA study. Two SNP-by-exposure interactions were replicated with nominal significant p-values ($p < 0.05$) and the same direction of interaction in the SAPALDIA study (table 3). SNP rs11950494 located in *actin, beta-like 2* (*ACTBL2*, 3'-UTR) was associated with a lower level of FEV_1 in subjects with ETS exposure (figure 2b). SNP rs2733727 in *zinc finger homeobox 4* (*ZFHX4;* intronic) was associated with a higher level of FEV_1 in subjects with ETS exposure (figure 2c), this SNP-by-ETS exposure interaction was replicated in the never smokers only (table 3).

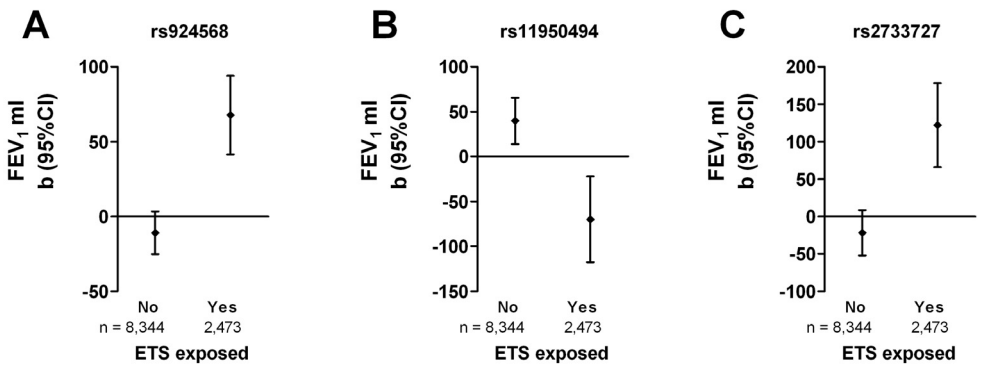

Figure 2. Additive SNP effects on the level of FEV_1 (ml) for subjects with and without ETS exposure in the identification sample (LifeLines). Linear regression models were adjusted for sex, age, height, ever smoking and packyears smoked.

Pathway analysis

Of all SNPs included in the SNP-by-ETS exposure interaction analysis in the LifeLines study, 165,298 were used for pathway analysis. These SNPs were mapped to 15,243 genes and 231 gene sets/pathways. Pathway analysis showed one significant (FDR p-value <0.05) and two suggestively enriched pathways (FDR p-value <0.25) (table 4). The most significant, i.e. the apoptosis pathway, includes 71 genes of which 54 were present in the LifeLines dataset and 23 were significantly associated with the outcome (table 5). The two suggestively associated pathways were the tumor necrosis factor (TNF) pathway (table 6) and p38 MAPK pathway (table 7), with 9 and 16 genes from the SNP-by-ETS exposure interaction analysis that were significantly associated with the outcome, respectively.

DISCUSSION

The current study is the first to explore gene-by-ETS interactions on the level of FEV_1 in a hypothesis-free genome-wide manner. We extended our findings to pathway level analysis and showed that several pathways, i.e. the apoptosis, p38 MAPK and TNF pathways, may be underlying susceptibility to ETS exposure in relation to the level of FEV_1.

The SNP with the strongest association with the level of FEV_1 in the identification cohort was located in *KCNH1,* also known as *ether-à-go-go (EAG1).* In addition there were several SNPs in this gene that were suggestively associated with a higher level of level of FEV_1 in subjects with ETS exposure ($p<10^{-4}$), most being in moderate/strong LD. KCNH1 is a voltage-gated potassium channel (Kv) that was highly expressed on mast cells and macrophages in germinal centers of reactive lymph nodes[14]. This SNP-by-ETS exposure interaction was, however, not replicated in the SAPALDIA study.

Two SNPs identified the LifeLines study were replicated with nominally significant p-values in the SAPALDIA study i.e. *actin, beta-like 2* (*ACTBL2*) and *zinc finger homeobox 4 (ZFHX4).* The SNP in *ACTBL2* was associated with a lower level of FEV_1 in subjects with ETS exposure. Little is known about the biological function and expression of *ACTBL2.* Recently a Korean GWA study on diastolic blood pressure identified one SNP in *ACTBL2.* The SNP identified in our study was not in LD with the SNP identified in the Korean study (r-squared = 0.002, CEU)[15]. GO annotations related to this gene include ATP binding. The second SNP located in *ZFHX4* was associated with a higher level of FEV_1 in subjects with ETS exposure, yet this positive interaction was replicated in never smokers only. More pronounced effects in never smokers were also found in a previous study showing interactions between ETS exposure and *GSTO1* SNPs, involved in oxidative stress reactions and the detoxification of xenobiotics[6].

Table 4. Significantly (FDR p-value <0.05) and suggestively (FDR p-value <0.25) enriched pathways based on genome-wide interaction SNP-by-ETS exposure p-values within 100 kb of the gene.

Pathway/ Gene set name	Description	FDR corrected p-value	Significant genes/ Selected genes/ All genes
Apoptosis	Apoptosis is a distinct form of cell death that is functionally and morphologically different from necrosis.	**0.003**	22/54/71
P38 MAPK pathway	The Rho family GTPases activate the p38 MAPKs under environmental stress or in the presence of pro-inflammatory cytokines.	0.063	16/33/40
Tumor Necrosis Factor pathway	Tumor necrosis factor is a pro-inflammatory cytokine that activates NF-kB and c-Jun.	0.081	9/22/29

Table 3. Replication of SNP-by-ETS exposure interactions in the SAPALDIA study, for all subjects and stratified for smoking status (never/ever).

LifeLines (identification)							All (10,817)				Never smokers (4,551)				Ever smokers (6,266)			
							95% CI				95% CI				95% CI			
CHR	SNP	Gene	A1	MAF	geno[a,b]	imp_q[c]	B	L95	U95	P	B	L95	U95	P	B	L95	U95	P
5	rs11950494	ACTBL2	G	0.08	1	NA	-110	-164	-55	**7.60E-05**	-142	-240	-44	**4.38E-03**	-115	-183	-47	**9.49E-04**
8	rs2733727	ZFHX4	A	0.05	1	NA	144	80	208	**1.02E-05**	191	75	308	**1.32E-03**	107	28	186	**7.94E-03**
SAPALDIA (replication)							**All (1,276)**				**Never smokers (573)**				**Ever smokers (703)**			
							95% CI				95% CI				95% CI			
CHR	SNP	Gene	A1	MAF	geno[a]	imp_q	B	L95	U95	P	B	L95	U95	P	B	L95	U95	P
5	rs11950494	ACTBL2	G	0.11	0	0.966	-162	-305	-18	**2.73E-02**	-310	-540	-81	**8.31E-03**	-93	-286	100	3.44E-01
8	rs2733727	ZFHX4	A	0.06	1	1.000	-24	-264	215	8.43E-01	373	13	733	**4.30E-02**	-276	-599	47	9.40E-02

[a] Genotyped, 1 = yes, 0 = no
[b] The LifeLines study consists of only genotypes SNPs.
[c] Imputation quality

This preferential effect in never smokers may be due to the fact that active smoking overrules the effect of ETS exposure. *ZFHX4* is a transcription factor that was recently shown to interact with the ATPase CHD4 (a.k.a. mi-2alpha) which is part of the nucleosome remodeling and deacetylase (NuRD) complex[16]. CHD4 and ZFHX4 co-localized at genomic sites of over 4,000 target genes[16], including genes involved in oxidative stress and detoxification of xenobiotic substances which have been previously associated with lung function levels and the prevalence of COPD, such as *ABCC1*, *GSTO1*, and *SOD2*[6,17,18]. This suggests that *ZFHX4* may affect the level of lung function via modulation of expression of genes important in decreasing the oxidative burden caused by ETS exposure[19].

Significant genes in the Apoptosis pathway			SNP-by-ETS exposure	
Gene Name	SNP	-log(*P*)	FEV_1 (ml)	*P*-value
APAF1	rs12581724	4.23	-111	5.88E-05
MYC	rs7829529	3.00	109	1.00E-03
IRF2	rs3775574	2.74	-47	1.81E-03
BNIP3L	rs3808578	2.63	-70	2.35E-03
CASP9	rs4645983	2.52	-52	3.00E-03
BCL2L11	rs1877330	2.47	85	3.40E-03
JUN	rs2716129	2.45	-43	3.52E-03
MAP2K4	rs2013868	2.11	-51	7.73E-03
TNFRSF10B	rs11784599	1.91	-48	1.24E-02
MAPK10	rs7688651	1.86	38	1.39E-02
FAS	rs1926189	1.75	54	1.76E-02
GZMB	rs1957519	1.75	-62	1.79E-02
NFKBIE	rs513688	1.72	37	1.91E-02
TNFRSF1B	rs235214	1.67	50	2.14E-02
BCL2L1	rs6060870	1.64	42	2.29E-02
FADD	rs10751209	1.57	-33	2.71E-02
BAK1	rs210138	1.55	42	2.80E-02
BCL2	rs1801018	1.50	32	3.17E-02
CASP8	rs3769823	1.47	35	3.40E-02
HRK	rs4767462	1.46	-63	3.43E-02
TNFRSF21	rs9463313	1.46	37	3.46E-02
NFKB1	rs2085548	1.38	33	4.17E-02

Table 5. Genes significantly enriched in the apoptosis pathway. Effect estimates (FEV_1 (ml)) and *p*-values from the SNP-by-ETS exposure interaction analysis are given.

In addition to SNP-by-ETS exposure interaction analysis we performed a pathway analysis based interaction p-values obtained from the identification analysis in the LifeLines study. Compared to SNP-by-ETS exposure analysis, pathway analysis may have increased power to detect genetic associations of the phenotype with a gene set/pathway[20]. Three pathways were significantly or suggestively enriched, i.e. the apoptosis, p38 MAPK and TNF pathways. Interestingly all three pathways have been implicated in the pathogenesis of COPD, and may mutually interact.

Apoptosis is a programmed form of cell death, and imbalance between apoptosis and proliferation of alveolar epithelial and endothelial cells has been observed in the lungs of COPD patients[21]. Previous investigations within the SAPALDIA study have found suggestive evidence that genetic variation in the apoptosis pathway modified the effect of packyears

smoked on the decline of FEV_1 [22]. Moreover, SNPs in apoptosis-related genes modified the effects of packyears smoked and exposure to ambient air pollution on the decline of lung function (i.e. FEV_1, FEV_1/FVC and $FEF_{25-75\%}$)[22,23]. Apoptosis is regulated by various pathways. One of the pathways is a response to extracellular signals by binding of members of the tumor necrosis family, such as TNF-alpha with death receptor TNF-receptor 1[21]. For example, cigarette smoke exposure was shown to increase TNF-alpha expression[24]. Another pro-apoptotic pathway responds to physical and chemical stressors via the release of cytochrome C by the mitochondria. Subsequent formation of an apoptosome activates several caspases which eventually initiate apoptosis. Interestingly, we identified an intronic SNP in *APAF1* that interacted with ETS exposure (p-value = $5.88*10^{-5}$), the expressed protein of this gene is part of this apoptosome initiating apoptosis (tables 2 and 5). However, this SNP-by-ETS exposure interaction was not replicated in the SAPALDIA study.

Significant genes in the Tumor Necrosis Factor			SNP-by-ETS exposure	
Gene Name	SNP	-log(*P*)	FEV_1 (ml)	*P*-value
JUN	rs2716129	2.45	-43	3.52E-03
MAP2K4	rs2013868	2.11	-51	7.73E-03
TNFAIP3	rs11970361	1.94	-95	1.14E-02
NFKBIE	rs513688	1.72	37	1.91E-02
TNFRSF1B	rs235214	1.67	50	2.14E-02
FADD	rs10751209	1.57	-33	2.71E-02
MAP3K7	rs205349	1.47	50	3.38E-02
CASP8	rs3769823	1.47	35	3.40E-02
NFKB1	rs2085548	1.38	33	4.17E-02

Table 6. Genes significantly enriched in the Tumor Necrosis Factor pathway. Effect estimates (FEV_1, ml) and *p*-values from the SNP-by-ETS exposure interaction analysis are given.

The TNF pathway was suggestively enriched in the pathway analysis. TNF-alpha is a cytokine playing an important role in inflammation through its activation of several downstream signaling cascades, amongst others the p38 MAPK pathway. Levels of TNF-alpha have been shown to be increased in sputum of COPD patients compared to both non-smoking and smoking controls, and in response to air pollution exposure[25,26]. There was quite some overlap in genes enriched in the TNF alpha (table 6) and the apoptosis pathway (table 5). The second suggestively enriched pathway was the P38 mitogen activated protein kinase (MAPK) pathway, this pathway has also been implicated in the development and/or maintenance of a number of chronic airway inflammatory diseases such as COPD[27]. The p38 MAPK pathway is activated by various environmental stressors, growth factors and cytokines and in turn regulates the expression of inflammatory cytokines such as TNF-alpha and may initiate apoptosis[28]. Increased activation of p38 MAPK was seen in alveolar walls and alveolar macrophages of COPD patients compared to non-smoking and smoking controls[29].

In the current study we used a large and well documented homogeneous sample of a general population, i.e. the LifeLines study, to assess SNP-by-ETS exposure interactions. However, none of the SNPs reached the genome-wide significance threshold (p-value = $2.19*10^{-7}$). We attempted to verify the SNP-by-ETS exposure interactions in a second independent sample, the SAPALDIA study. The SAPALDIA study had similar mean age, level of lung function and smoking

history compared to the LifeLines study, importantly exposure assessment was done in the same manner. The main difference between the two studies was that the SAPALDIA study was enriched in asthmatics (40%), which could have affected the results. Only 2 SNP-by-ETS exposure interactions were replicated with the same direction of effects, which is less than expected based on chance only (i.e. 5% of 44 SNPs = 2.2). The SAPALDIA study sample was relatively small, which may have limited the power to significantly replicate our findings.

To summarize, with the current study we explored gene-by-ETS interactions in association with the level of FEV_1 in a hypothesis-free genome-wide manner. Our results show that pathways previously implicated in COPD pathology may underlie susceptibility to ETS exposure as well.

Significant genes in the p38 MAPK pathway			SNP-by-ETS exposure	
Gene Name	**SNP**	**-log(*P*)**	**FEV_1 (ml)**	***P*-value**
MYC	rs7829529	4.23	109	1.00E-03
TGFBR1	rs12686783	3.00	71	1.39E-03
MAP2K6	rs2716227	2.74	-45	2.36E-03
MAP2K4	rs2013868	2.63	-51	7.73E-03
CREB1	rs722761	2.52	-38	1.38E-02
MAPK14	rs851023	2.47	52	1.47E-02
MEF2A	rs7164257	2.45	-55	1.80E-02
MAP3K9	rs731571	2.11	42	2.01E-02
TGFB2	rs2798631	1.91	35	2.05E-02
HSPB1	rs2908201	1.86	-39	2.22E-02
MAX	rs2763887	1.75	-67	3.32E-02
MAP3K7	rs205349	1.75	50	3.38E-02
MAP3K5	rs2237268	1.72	32	3.41E-02
PLA2G4A	rs10489409	1.67	-53	3.81E-02
MAPKAPK2	rs11119447	1.64	-32	3.87E-02
HMGN1	rs2836992	1.57	-31	3.93E-02

Table 7. Genes significantly enriched in the p38 MAPK pathway. Effect estimates (FEV_1 (ml)) and *p*-values from the SNP-by-ETS exposure interaction analysis are given.

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7

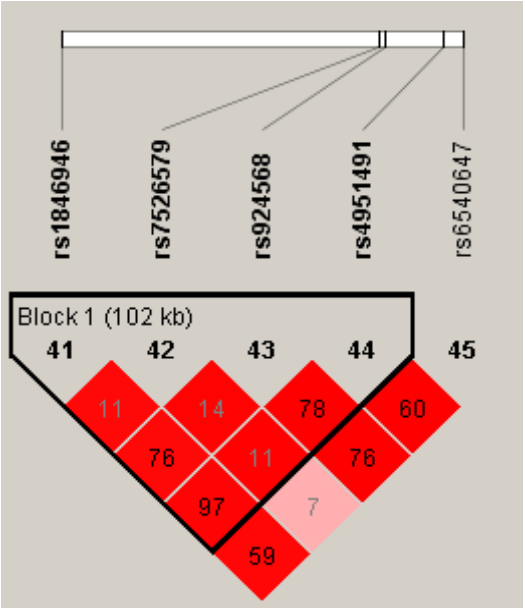
SUPPLEMENTARY MATERIAL

Supplementary table 1. SNPs-by-ETS exposure interactions stratified by smoking status (never/ever) for SNPs with interaction p-values <10^{-4} from the identification analysis in the whole LifeLines sample. Linear regression models were adjusted for sex, age, height, and packyears smoked (in ever smokers).

						Never smokers				Ever smokers			
							95% CI				95% CI		
CHR	SNP	A1	Gene	Functional annotation	LD R2	B	L95	U95	P	B	L95	U95	P
1	rs2859741	T	9.5kb 5' of GRIK3			69	19	119	7.05E-03	73	36	110	1.12E-04
1	rs1846946	T	KCNH1	intronic		44	-10	98	1.07E-01	76	36	115	1.54E-04
1	rs7526579	C	KCNH1	intronic		-51	-115	12	1.13E-01	-80	-126	-35	5.25E-04
1	rs924568	G	KCNH1	intronic	LD block	63	11	114	1.65E-02	88	50	126	5.03E-06
1	rs4951491	C	KCNH1	intronic		47	-6	101	8.39E-02	79	40	118	7.80E-05
1	rs6540647	G	KCNH1	intronic		56	6	107	2.98E-02	67	30	104	3.57E-04
2	rs17031275	A	31kb 3' of LOC285000			142	45	238	4.07E-03	114	44	184	1.49E-03
2	rs4954603	G	185kb 5' of CXCR4		0.54	91	15	166	1.88E-02	93	35	150	1.59E-03
2	rs7570134	G	210kb 5' of CXCR4			107	44	169	8.28E-04	63	16	110	9.22E-03
2	rs10497902	C	77kb 3' of PTH2R			97	27	166	6.21E-03	73	24	122	3.50E-03
3	rs528581	C	75kb 5' of RAP2B			87	34	140	1.31E-03	50	12	87	1.03E-02
3	rs2084386	C	PAK2	intronic		40	-36	115	3.01E-01	121	64	177	2.92E-05
4	rs17062990	G	118kb 3' of SPCS3			-90	-174	-6	3.56E-02	-109	-170	-49	4.21E-04
4	rs4146433	T	147kb 3' of SPCS3		LD block	-74	-152	4	6.16E-02	-96	-150	-41	5.88E-04
4	rs11133161	A	138kb 3' of VEGFC			-63	-142	15	1.12E-01	-106	-163	-49	2.71E-04
4	rs4861505	G	ODZ3	intronic		-99	-158	-40	1.04E-03	-57	-99	-15	8.28E-03
5	rs11950494	G	ACTBL2	3'-UTR		-142	-240	-44	4.38E-03	-115	-183	-47	9.49E-04
5	rs1393082	A	H2AFY	intronic		87	34	141	1.31E-03	48	8	88	1.91E-02
6	rs4421160	T	144kb 3' of CD109			-74	-148	-1	4.73E-02	-101	-159	-43	6.38E-04
6	rs982124	A	KLHL32	intronic		-406	-670	-141	2.65E-03	-250	-431	-69	6.84E-03
6	rs9386622	T	PDSS2	intronic	0.60	73	17	129	1.11E-02	58	18	99	4.89E-03
6	rs11153056	C	PDSS2	intronic		79	23	135	5.86E-03	64	23	105	2.08E-03
7	rs1533956	G	12kb 5' of MIR3147		0.74	71	20	122	6.37E-03	81	43	119	3.19E-05
7	rs11135646	T	18kb 5' of ZNF716			61	7	114	2.64E-02	71	31	112	5.38E-04
8	rs2090789	A	LOC100128993	intronic		-47	-97	4	6.99E-02	-65	-102	-28	5.68E-04
8	rs2733727	A	ZFHX4	intronic		191	75	308	1.32E-03	107	28	186	7.94E-03

Continuation supplementary table 1. SNPs-by-ETS exposure interactions stratified by smoking status (never/ever) for SNPs with interaction p-values $<10^{-4}$ from the identification analysis in the whole LifeLines sample. Linear regression models were adjusted for sex, age, height, and packyears smoked (in ever smokers).

						Never smokers				Ever smokers			
							95% CI				95% CI		
CHR	SNP	A1	Gene	Functional annotation	LD R2	B	L95	U95	P	B	L95	U95	P
8	rs16894633	C	6.2kb 5' of SDC2		0.92	153	65	241	6.69E-04	113	47	179	8.10E-04
8	rs16894649	A	SDC2	intronic		136	49	224	2.32E-03	111	46	176	8.25E-04
8	rs12056723	G	SDC2	intronic		-163	-265	-61	1.83E-03	-123	-198	-47	1.42E-03
8	rs7831729	C	15kb 5' of SNX31			-64	-130	3	6.06E-02	-86	-133	-39	3.72E-04
8	rs13282467	A	LRRC6	intronic		-184	-347	-21	2.68E-02	-173	-293	-54	4.51E-03
9	rs7030493	T	TMEM2	intronic	0.93	-91	-182	1	5.17E-02	-122	-189	-55	3.52E-04
9	rs1552708	G	TMEM2	intronic		-90	-180	0	5.08E-02	-130	-195	-64	9.83E-05
10	rs2174257	G	PRKG1	intronic		-34	-85	18	2.01E-01	-71	-108	-33	2.31E-04
10	rs2593163	G	PSAP	intronic		101	19	182	1.53E-02	104	41	166	1.18E-03
12	rs12581724	C	APAF1	intronic		-227	-317	-137	8.02E-07	-63	-132	6	7.50E-02
12	rs225574	T	LOC400084	intronic		-61	-128	5	6.80E-02	-89	-137	-41	2.88E-04
15	rs6496799	G	124kb 3' of SV2B			-56	-125	13	1.14E-01	-96	-144	-48	8.27E-05
16	rs8052564	A	RBFOX1	intronic		152	47	256	4.39E-03	105	37	173	2.35E-03
17	rs8067644	T	PIK3R6	intronic		-15	-67	38	5.88E-01	-86	-125	-47	1.31E-05
18	rs7233554	C	20kb 3' of CYB5A			-106	-199	-13	2.56E-02	-103	-169	-36	2.49E-03
19	rs7976	T	KRTDAP	3'-UTR		42	-18	101	1.68E-01	82	37	127	3.34E-04
19	rs311384	G	ARHGAP35	intronic		70	17	124	9.69E-03	71	31	111	5.58E-04
20	rs753320	A	27kb 5' of SCRT2			0	-77	76	9.96E-01	-119	-172	-66	9.81E-06
22	rs743262	A	206kb 3' of MN1			274	65	482	1.02E-02	266	98	433	1.87E-03



Supplementary figure 1. LD structure for SNPs located in *KCHN1* in the identification sample (LifeLines).

8

Genome-wide interaction study of gene-by-occupational exposures on the level of FEV_1

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ABSTRACT

Rationale

Chronic obstructive pulmonary disease (COPD) is a complex disease characterized by a low level of lung function and airway obstruction resulting from interactions between multiple genes and multiple environmental exposures. So far, genome-wide association studies have largely disregarded environmental factors that may trigger the development of this disease, like occupational exposures that are thought to contribute to 15-20% of the COPD prevalence.

Objectives

We performed a genome-wide interaction study to identify novel susceptibility loci for occupational exposure to biological dust, mineral dust and gases and fumes in relation to the level of FEV_1.

Methods and Measurements

We performed an identification analysis in 12,400 subjects from the LifeLines cohort study, and verified our findings in 1,436 subjects from a second independent cohort, i.e. Vlagtwedde-Vlaardingen. Additionally we assessed whether replicated SNPs were cis-acting expression (mRNA) quantitative trait loci in lung tissue.

Main Results

Of the 7 replicated SNPs that interacted with one of the occupational exposures, several identified loci were plausible candidates that may be involved in biological pathways leading to lung function impairment, for example *PCDH9* and *GALNT13*. Two of the 7 replicated SNPs were cis-acting eQTL associated with gene expression of *PDE4D* and *TMEM176A* in lung tissue.

Conclusions

This genome-wide interaction study on occupational exposures in relation to the level of lung function identified several novel genes. Further research should determine whether the identified genes are true susceptibility loci for occupational exposures, and whether these SNP-by-exposure interactions consequently contribute to the development of COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common disease with a large morbidity and mortality worldwide[1]. Despite the recognition of COPD as a major and increasing public health problem, there is still limited understanding about the cellular and molecular pathways driving the development of this disease[2]. COPD is a complex disease characterized by a low level of lung function and airway obstruction resulting from interactions between multiple genes and multiple environmental exposures[3].

Previous candidate gene approaches have shown that genetic variants may affect the level of lung function, decline of lung function and the risk for COPD[4]. For example single nucleotide polymorphisms (SNPs) in *MRP1* were associated with the level and decline of lung function[5] and SNPs in *ADAM33* were associated with the decline of lung function and COPD development[6]. Moreover, genetic variation, for example in the gene coding for the antioxidant enzyme glutamate cysteine ligase (GCL) has been shown to increase susceptibility to environmental exposures with known detrimental effects, such as tobacco smoke[7]. These candidate gene studies have mostly been driven by hypotheses relying on known biological pathways. More recently, hypothesis free genome-wide association (GWA) studies have identified novel genetic loci associated with lung function levels and risk for COPD[8-12]. However, these studies have so far disregarded environmental factors that may trigger the development of the disease [3]. Therefore the next step is to perform genome-wide interaction (GWI) studies to identify genetic loci that affect the susceptibility for the effects of known harmful exposures. Such findings may consequently contribute to the understanding of biological pathways driving lung function impairment and the development of COPD. Moreover, they may shed light on the identification of susceptible subgroups within the general population and eventually may help to set exposure limits based on the most susceptible subgroups.

Although tobacco smoking is still considered the main risk factor for reduced lung function level, accelerated lung function decline and development of COPD, about 15-20% of all COPD cases have been attributed to occupational exposures[13]. Occupational exposure to vapors, gases, dusts, and fumes is common and has been associated with decreased levels of lung function and increased risk for COPD[14-20]. Recently a genome-wide study was published that investigated genetic susceptibility to dust exposure in the general population[21]. This study identified one genome-wide significant SNP in *SLC38A8,* but did not replicate findings in a second independent cohort.

The current GWI study aimed to identify novel susceptibility loci for several types of occupational exposures, i.e. biological dust, mineral dust and gases and fumes, in relation to the level of FEV_1 in a general population cohort. We used a second independent cohort to verify our initial findings. Furthermore, the functional meaning of newly identified SNPs interacting with occupational exposure on the level of lung function was extended to gene expression in lung tissue.

METHODS

Identification sample

Genotyped individuals from the LifeLines cohort study with full data on all covariates were included (n = 12,400). The LifeLines cohort study is a general population based cohort that started in 2006, including subjects from the three Northern provinces of the Netherlands[22]. At baseline all participants filled in a standardized questionnaire and were subject to a medical examination including spirometry following ATS guidelines.

Occupational exposure

Self-reported job title and description were coded according to the International Standard Classification of Occupations version 1988 (ISCO-88)[23]. These four-digit codes were used to estimate job-specific exposure to biological dust, mineral dust and gases and fumes using the ALOHA+ Job Exposure Matrix (JEM)[18, 20]. The ALOHA+ JEM classifies subjects based on the ISCO-88 job codes into no, low, and high exposure categories (0,1,2).

Genotyping and quality control

Genotyping was performed using IlluminaCytoSNP-12 arrays. SNPs that fulfilled the quality control criteria were included: genotype call-rate ≥95%, minor allele frequency ≥1%, and Hardy-Weinberg equilibrium cut-off p-value ≥10^{-4}. Non-Caucasian samples and first-degree relatives were excluded.

Statistical analysis

SNPs were tested in an additive genetic model. Effects of SNP-by-exposure interactions (i.e. SNP-by-low and SNP-by-high exposure) on the level of FEV_1 were tested using a linear regression model adjusted for sex, age, height and ever smoking (no/yes) in the software package PLINK (PLINK version 1.07[24]). We selected SNPs for replication based on the SNP-by-high exposure interaction.

Replication sample

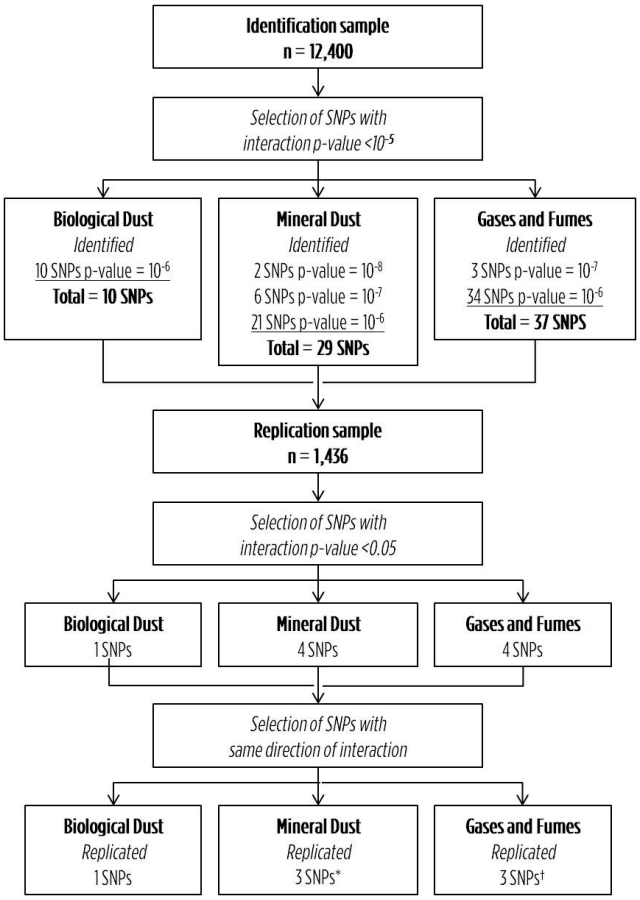
We included 1,436 subjects, having full data on genotypes and covariates from the Vlagtwedde-Vlaardingen cohort, a prospective general population based cohort including Caucasians from Dutch decent, to verify our initial findings. We used data from the last survey in 1989/1990. Genotyping, exposure assessment and statistical analysis were all performed as described in the identification cohort.

Selection of SNPs

Identification

We selected SNPs that interacted with high exposure to biological dust, mineral dust or gases/fumes in the identification analysis (p-values $<10^{-5}$; figure 1). Subsequently, we assessed whether the selected SNPs interacted with high occupational exposure in the replication cohort (p-values <0.05). Finally, SNPs that had an interaction effect in the same direction in both cohorts were meta-analyzed and further investigated in the gene expression analysis.

Figure 1. Flow chart showing the selection of SNPs.



* One SNP reached genome-wide significance after meta-analysis.

† Two SNPs reached genome-wide significance after meta-analysis.

Gene expression analysis

We assessed whether the replicated SNPs were cis-acting expression (mRNA) quantitative trait loci (cis-eQTLs) in lung tissue. Lung tissue was collected from patients who underwent lung resectional surgery at three participating sites; University of Groningen, Laval University and University of British Columbia[25]. DNA samples were genotyped with Illumina Human1M-Duo BeadChip arrays, and gene expression profiles were obtained using a custom Affymetrix array[25]. Gene expression levels were log transformed (2-Log) and adjusted for the first 25 principal components.

Linear regression analysis was used to test for association between the SNP genotypes and gene expression levels. We defined a cis-eQTL as a SNP that was significantly associated with expression levels of a gene within a 2 Mb distance of that SNP, with a p-value below the Bonferroni corrected threshold (p = 0.05/number of probe sets within the 4 Mb window).

RESULTS

Characteristics of the study populations and the prevalence of exposures can be found in table 1. The genomic inflation factor for the identification sample suggests little population stratification (λ = 1.05, online supplement figure E1). The number of subjects in each exposure category (no/low/high), minor allele frequencies (MAF) (tables E1 and E2) and more detailed information about each model, i.e. the SNP and exposure main effects (tables E3-E5) can be found in the online supplement.

Table 1. Characteristics of the subjects included in the identification (LifeLines) and replication (Vlagtwedde-Vlaardingen) cohorts.

	LifeLines	Vlagtwedde-Vlaardingen
N with non-missing data	12400	1436
Males, n (%)	5123 (41)	772 (54)
Age (yrs), median (min-max)	47 (18-89)	53 (35-79)
Smoking status		
Never, n (%)	5070 (41)	431 (30)
Ever, n (%)	7330 (59)	1005 (70)
Lung function, mean (sd)		
FEV_1%predicted (%)*	102 (14)	93 (16)
FEV_1/VC	76 (7)	74 (9)
Exposure, high level of, n (%)		
Biological dust	505 (4)	126 (9)
Mineral dust	590 (5)	307 (21)
Gases and Fumes	739 (6)	140 (10)

* FEV_1 as percentage of predicted following the reference equations of Quanjer et al (1993)[38].

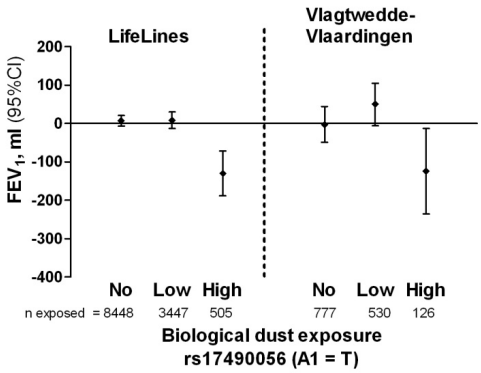
SNP by exposures interactions

Biological dust

10 SNPs interacted with high exposure to biological dust on the level of FEV_1 in de identification sample (p-values for interaction $<10^{-5}$) (figure 1). The interaction with one SNP was replicated significantly in the second sample ($p < 0.05$), and this interaction was in the same direction in both cohorts (table 2). SNP rs17490056 is located at an intergenic region at chromosome 13 nearby *protocadherin-9* (*PCDH9)*. The minor allele (A1) of this SNP is associated with a lower FEV_1 in subjects with high exposure to biological dust (figure 2).

The interaction effect did not reach genome-wide significance after meta-analysis of the effect estimates from both cohorts (table 2).

Figure 2. Additive associations (for minor allele A1) between the SNP and the level of FEV_1 in subjects with no, low and high exposure to biological dust.



Mineral dust

We identified 29 SNPs that interacted with high exposure to mineral dust on the level of FEV_1 (figure 1). Interactions with 4 SNPs were significantly replicated ($p < 0.05$), of which 3 interactions were in the same direction in both cohorts (table 2). Of the 3 SNPs, there was one intronic SNP located in *GALNT13* (table 2). The minor allele of SNP rs6751439 in *GALNT13* was associated with a lower level of FEV_1 in the subjects with high exposure to mineral dust (figure 3c). The other 2 SNPs that interacted with high exposure to mineral dust on the level of FEV_1 were both intergenic variants located nearby genes *ZMAT4* and *OLIG3*. The minor alleles of rs13278529 (nearby *ZMAT4*) and rs473892 (nearby *OLIG3*) were associated with a higher level of FEV_1 in subjects with high exposure to mineral dust (figure 3a and b). SNP rs13278529 nearby *ZMAT4* reached genome-wide significance after meta-analysis of the effects from both cohorts, meta-analyzed interaction effects of the other two SNPs were borderline significant (table 2).

Gases and fumes

We identified 37 SNPs that interacted with high exposure to gases and fumes on the level of FEV_1 (figure 1). Interactions with 4 SNPs were significantly replicated ($p < 0.05$), of which 3 SNPs had interactions with gases and fumes in the same direction in both cohorts (table 2). These 3 SNPs were located in intergenic regions nearby genes *PDE4D, ODZ2 and TMEM176A*. Effects of the SNPs in the groups with no, low and high exposure are shown in figure 4. The minor alleles of rs159497 (nearby *PDE4D*) and rs2888674 (nearby *TMEM176A*) were associated with a higher level of FEV_1 in subjects with high exposure to gases and fumes (figure 4a and c), the minor allele of rs516732 (nearby *ODZ2*) was associated with a lower FEV_1 in subjects with high exposure to gases and fumes (figure 4b). The interaction effects of SNP rs159497 nearby *PDE4D* and rs516732 nearby *ODZ2* reached genome-wide significance after meta-analysis, the meta-analyzed interaction effect of rs2888674 nearby *TMEM176A* was borderline significant (table 2).

Table 2. Interactions between SNPs (additive effect for minor allele A1) and high exposure to biological dust, mineral dust and gases and fumes on the level of FEV_1 (ml). Linear regression models were adjusted for gender, age, height and smoking status (never/ever). Minor allele frequency (MAF) is given for A1.

Biological dust				Annotation	Identification					Replication					Meta-analysis			
SNP	**CHR**	**A1**	**A2**	**Nearest gene**	**N**	**B_{int}**	**SE_{int}**	**P_{int}**	**MAF**	**N**	**B_{int}**	**SE_{int}**	**P_{int}**	**MAF**	**I**	**Q**	**B_f**	**P_f***
rs1749005	13	T	C	PCDH9 (150 Kb 3')	12400	-136.9	30.7	8.26E-06	0.50	1433	-121.2	61.5	4.89E-02	0.48	0	0.82	-133.8	1.12E-06
Mineral dust				Annotation	Identification					Replication					Meta-analysis			
SNP	**CHR**	**A1**	**A2**	**Nearest gene**	**N**	**B_{int}**	**SE_{int}**	**P_{int}**	**MAF**	**N**	**B_{int}**	**SE_{int}**	**P_{int}**	**MAF**	**I**	**Q**	**B_f**	**P_f***
rs1327852	8	G	T	ZMAT4 (55 Kb 3')	12400	178.4	38.4	3.49E-06	0.15	1436	164.8	59.7	5.87E-03	0.19	0	0.85	174.4	**6.77E-08**
rs473892	6	T	C	OLIG3 (136 Kb 5')	12400	126.3	27.3	3.89E-06	0.46	1436	93.9	43.4	3.06E-02	0.49	0	0.53	117.1	4.15E-07
rs6751439	2	A	G	GALNT13 (intronic)	12400	-184.8	40.5	5.16E-06	0.13	1415	-134.8	65.1	3.85E-02	0.12	0	0.51	-170.8	6.86E-07
Gases and Fumes				Annotation	Identification					Replication					Meta-analysis			
SNP	**CHR**	**A1**	**A2**	**Nearest gene**	**N**	**B_{int}**	**SE_{int}**	**P_{int}**	**MAF**	**N**	**B_{int}**	**SE_{int}**	**P_{int}**	**MAF**	**I**	**Q**	**B_f**	**P_f***
rs159497	5	C	T	PDE4D (57 Kb 3')	12400	131.1	26.3	6.44E-07	0.46	1435	136.3	62.4	2.90E-02	0.46	0	0.94	131.9	**5.42E-08**
rs516732	5	C	T	ODZ2 (1.6 Mb 5')	12400	-115.4	25.0	3.76E-06	0.47	1436	-158.5	61.1	9.61E-03	0.47	0	0.51	-121.6	**1.42E-07**
rs2888674	7	A	G	TMEM176A (8.7 kb 3')	12400	122.2	26.6	4.36E-06	0.46	1436	130.5	62.1	3.58E-02	0.45	0	0.90	123.5	4.33E-07

* Genome-wide significance p-value = 2.26E-07 (Number of SNPs in meta-analysis: biological dust = 221,593; mineral dust = 221,663; gases and fumes = 221,638).

Expression Quantitative Trait Loci (eQTL)

We investigated whether the 7 identified and replicated SNPs were associated with gene expression levels in lung tissue from 1,095 patients. Two SNPs showed cis-eQTL associations with p-values below the Bonferroni corrected threshold (supplementary table E6). Rs159497, the SNP that significantly interacted with high exposure to gases and fumes was associated with expression of *PDE4D* ($p = 1.81*10^{-4}$) (figure 5a). SNP rs2888674, that also interacted with high exposure to gases and fumes was significantly associated with expression levels of both its left and right neighboring genes *TMEM176A* and *ABP1*, p-values were $1.73*10^{-16}$ and $1.54*10^{-5}$ respectively (figures 5b and c).

DISCUSSION

This is the first genome-wide gene-by-occupational exposure interaction study on the level of lung function with replication in an independent cohort. We identified 7 SNPs that were associated with the level of FEV_1 in subjects with high exposure to one of the occupational exposures biological dust, mineral dust or gases and fumes. All SNPs had relevant main effects in the highly exposed individuals, and in most cases there were no effects in the individuals with no exposure. This supports the hypothesis that there are individual differences in genetic susceptibility to occupational exposure to biological dust, mineral dust and gases and fumes.

Most loci are novel and have not been studied in relation to lung function impairment or COPD to date (table 3). Several identified SNPs were located in or nearby genes that may be involved in biological pathway leading to lung function impairment (i.e. *GALNT13* and *PCDH9* respectively). GALNT13 belongs to the GalNAcT family of enzymes which initiate O-glycosylation of mucins. Terminal sugars may affect the physical and/or biological properties of mucins, and altered glycosylation has been found in airways disease like cystic fibrosis[26, 27]. Polymorphisms in *GALNT13* have been associated with survival time among mice with acute lung injury induced by acrolein[28].

Moreover, a recent genome-wide interaction study identified 2 intronic SNPs in *GALNT13* that interacted with *in utero* tobacco smoke exposure on childhood asthma, however these interactions could not be replicated[29]. *PCDH9* is a protocadherin belonging to the cadherin superfamily of adhesion molecules. Family member *PCDH1* has been identified as a susceptibility gene for bronchial hyperresponsiveness[30]. In a recent GWA study two SNPs nearby *PCDH9* (rs17077331 and rs17077335) were associated with FEV_1/FVC decline in non-asthmatics, although this association was only driven by a single study[31]. The identified SNPs were not cis-eQTLs for *GALNT13* and *PCDH9* in lung tissue in our study, but may be associated with lung function impairment via other biological mechanisms. For example SNPs may change protein structure and consequently alter GALNT13 enzyme activity or functionality of PCDH9.

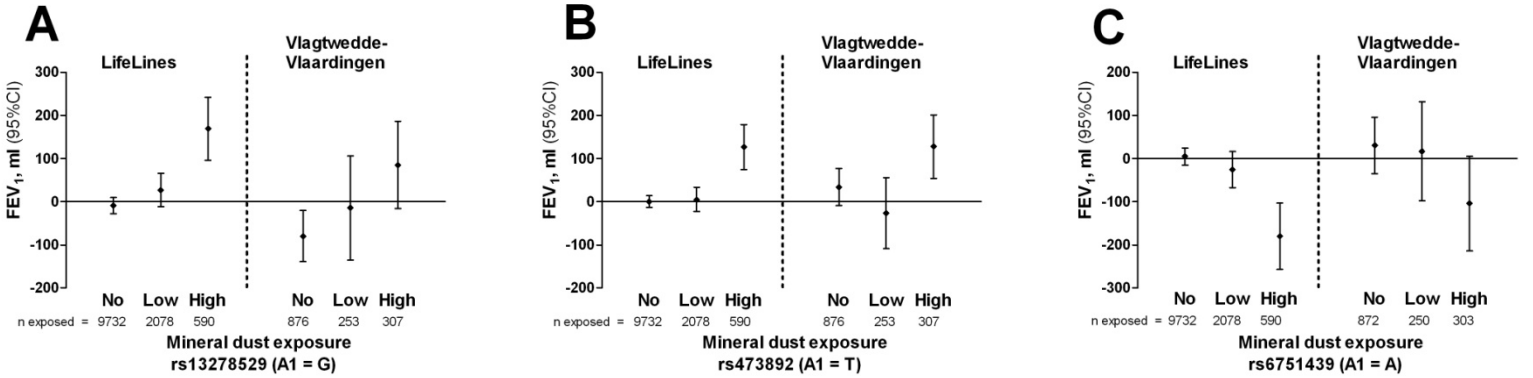


Figure 3. Additive associations (for minor allele A1) between the SNPs and the level of FEV_1 in subjects with no, low and high exposure to mineral dust.

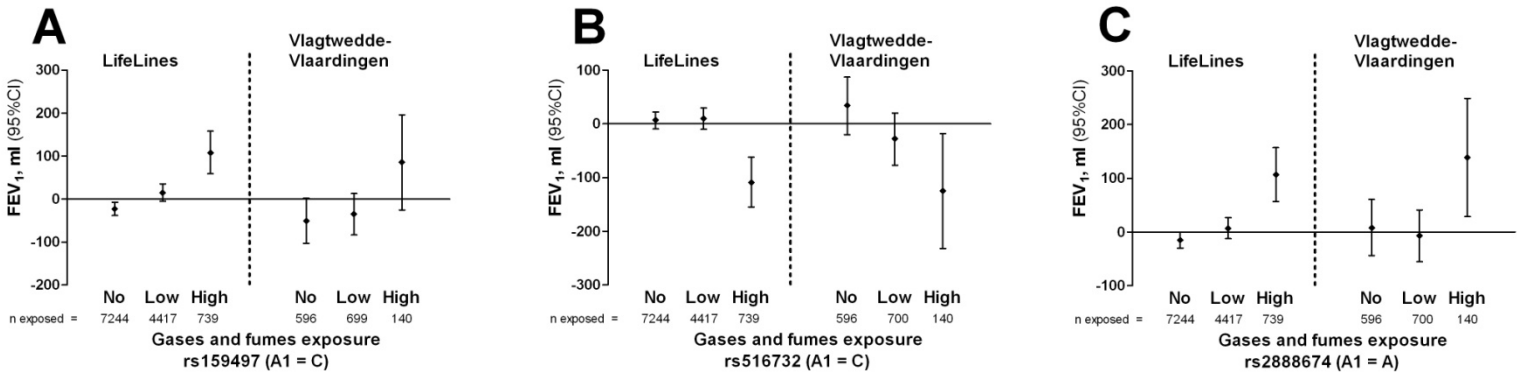


Figure 4. Additive associations (for minor allele A1) between the SNPs and the level of FEV_1 in subjects with no, low and high exposure to gases and fumes.

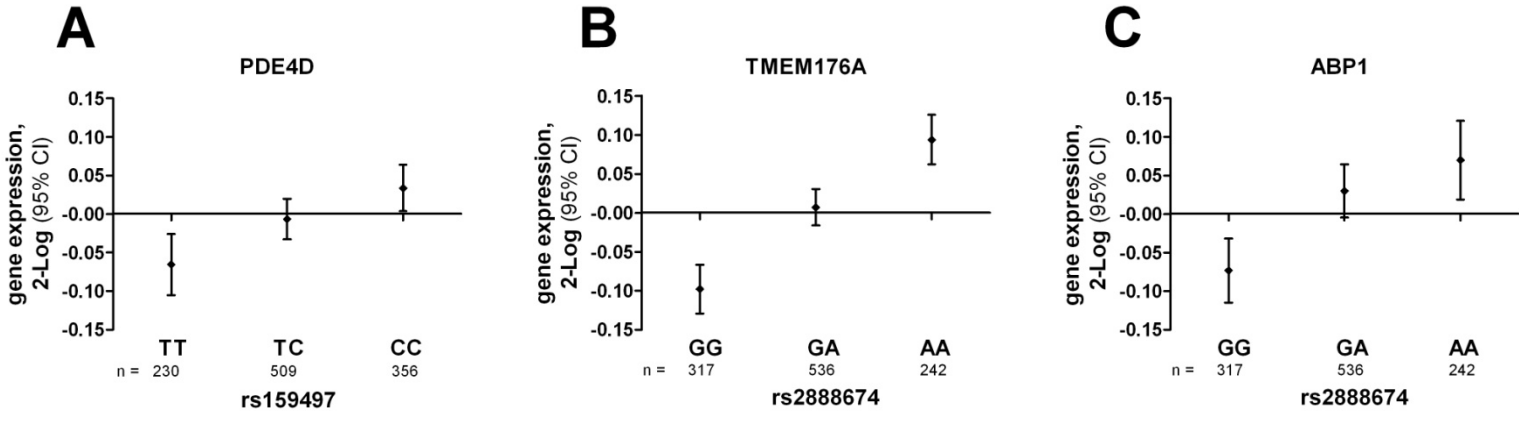


Figure 5. Gene expression levels stratified by genotype for cis-eQTL SNPs.

In the cis-eQTL analysis we identified two intergenic SNPs that were associated with expression of nearby genes in lung tissue. The strongest cis-eQTL association was found for rs2888674 associated with expression of *TMEM176A*. Decreased expression of *TMEM176A* has been shown to increase expression of co-stimulatory molecules CD86 and CD40 in mice bone-marrow dendritic cells (DCs), thereby causing DC maturation and consequently immune response stimulation like T cell differentiation and survival[32]. In humans, Freeman et al found a positive correlation between COPD severity and increased expression of these co-stimulatory molecules on pulmonary DCs[33]. In the current study the minor allele for rs2888674 was associated with higher expression of *TMEM176A*. Higher expression of *TMEM176A* may lead to lower expression of co-stimulatory molecules on DCs and consequently decreased immune and inflammatory response to environmental stimuli like gases and fumes, as was suggested by the higher level of FEV_1 associated with the minor allele of rs2888674 found in our study.

The second cis-eQTL is rs159497 associated with higher expression of the nearby gene *PDE4D*. PDE4D is a regulator of airway smooth-muscle contractility and there is evidence from the literature supporting a role for PDE4D in lung pathology, i.e. *PDE4D* has been shown as susceptibility gene for asthma[34] and for a lower level of FEV_1 in ever smokers[12]. PDE4 enzymes metabolize cAMP to 5 ` AMP, and inhibition of these PDE4 enzymes decreases the activity of inflammatory cells in association with bronchodilation. This seems contradictory to our finding that the minor allele for rs2221132 was associated with increased expression of *PDE4D* in lung tissue as well as with a higher level of FEV_1 in subjects with high exposure to gases and fumes. We have studied expression in whole lung tissue and expression may be different in specific cell types, such as epithelial or alveolar cells, where gases and fumes might act on. Moreover, the SNP could be associated with a specific alternative splice variant of *PDE4D*. Alternative splice variants have been shown to be differentially expressed and regulated in lung tissue[35, 36].

Compared to the recently published GWI study on general dust exposure[21], we investigated several types of occupational exposures, i.e. biological dust, mineral dust and gases and fumes. Job exposure was estimated using the ALOHA+ JEM that is specifically designed for population-based studies. In general, job exposure matrix-based exposure estimates are less likely to be affected by recall bias and differential misclassification compared to self-reports[37]. Moreover, to our knowledge this is the first GWI study on occupational exposures that replicated findings in a second independent cohort. Both the identification and replication cohorts included only Caucasian individuals from Dutch decent, and occupational exposure assessment was performed using the same method in both samples. Finally we extended our findings to gene expression analysis in lung tissue.

The major difference between the two samples included in the current study were the higher prevalence of occupational exposures and current smokers in the replication sample (Vlagtwedde-Vlaardingen) compared to the identification sample (LifeLines). This may be explained by historical timing of both studies, i.e. the identification sample included data

from measurements performed between 2006 and 2011, whereas the replication sample included data from measurements performed in 1989/1990.

Because our GWI study is explorative in character, we wanted to keep the risk low of not detecting a true association and therefore used a more liberal p-value threshold for identification of SNPs in the first sample ($p < 10^{-5}$). When we assessed these interactions in the second independent sample we found more significant interactions than expected based on chance only. Moreover, the additive SNP effects in the highly exposed subjects were of clinical relevance, with effects between 100 and 200 ml FEV_1 per minor allele for most replicated SNPs. Finally, the cis-eQTL analysis showed that 2 SNPs identified in the interaction analysis were significantly associated with expression levels of neighboring genes. This are unique data and provides additional support for a possible role of these genes in lung function impairment.

In conclusion, this is the first genome-wide study with replication in a second independent cohort that investigated interactions of SNPs with several types of occupational exposures on the level of lung function. We identified several plausible candidates that may be involved in biological pathways leading to lung function impairment, i.e. *PCDH9*, *GALNT13*, *PDE4D and TMEM176A*. Further research should determine whether the identified (novel) genes are true susceptibility loci for lung function impairment due to occupational exposure to biological dust, mineral dust and gases and fumes, and whether these SNP-by-exposure interactions consequently increase the risk to develop COPD. This information may eventually contribute to the understanding of cellular and molecular pathways driving the development of COPD. Moreover, since occupational exposures are common, but modifiable, this knowledge may be used to set exposure limits considering susceptible subgroups.

Table 3. Putative function of the identified and replicated genes.

SNP	CHR	Nearest gene	Putative function of nearest gene
rs17490056	13	*PCDH9* (150 Kb 3')	*Protocadherin 9 (PCDH9).* PCDH9 expression was shown in epithelial regions of the nasal cavity[39]. PCDH9 is a member of the family δ1-protocadherins that are thought to be involved in intracellular signaling. Family member *PCDH1* has been identified as a susceptibility gene for bronchial hyperresponsiveness[30]. In a recent GWA study, two SNPs nearby *PCDH9* (rs17077331 and rs17077335) were associated with FEV_1/FVC decline in non-asthmatics, although this association was only driven by a single study (Imboden et al, 2012, online supplement)[31].
rs13278529	8	*ZMAT4* (55 Kb 3')	*zinc finger, matrin-type 4 (ZMAT4).* SNPs in *ZMAT4* have been associated with fasting blood glucose[40] and refractive error, a leading cause of visual impairment[41].
rs473892	6	*OLIG3* (136 Kb 5')	*Oligodendrocyte Transcription Factor 3 (OLIG3).* OLIG3 is involved in specification of class A and B neurons. The OLIG3/TNFAIP3 locus is associated with autoimmune diseases, like rheumatoid arthritis[42] and coeliac disease[43]
rs6751439	2	*GALNT13* (intronic)	*UDP-N-Acetyl-Alpha-D-Galactosamine:Polypeptide N-Acetylgalactosaminyltransferase 13 (GALNT13, GalNAcT13).* GALNT13 belongs to the GalNAcT family of enzymes which initiate O-glycosylation of mucins. O-glycosylation may affect the physical and/or biological properties of mucins. Sites for O-glycosylation vary between tissues, during inflammation and in cancer[44]. Polymorphisms in *GALNT13* have been associated with survival time among mice with acute lung injury induced by acrolein[28]. A recent genome-wide interaction study identified 2 intronic SNPs in *GALNT13* that interacted with *in utero* tobacco smoke exposure on childhood asthma, however these interactions could not be replicated[29].
rs159497	5	*PDE4D* (57 Kb 3')	*Phosphodiesterase-4D (PDE4D). PDE4D* is an important cAMP-metabolizing enzyme expressed in immune and inflammatory cells, airway smooth muscle and pulmonary nerves. The PDE4 enzyme plays a significant role in modulating the activity of cAMP, an important second messenger that mediates the relaxation of airway smooth muscle[45]. Evidence from literature supports a role for PDE4D in lung pathogeneses, i.e. *PDE4D* has been shown as susceptibility gene for asthma[34] and was associated with FEV_1 in ever smokers[12].

Continuation Table 3. Putative function of the identified and replicated genes.

rs516732	5	*ODZ2* (1.6 Mb 5')	Encodes *Teneurin-2.* Teneurin-2 is a transmembrane glycoprotein. Teneurins have been mainly studied in relation to neural development where they seem to regulate the establishment of proper connectivity within the nervous system by promoting neurite outgrowth and cell adhesion. Recently Teneurin-2 was shown to be expressed in the majority of malignant mesothelioma cell lines, but not in lung adenocarcinoma cell lines investigated[46, 47]. Mesothelioma is a rare form of cancer developing in the mesothelium, the protective body that covers many of the internal organs of the body, like the pleura (the outer lining of the lungs and internal chest wall). Mesothelioma is most commonly caused by (occupational) exposure to asbestos.
rs2888674	7	*TMEM176A* (8.7 Kb 3')	*Transmembrane Protein 176A.* TMEM176A was shown to be highly expressed in the lungs, and more specifically to be highly expressed in bone-marrow derived dendritic cells (BMDCs) and in classical dendritic cells (cDC). Moreover, TMEM176A was down-regulated in mice BMDCs after inflammatory stimulation by LPS or Poly I:C. Additionally, inhibition of TMEM176A expression in immature BMDCs increased the expression of co-stimulatory molecules CD86 and CD40. These findings suggested that TMEM176A, together with TMEM176B, plays a role in maintaining the immature state of DC (which seems not important anymore in more matured DCs)[32]. Another study showed that TMEM16A expression levels in the lungs were low, yet proteins levels were increased in lung carcinoma[48].

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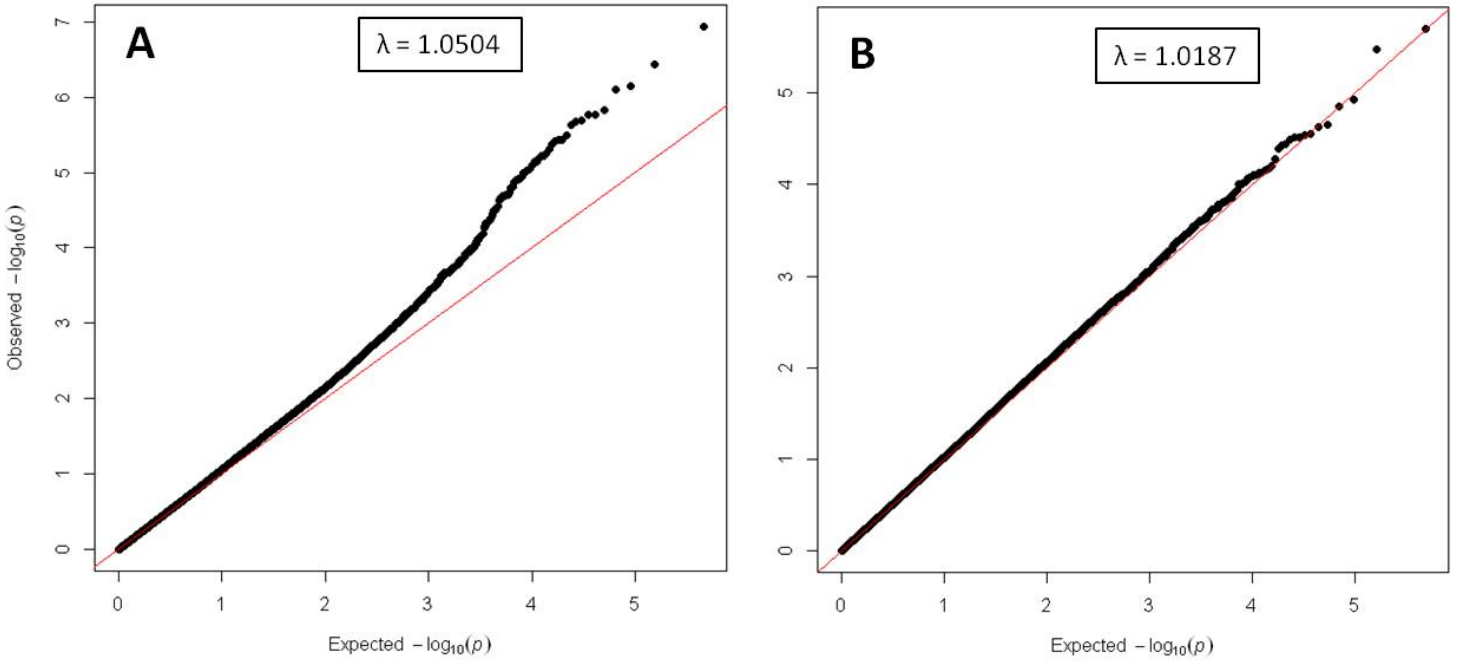
8

SUPPLEMENTARY MATERIAL



Supplementary Figure E1. QQ-plots for the marginal association between the SNPs and level of FEV_1 in the identification (A) and replication sample (B). The analysis was adjusted for sex, age, height and ever smoking (no/yes).

Supplementary Table E1. Genotype frequencies in non-exposed, low exposed and high exposed subjects included in the identification analyses. A1 is the minor (mutant) allele.

Identification													
Biological dust		Non-exposed				Low exposed				High exposed			
SNP	**A1**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**
rs17490056	T	8448	2129 (25)	4207 (50)	2112 (25)	3447	905 (26)	1646 (48)	896 (26)	505	129 (26)	257 (51)	119 (24)
Mineral dust		Non-exposed				Low exposed				High exposed			
SNP	**A1**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**
rs13278529	G	9732	7124 (73)	2423 (25)	185 (2)	2078	1498 (72)	524 (25)	56 (3)	590	421 (71)	153 (26)	16 (3)
rs473892	T	9732	2888 (30)	4838 (50)	2006 (21)	2078	607 (29)	1025 (49)	446 (22)	590	191 (32)	270 (46)	129 (22)
rs6751439	A	9732	7424 (76)	2149 (22)	159 (2)	2078	1545 (74)	495 (24)	38 (2)	590	437 (74)	141 (24)	12 (2)
Gases and fumes		Non-exposed				Low exposed				High exposed			
SNP	**A1**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**
rs159497	C	7244	2053 (28)	3652 (50)	1539 (21)	4417	1238 (28)	2273 (51)	906 (21)	739	215 (29)	383 (52)	141 (19)
rs516732	C	7244	2032 (28)	3586 (50)	1626 (22)	4417	1235 (28)	2230 (50)	952 (22)	739	212 (29)	346 (47)	181 (24)
rs2888674	A	7244	2098 (29)	3585 (49)	1561 (22)	4417	1320 (30)	2149 (49)	948 (22)	739	200 (27)	394 (53)	145 (20)

WT = wild type, HZ = heterozygote, HM = homozygote mutant for allele A1.

Supplementary Table E2. Genotype frequencies in non-exposed, low exposed and high exposed subjects included in the replication analyses. LD (r-squared) was also shown if applicable. A1 is the minor (mutant) allele.

Replication													
Biological dust		Non-exposed				Low exposed				High exposed			
SNP	**A1**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**
rs17490056	T	777	179 (23)	403 (52)	195 (25)	530	131 (25)	269 (51)	130 (25)	126	32 (25)	60 (48)	34 (27)
Mineral dust		Non-exposed				Low exposed				High exposed			
SNP	**A1**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**
rs13278529	G	876	646 (74)	206 (24)	24 (3)	253	197 (78)	51 (20)	5 (2)	307	224 (73)	75 (24)	8 (3)
rs473892	T	876	281 (32)	428 (49)	167 (19)	253	82 (32)	128 (51)	43 (17)	307	104 (34)	148 (48)	55 (18)
rs6751439	A	862	655 (76)	194 (23)	13 (2)	250	188 (75)	56 (22)	6 (2)	303	239 (79)	57 (19)	7 (2)
Gases and fumes		Non-exposed				Low exposed				High exposed			
SNP	**A1**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**	**Total**	**WT**	**HZ**	**HM**
rs159497	C	596	165 (28)	297 (50)	134 (22)	699	207 (30)	344 (49)	148 (21)	140	37 (26)	73 (52)	30 (21)
rs516732	C	596	163 (27)	307 (51)	126 (21)	700	200 (29)	348 (50)	152 (22)	140	42 (30)	68 (49)	30 (21)
rs2888674	A	596	186 (31)	291 (49)	119 (20)	700	207 (30)	342 (49)	151 (22)	140	37 (26)	72 (51)	31 (22)

WT = wild type, HZ = heterozygote, HM = homozygote mutant for allele A1.

Supplementary Table E3. Estimates for the effects of SNP, biological dust exposure and their interaction on the level of FEV_1 (ml) using linear regression models adjusted for gender, age, height and smoking status (never/ever).

Biological dust			Identification				Replication			
				95% CI				95% CI		
SNP	**A1**	**Predictor**	**B**	**Lower**	**Upper**	**P**	**B**	**Lower**	**Upper**	**P**
rs17490056	T	SNP (additive)	7.3	-6.8	21.4	3.12E-01	-2.7	-49.5	44.0	9.09E-01
		Low exposure	-20.5	-52.6	11.6	2.11E-01	-138.2	-227.3	-49.0	2.43E-03
		High exposure	56.4	-16.4	129.2	1.29E-01	33.4	-116.7	183.6	6.63E-01
		SNP*Low exposure	1.0	-24.9	26.9	9.41E-01	52.4	-20.4	125.1	1.58E-01
		SNP*High exposure	-136.9	-197.1	-76.8	8.26E-06	-121.2	-241.7	-0.7	4.89E-02

Supplementary Table E4. Estimates for the effects of SNP, mineral dust exposure and their interaction on the level of FEV_1 (ml) using linear regression models adjusted for gender, age, height and smoking status (never/ever).

Mineral dust			Identification				Replication			
			95% CI				95% CI			
SNP	**A1**	**Predictor**	**B**	**Lower**	**Upper**	**P**	**B**	**Lower**	**Upper**	**P**
rs13278529	G	SNP (additive)	-9.4	-28.3	9.5	3.30E-01	-79.7	-139.4	-20.0	8.94E-03
		Low exposure	-65.7	-91.8	-39.6	8.07E-07	-16.4	-89.8	57.0	6.62E-01
		High exposure	-173.8	-220.2	-127.3	2.42E-13	-119.5	-191.3	-47.6	1.14E-03
		SNP*Low exposure	36.2	-7.3	79.6	1.03E-01	65.4	-68.9	199.7	3.40E-01
		SNP*High exposure	178.4	103.0	253.7	3.49E-06	164.8	47.7	281.8	5.87E-03
rs473892	T	SNP (additive)	0.4	-12.8	13.7	9.48E-01	33.7	-9.5	76.9	1.26E-01
		Low exposure	-59.3	-95.9	-22.6	1.53E-03	55.6	-46.7	157.9	2.87E-01
		High exposure	-230.8	-293.3	-168.3	4.75E-13	-147.7	-242.9	-52.6	2.39E-03
		SNP*Low exposure	5.0	-26.5	36.4	7.57E-01	-60.2	-153.3	32.9	2.06E-01
		SNP*High exposure	126.3	72.7	179.9	3.89E-06	93.9	8.9	178.9	3.06E-02
rs6751439	A	SNP (additive)	4.5	-15.3	24.3	6.55E-01	30.7	-35.1	96.4	3.61E-01
		Low exposure	-46.9	-72.6	-21.2	3.51E-04	7.8	-67.1	82.7	8.39E-01
		High exposure	-66.6	-112.3	-20.9	4.27E-03	-38.5	-109.5	32.6	2.89E-01
		SNP*Low exposure	-29.5	-75.5	16.5	2.08E-01	-13.7	-146.4	119.1	8.40E-01
		SNP*High exposure	-184.8	-264.2	-105.4	5.16E-06	-134.8	-262.4	-7.2	3.85E-02

Supplementary Table E5. Estimates for the effects of SNP, gases and fumes exposure and their interaction on the level of FEV_1 (ml) using linear regression models adjusted for gender, age, height and smoking status (never/ever).

Gases and Fumes			Identification				Replication			
				95% CI				95% CI		
SNP	**A1**	**Predictor**	**B**	**Lower**	**Upper**	**P**	**B**	**Lower**	**Upper**	**P**
rs159497	C	SNP (additive)	-22.7	-38.1	-7.3	3.94E-03	-50.8	-103.1	1.5	5.73E-02
		Low exposure	-71.5	-100.8	-42.2	1.71E-06	-67.0	-150.7	16.8	1.17E-01
		High exposure	-222.8	-282.1	-163.5	1.91E-13	-167.3	-313.6	-20.9	2.53E-02
		SNP*Low exposure	37.6	12.3	62.8	3.54E-03	15.6	-55.5	86.7	6.68E-01
		SNP*High exposure	131.1	79.5	182.7	6.44E-07	136.3	14.1	258.6	2.90E-02
rs516732	C	SNP (additive)	6.6	-8.7	21.8	3.99E-01	33.6	-19.8	87.0	2.17E-01
		Low exposure	-39.8	-69.1	-10.5	7.71E-03	7.6	-76.8	92.1	8.59E-01
		High exposure	6.3	-53.1	65.6	8.36E-01	107.3	-34.2	248.8	1.38E-01
		SNP*Low exposure	3.4	-21.5	28.4	7.87E-01	-62.1	-134.1	10.0	9.15E-02
		SNP*High exposure	-115.4	-164.4	-66.5	3.76E-06	-158.5	-278.2	-38.7	9.61E-03
rs2888674	A	SNP (additive)	-14.9	-30.2	0.4	5.58E-02	8.4	-43.9	60.8	7.52E-01
		Low exposure	-57.1	-85.8	-28.3	1.02E-04	-36.4	-118.3	45.6	3.85E-01
		High exposure	-217.0	-277.6	-156.4	2.31E-12	-163.8	-309.4	-18.1	2.77E-02
		SNP*Low exposure	22.1	-2.6	46.9	7.95E-02	-15.3	-86.3	55.8	6.73E-01
		SNP*High exposure	122.2	70.1	174.3	4.36E-06	130.5	8.8	252.1	3.58E-02

Supplementary Table E6. SNPs acting as cis-eQTLs on genes within a 2 Mb distance.

SNP	Interaction with	Cis-gene	p-value*	Threshold‡
rs159497	Gases and fumes	*PDE4D*	**1.81E-04**	1.35E-03
rs2888674	Gases and fumes	*TMEM176A*	**1.73E-16**	3.50E-04
		ABP1	**1.54E-05**	3.50E-04

*p-value from the linear regression model.

‡Bonferroni corrected threshold calculated as p=0.05/number of probe sets within the 4 Mb window.

9

NOS1 : a susceptibility gene for pesticide exposure in relation to the level of FEV_1?

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To the editor,

Chronic obstructive pulmonary disease (COPD) is a common disease characterized by airway obstruction caused by complex interactions between genes and environmental factors[1]. Recently, there has been growing evidence showing that pesticide exposure is associated with poorer respiratory outcomes, such as chronic bronchitis and an impaired level of lung function compatible with airway obstruction[2,3]. Different types of pesticides have distinct physicochemical properties, and may cause direct damage to the cells of the bronchial mucosa or may cause indirect damage via interaction with pro-inflammatory receptors, for example transient receptor potential (TRP) ion channels on airway chemosensory nerves[4]. Genetic susceptibility to the adverse effects of pesticide exposure has been implicated in several diseases like cancer[5] and Parkinson's disease[6]. Whether genetic susceptibility is of importance for the effects of pesticide exposure in the lungs is largely unknown. Therefore, in the current study we performed a genome-wide interaction study to assess genetic susceptibility loci for occupational exposure to pesticides in relation to the forced expiratory volume in one second (FEV_1), a measurement of lung function.

We used data from two general population based cohorts from the Netherlands, the LifeLines and the Vlagtwedde-Vlaardingen general population-based cohort studies. In these cohorts we have previously shown adverse effects of occupational pesticide exposure on the levels of FEV_1 and FEV_1/FVC[3]. The LifeLines cohort study started in 2006 and includes subjects from the three Northern provinces of the Netherlands[7]. The Vlagtwedde-Vlaardingen longitudinal cohort study started in 1965, and subjects were followed for 25 years until the last survey in 1989/1990[8]. In the current study we used data from the last survey. Occupational pesticide exposure in both cohorts was assessed using the ALOHA+ job exposure matrix (JEM) estimating no, low or high exposure to pesticides (including herbicides and insecticides) based on self-reported job titles and functions[3]. Genome-wide genotyping in both cohorts was performed using IlluminaCytoSNP-12 arrays (for detailed information on the measurements, genotyping platform, quality control and genomic inflation factors, see supplementary material). The LifeLines and the Vlagtwedde-Vlaardingen cohorts include only Caucasian subjects from Dutch decent.

First, in both cohorts separately, SNP-by-exposure interactions on the level of FEV_1 were assessed using linear regression adjusted for sex, age, height and smoking status (never/ever smoker). Pesticide exposure was coded by dummy variables and both the SNP-by-low exposure and SNP-by-high exposure interactions were included in the model. To have a clear exposure contrast we focussed on SNP-by-high exposure interactions only. SNPs were tested in an additive genetic model. Subsequently, we meta-analyzed all SNP-by-high pesticide exposure interactions using the software package PLINK version 1.07[9]. Meta-analyzed SNP-by-high exposure interactions with a p-value $<2.26*10^{-7}$ (i.e. 0.05/221,444 SNPs available in both cohorts) were considered genome-wide significant.

Table 1. SNP (additive)-by-high occupational pesticide exposure interactions in relation to the level of FEV_1 (ml), adjusted for sex, age, height and ever smoking (no/yes). Interaction effects are shown for the LifeLines cohort, Vlagtwedde-Vlaardingen cohort and the two cohorts meta-analyzed. Minor allele frequency (MAF) is given for minor allele A1.

					LifeLines			Vlagtwedde-Vlaardingen			Meta-analysis					
SNP	**CHR**	**A1**	**A2**	**Annotation**	**B**	**P**	**MAF**	**B**	**P**	**MAF**	**P *f***	**P *r***	**B *f***	**B *r***	**Q**	**I^2**
rs4764419	12	G	T	PLCZ1 (intronic)	286	1.37E-06	0.46	151	2.37E-03	0.45	5.51E-08	1.51E-03	206	215	0.079	68
rs10459067	12	T	C	PLCZ1 (intronic)	290	1.29E-06	0.43	145	4.32E-03	0.42	1.11E-07	3.26E-03	205	214	0.064	71
rs482555	12	C	T	NOS1 (intronic)	-283	8.05E-05	0.25	-217	3.60E-04	0.23	1.30E-07	1.30E-07	-244	-244	0.484	0
rs2145067	6	T	C	MANEA (769kb 5')	-310	8.60E-05	0.17	-242	5.87E-04	0.19	2.15E-07	2.15E-07	-271	-271	0.521	0

f: fixed effects model; *r*: random effects model; Q: p-value for Cochrane's Q[10]; I^2: percentage of variation across studies

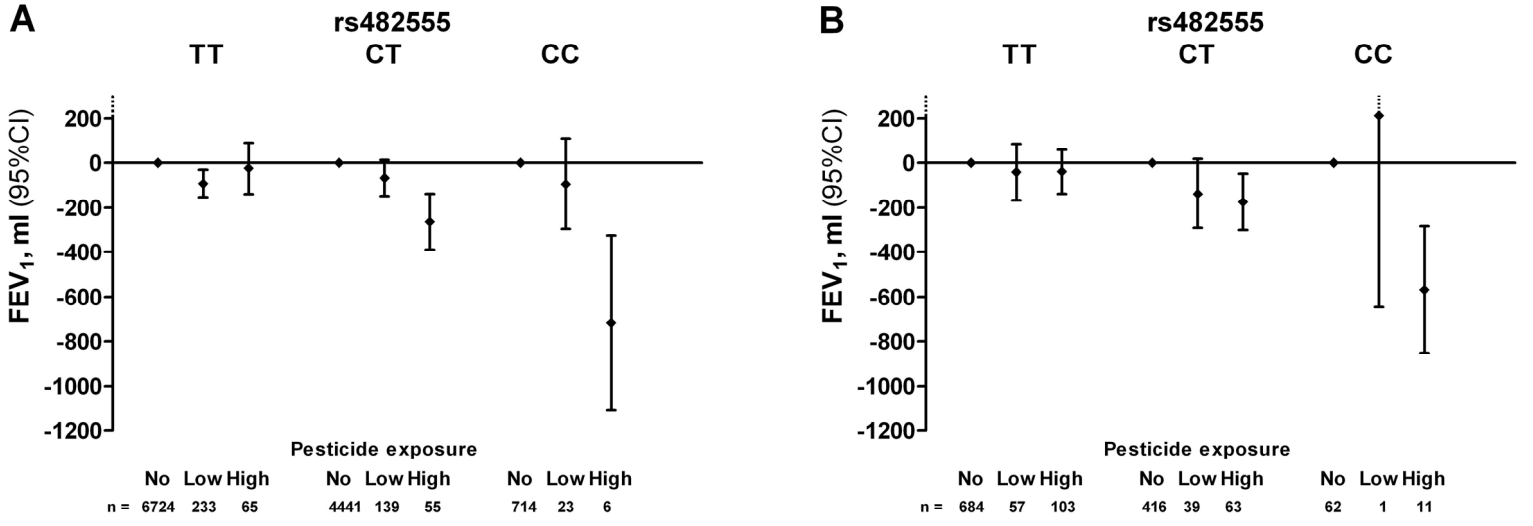

Figure 1. Associations of no, low and high occupational exposure to pesticides and the level of FEV_1 (ml) in the LifeLines cohort (A) and Vlagtwedde-Vlaardingen cohort (B) stratified for *NOS1*rs482555 genotype. No exposure was set as reference category. Associations were adjusted for sex, age, height and ever smoking (no/yes).

Within the LifeLines cohort data were available for 12,400 subjects with a median age of 47 years (range 18-89) in 2006/2011, 41% were males, 59% ever smokers, and only 1% had high occupational exposure to pesticides. Within the Vlagtwedde-Vlaardingen cohort data were available for 1,436 subjects, median age was 53 years (range 35-79) in 1989/1990, 54% males, 70% ever smokers, and 12% had high occupational exposure to pesticides.

Interactions of 4 SNPs with high occupational exposure to pesticides reached genome-wide significance in the meta-analysis (table 1). There were no significant main effects of these SNPs or interaction effects with low exposure to pesticides on the level of FEV_1 (supplementary table 1). Two SNPs were located in *phospholipase C, zeta 1* (*PLCZ1*). This gene has been mainly studied in relation to embryonic development i.e. expression of this protein increases calcium release during fertilization of mammalian eggs[11]. The two SNPs *PLCZ1* were in high LD (R-squared 0.99), and effect estimates from the two samples, i.e. LifeLines and Vlagtwedde-Vlaardingen, were quite heterogeneous (I-squared 68 and 71%)[10]. Meta-analysis using the random effects model showed less significant p-values. Another significant SNP after meta-analysis was located nearby *mannosidase, endo-alpha (MANEA)*, a protein that processes oligosaccharides in the Golgi apparatus[12].

The most plausible SNP involved in the adverse effects of pesticide exposure in the lungs was located in the gene *nitric oxide synthase 1* (*NOS1*). Subjects carrying the minor allele of the SNP were more susceptible to high levels of occupational exposure to pesticides, indicated by a lower level of FEV_1 (figure 1). Interestingly, a recent study showed that 3 SNPs in *NOS1* increased the susceptibility for the effects of pesticide exposure on the risk for Parkinson's disease[13]. These SNPs (rs12829185, rs10774910 and rs2682826) were not in Linkage Disequilibrium (LD) with the SNP (rs482555, *R*-squared < 0.1) identified in the current study. SNP rs482555 was not associated with the level of FEV1 independently of pesticide exposure (b = -5 ml FEV1, p-value 0.471; model adjusted for sex, age, height and smoking status), which is in line with a study that showed no associations between NOS1 SNP rs41279104 (LD with rs482555 R-squared < 0.1) with baseline lung function level and 5-year decline in a cohort of smokers[14].

The *NOS1* gene encodes for neuronal nitric oxide synthase (nNOS) that synthesizes endogenous nitric oxide (NO) from arginine. In the human lung, NOS1 was found in submucosal nerves and endothelial cells[15]. Messenger RNA and protein levels of *NOS1* were increased in peripheral lung tissue of moderate to very severe COPD patients compared to healthy smokers and non-smoking controls, and expression levels increased with increasing COPD severity, as defined by the Global initiative for chronic Obstructive Lung Disease criteria[16]. NO has been implicated in several ways in the toxicity of paraquat, a commonly used herbicide. Paraquat may activate NF-$\kappa\beta$, thereby inducing an increased expression of *inducible NOS*, which subsequently increases cytoplasmic NO and enhances nitrosative stress[17]. Secondly, NO may react with the superoxide anion ($O2^{\cdot-}$) generated by the redox cycling of paraquat, thereby increasing peroxinitrite ($ONOO^-$) levels[17]. Interestingly, increased production of NO was shown to be nNOS dependent in COPD patients and potentially increases the production of peroxinitrite[16]. Increased production of peroxinitrite amplifies nitrosative stress and thereby

induces expression of other *NOS* isoforms resulting in increased expression of NO[16]. This mechanism was suggested to be involved in inflammation and progression of disease pathogenesis in COPD[16] and to underlie the effects of pesticide exposure in the lungs[17].

In the current GWI study we aimed identify novel susceptibility loci for occupational exposure to pesticides in relation to FEV_1. We have used the ALOHA+ JEM to estimate pesticide exposure as no, low or high. Using JEM-based estimates is an efficient way of exposure assessment in large samples, yet chemical specificity is lacking. Further research should elucidate the role of *NOS1* in the development of impaired lung and consequently the development of COPD, and the role of exposure to specific chemicals in this process.

To conclude, with the current study using a hypothesis free approach and meta-analyzing data from two independent cohorts, we identified 4 SNPs in 3 genes. The most plausible candidate was *NOS1* as a novel susceptibility gene for the adverse effects of pesticide exposure in relation to the level of lung function. Interestingly this gene has been implicated in pesticide toxicity as well as in COPD pathogenesis. Excess release of NO may underlie pesticide toxicity in the lungs and subsequently lead to an impaired lung function, a hallmark of COPD.

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9

SUPPLEMENTARY MATERIAL

METHODS

LifeLines cohort

Genotyped individuals from the LifeLines cohort study with full data on all covariates were included (n = 12,400). At baseline all participants filled in a standardized questionnaire and were subject to a medical examination. The questionnaire included questions regarding personal characteristics, smoking habits, job title and description of current or last held job. The medical examination included pre-bronchodilator spirometry according to a standardized protocol following ATS guidelines using a Welch Allyn Version 1.6.0.489, PC-based SpiroPerfect with Ca Workstation software.

Vlagtwedde-Vlaardingen cohort

For replication of our initial finding we included 1,436 subjects with full data on genotypes and covariates from the Vlagtwedde-Vlaardingen cohort, a prospective general population based cohort including Caucasians from Dutch decent only. Pre-bronchodilator spirometry was performed according to a standardized protocol following ERS guidelines with a water-sealed spirometer (Lode Instruments, the Netherlands). Reported jobs were coded according to ISCO-88 classification and job specific exposures were estimated with the ALOHA+ JEM, similar to the identification sample.

Occupational exposure

Job title and description were coded according to the International Standard Classification of Occupations version 1988 (ISCO-88)(1). These four-digit classification codes were used to estimate job-specific exposures to pesticides using the ALOHA+ Job Exposure Matrix (JEM) (2, 3). The ALOHA+ JEM classifies subjects based on the ISCO-88 job codes into no, low, and high exposure categories (0/1/2, respectively). Jobs were averaged and rounded to the nearest integer in case a participant reported two different jobs simultaneously (0.5 = 1 and 1.5 = 2).

Genotyping and quality control

In both cohorts, genotyping was performed using IlluminaCytoSNP-12 arrays. The IlluminaCytoSNP-12 is an oligonucleotide chip designed to have a uniform spacing of markers across all chromosomes, with the far majority of the markers on this chip reflecting common SNPs: 93% of the 301,232 markers on this chip reflect bi-allelic SNP markers with callable polymorphic genotypes. Applicability of the CytoSNP12 LifeLines data has been shown before (4).

In the LifeLines cohort 227,981 SNPs fulfilled the quality control criteria: genotype call-rate ≥95%, minor allele frequency ≥1%, and Hardy-Weinberg equilibrium cut-off p-value $\geq 10^{-4}$. Non-Caucasian samples and first-degree relatives were excluded. In the Vlagtwedde-Vlaardingen cohort, 242,926 SNPs fulfilled the quality control criteria.

Statistical model

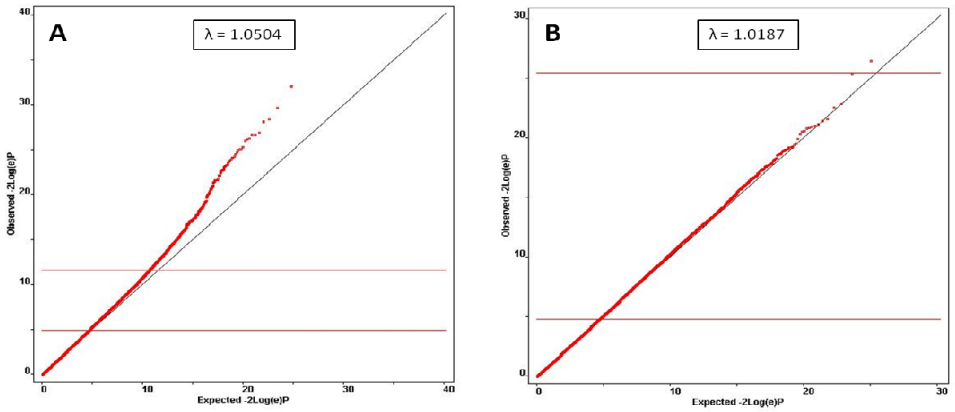
The statistical model was specified as: FEV_1 = SNP + low exposure (no/yes) + high exposure (no/yes) + SNP*low exposure + SNP*high exposure + covariates. Thus both SNP-by-low exposure and SNP-by-high exposure interactions were included in the model. In order to have a clear exposure contrast we focussed on SNP-by-high exposure interactions only. SNPs were tested in an additive genetic model. All SNP-by-high pesticide exposure interactions from both cohorts were meta-analysed using the software package PLINK version 1.07 (5). Meta-analyzed SNP-by-high exposure interactions with a p-value $<2.26*10^{-7}$ (i.e. 0.05/221,444 SNPs available in both cohorts) were considered genome-wide significant.

Ethical approval

Both the LifeLines cohort study and the Vlagtwedde-Vlaardingen studies were approved by the Medical Ethics Committee of the University Medical Center Groningen, Groningen, The Netherlands. All subjects gave written informed consent.

Genomic inflation factors for marginal associations

QQ-plots for the marginal association between the SNPs and level of FEV_1 in the LifeLines (A) and Vlagtwedde-Vlaardingen samples (B), adjusted for sex, age, height and ever smoking (no/yes).



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Supplementary Table 1. Estimates for the effects of SNPs, pesticide exposure (low and high versus no exposure) and their interaction on the level of FEV_1 (ml) using linear regression models adjusted for sex, age, height and smoking status (never/ever).

						LifeLines cohort (n = 12,400)				Vlagtwedde-Vlaardingen cohort (n = 1,436)			
SNP	**CHR**	**A1**	**A2**	**Annotation**	**Predictor**	**B**	**95% CI**		**P**	**B**	**95% CI**		**P**
rs4764419	12	G	T	PLCZ1 (intronic)	SNP (additive)	-10	-22	2	1.19E-01	-24	-63	15	2.22E-01
					Low exposure	-65	-140	11	9.18E-02	-154	-313	5	5.84E-02
					High exposure	-430	-567	-293	7.33E-10	-255	-369	-141	1.25E-05
					SNP*Low exposure	-22	-90	45	5.16E-01	84	-54	221	2.33E-01
					SNP*High exposure	286	170	402	1.37E-06	151	54	248	2.37E-03
rs10459067	12	T	C	PLCZ1 (intronic)	SNP (additive)	-11	-23	1	6.54E-02	-24	-63	15	2.34E-01
					Low exposure	-56	-128	16	1.27E-01	-163	-314	-11	3.57E-02
					High exposure	-408	-538	-279	6.74E-10	-242	-353	-130	2.26E-05
					SNP*Low exposure	-35	-103	32	3.04E-01	99	-36	234	1.52E-01
					SNP*High exposure	290	173	407	1.29E-06	145	45	244	4.32E-03
rs482555	12	C	T	NOS1 (intronic)	SNP (additive)	-3	-17	11	6.91E-01	31	-14	75	1.77E-01
					Low exposure	-90	-150	-30	3.31E-03	-43	-166	80	4.96E-01
					High exposure	-12	-123	100	8.39E-01	-17	-110	77	7.25E-01
					SNP*Low exposure	14	-64	91	7.33E-01	-78	-261	105	4.02E-01
					SNP*High exposure	-283	-423	-142	8.05E-05	-217	-336	-98	3.60E-04
rs2145067	6	T	C	MANEA (769kb 5')	SNP (additive)	-3	-18	13	7.51E-01	38	-10	87	1.23E-01
					Low exposure	-112	-168	-56	8.36E-05	-86	-198	26	1.34E-01
					High exposure	-59	-156	39	2.37E-01	-33	-122	57	4.74E-01
					SNP*Low exposure	90	-2	181	5.48E-02	21	-131	173	7.85E-01
					SNP*High exposure	-310	-463	-155	8.60E-05	-242	-379	-104	5.87E-04

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Summary, discussion and future perspectives

SUMMARY

The aim of this PhD project was to assess whether environmental exposures, such as occupational exposures and environmental tobacco smoke (ETS) exposure, are associated with the level of lung function and the prevalence of COPD, and to assess inter-individual differences in genetic susceptibility to these exposures. The studies described in this thesis have shown that environmental exposures are associated with different measures of lung function and a higher prevalence of COPD. We identified several factors that affect inter-individual susceptibility to these exposures, such as personal smoking and polymorphisms in (novel) genes.

Chapter 2 describes a study in which we assessed cross-sectional associations between occupational exposure to vapors, gases, dusts and fumes and their composite measure VGDF, pesticides, herbicides, insecticides and the level of FEV_1 and FEV_1/FVC and prevalence of COPD. Additionally we assessed whether these associations were different for never and ever smokers or males and females. In line with previous findings described in the literature we showed that occupational exposure to VGDF was clearly associated with lower levels of FEV_1 and FEV_1/FVC as well as with a higher prevalence of COPD. This study added to the current knowledge by showing that occupational exposure to pesticides, including herbicides and insecticides, was associated with a lower level FEV_1 and FEV_1/FVC and a higher prevalence of COPD. For both VGDF and pesticides, the associations with the level of FEV_1 were significantly stronger in ever smokers compared to never smokers, suggesting a synergistic effect between smoking and occupational exposure. There were no differences between males and females.

Chapter 3 describes a study in which we assessed cross-sectional associations between occupational exposure to vapors, gases, dusts and fumes and their composite measure VGDF, pesticides, herbicides, insecticides and obstruction of the small airways, as measured with $FEF_{25-75\%}$. Because we hypothesized that small airways obstruction could be a secondary phenomenon associated with obstruction of the large airways, we additionally assessed these associations in subjects without large airway obstruction (FEV_1/FVC≥70%, FEV_1≥80% of predicted). We found that exposure to the composite measure VGDF and to the subcategories biological dust and gases and fumes was associated with lower FEF_{25-75} levels. These associations remained present when we restricted our analysis to subjects without large airways obstruction, indicating that effects of exposure to vapors, gases, dusts and fumes on the small airways are a primary response and independently from effects on the large airways. In contrast to earlier observed effects on the large airways, associations between VGDF and FEF_{25-75} were similar in ever and never smokers. Although we previously showed consistent association between pesticides and the large airways, the trend for an association with the FEF_{25-75} did not reach statistical significance and disappeared when analyses were restricted to subjects without large airways obstruction. It may be that the aerodynamic diameter of the pesticides aerosols results in deposition predominantly in the larger

airways, whereas for example fibrous dust and welding aerosols have smaller aerodynamic diameters and may deposit in the smaller airways as well.

Chapter 4 describes a study in which we assessed associations between occupational exposures to vapors, gases, dusts and fumes and their composite measure VGDF, pesticides, herbicides, insecticides and the longitudinal decline in FEV_1 and FEV_1%VC. Additionally we assessed whether these associations were different in never and ever smokers, as was suggested by the cross-sectional analysis described in chapter 2. In this study we showed that occupational exposure to pesticides was associated with clinically relevant accelerated annual decline of FEV_1 and FEV_1%VC. In line with our cross-sectional analysis we found that these associations were significantly stronger in ever smokers, providing additional evidence for a synergistic effect of smoking and occupational exposure to pesticides. There were no significant associations between exposure to VGDF and decline of FEV_1 and FEV_1%VC independently of exposure to pesticides.

Chapter 5 describes a study in which we assessed risk factors for chronic mucus hypersecretion (CMH) in subjects with and without COPD. We found that environmental exposures, such as ETS and occupational exposures contribute differentially to CMH in subjects with and without COPD. In COPD patients, a higher risk for CMH was associated with higher pack-years smoked and exposure to ETS. In individuals without COPD a higher risk for CMH was associated with male gender, higher body mass index, higher pack-years smoked, current smoking, and occupational exposure to mineral dust, gases and fumes.

Chapter 6 describes a candidate-gene study in which we assessed associations between ETS exposure during different periods throughout the life-span and the level of FEV_1 and FEV_1/FVC during adulthood. The main aim of this study was to assess whether these associations were modified by genetic variation in *Gluthatione-S-Transferases Omega (GSTO) 1* and *2*. These genes were chosen a-priori based on their known role in oxidative stress reactions and detoxification of xenobiotic substances. *In utero* ETS exposure was associated with a lower level of FEV_1/FVC, and this association was similar for never and ever smokers. Daily ETS exposure of at least one hour per day was associated with a lower level of FEV_1 and exposure to ETS at the workplace was associated with a lower FEV_1 and FEV_1/FVC. Associations of daily and workplace ETS exposure were more pronounced in never smokers. We found significant interactions between the *GSTO* SNPs and ETS exposure in relation to the level of FEV_1, yet effects for *in utero* ETS exposure and ETS exposure during adulthood (daily and workplace ETS) were in the opposite direction. Being homozygote for the minor alleles of the *GSTO* SNPs was associated with a higher FEV_1 in subjects that were exposed to ETS *in utero*. Contrary, being homozygote for the minor alleles of the *GSTO* SNPs was associated with a lower level of FEV_1 in subjects that were exposed during adulthood. Interactions were generally more pronounced in never smokers. We did not find consistent significant interaction effects of the *GSTO* SNPs and ETS exposure on the level of FEV_1/FVC, suggesting a restrictive rather than an obstructive effect on lung function.

Chapter 7 describes a genome-wide interaction study in which we aimed to identify novel genetic loci and pathways that affect individual susceptibility to the effects of ETS exposure on the level of FEV_1. ETS exposure was based on the number of hours a person reports to be exposed to other people's tobacco smoke per day. Subjects were classified as non-exposed when self-reported ETS exposure was 0 hour/day and as ETS exposed when self-reported exposure was at least 1 hour/day (≥1 hour/day). Subjects with self-reported ETS exposure between 0 and 1 hour/day were excluded in order to have a distinct exposure contrast. SNP-by-ETS exposure interactions were assessed in 10,817 subjects from the LifeLines cohort study and verified in 1,276 subjects from the Swiss SAPALDIA study, both population based cohorts. Subsequently we used the SNP-by-ETS exposure p-values obtained from the identification analysis in LifeLines to perform a pathway analysis. The top interacting SNP from the identification analysis was located in *potassium voltage-gated channel, subfamily H member 1* (*KCNH1*). Other potassium channels (K+) have been found on cells involved in the disease pathogenesis of asthma and COPD, such as airway smooth muscle and inflammatory cells. However this SNP-by-ETS exposure interaction did not replicate in an independent sample from the SAPALDIA study. Two other SNP-by-ETS exposure interactions were replicated with nominally significant p-values (<0.05), i.e. SNPs in *actin, beta-like 2* (*ACTBL2*) and *zinc finger homeobox 4 (ZFHX4)* respectively. The latter SNP-by-ETS exposure interaction was replicated in never smokers only. In the pathway-level analysis we found three pathways that were significantly or suggestively enriched, i.e. the apoptosis, p38 MAPK and TNF pathways. Interestingly all three pathways have been previously implicated in the pathogenesis of COPD and may underlie susceptibility to ETS exposure.

Chapter 8 describes a genome-wide interaction study in which we aimed to identify novel genetic loci that affect individual susceptibility to common occupational exposures, i.e. biological dust, mineral dust and gases and fumes, on the level of FEV_1. We performed an identification analysis in 12,400 subjects from the LifeLines cohort study, and verified our findings in 1,436 subjects from a second independent cohort, i.e. the Dutch Vlagtwedde-Vlaardingen cohort. Additionally we assessed whether these SNPs were cis-acting expression quantitive trait loci (cis-eQTLs) in lung tissue. We identified and replicated 7 SNPs that interacted with one of the occupational exposures. Several identified loci were plausible candidates involved in biological pathways leading to lung function impairment, for example *GALNT13* and *PCDH9*. Of these 7 replicated SNPs, 2 SNPs were cis-acting eQTLs associated with gene expression of their neighboring genes *TMEM176A* and *PDE4D* in lung tissue. This is unique data and provides additional support for a possible role of these genes in lung function impairment. Further research should determine whether the identified (novel) genes are true susceptibility loci for lung function impairment due to occupational exposure to biological dust, mineral dust and gases and fumes, and whether these SNP-by-exposure interactions consequently increase the risk to develop COPD.

Chapter 9 describes a genome-wide interaction study in which we aimed to identify novel genetic loci that affect individual susceptibility to exposure to pesticides on the level of FEV_1. First, we performed separate analyses in 12,400 subjects from the LifeLines study and in 1,436 subjects from the Vlagtwedde-Vlaardingen cohort study. Subsequently we

meta-analyzed the interaction effects from the two studies. Four SNPs that interacted with high occupational pesticide exposure on the level of FEV_1 reached genome-wide significance after meta-analysis. The most interesting SNP was located in the gene *nitric oxide synthase 1* (*NOS1*). We found that subjects carrying the minor allele of the SNP were more susceptible to high levels of exposure to pesticides, indicated by a lower level of FEV_1. SNPs in *NOS1* were previously shown to increase the susceptibility to the effects of pesticide exposure as a risk factor for Parkinson's disease, and *NOS1* has been implicated in inflammation and progression of disease pathogenesis in COPD. Future studies should determine whether *NOS1* is a true susceptibility locus associated with the development of COPD in subjects exposed to pesticides.

DISCUSSION

Environmental exposures and COPD

The studies described in this thesis provide evidence that environmental exposures independently from personal smoking are associated with a lower level of lung function as reflected by a lower FEV_1, FEV_1/FVC and FEF_{25-75}, an accelerated decline in FEV_1 and FEV_1/VC and an increased prevalence of COPD (table 1).

Table 1. Summary of associations between environmental exposures and the level of FEV_1, FEV_1/FVC, FEF_{25-75}, the decline of FEV_1 and FEV_1/VC and the prevalence of COPD and CMH studied in this thesis.

		Lung function level			Lung function decline		COPD	CMH	
Environmental Exposures		FEV_1	FEV_1/FVC	‡FEF_{25-75}	FEV_1	FEV_1/VC	prevalence	COPD	Non-COPD
Occupational	VGDF	↓	↓	↓	=	=	↑	=	↑
	Biological dust	=	=	↓	=	=	=	=	=
	Mineral dust	↓	=	=	=	=	↑	=	↑
	Gases/Fumes	↓	↓	↓	↑	=	↑	=	↑
	Pesticides	↓	↓	=	↑	↑	↑	NA	NA
	Herbicides	↓	↓	=	↑	↑	↑	NA	NA
	Insecticides	↓	↓	=	↑	↑	↑	NA	NA
ETS	*In utero*	=	↓	NA	NA	NA	NA	NA	NA
	Daily	↓	=	NA	NA	NA	NA	↑	=
	Workplace	↓	↓	NA	NA	NA	NA	=	=

↓ significantly lower, ↑ significantly higher, = not significant, NA not assessed,

↓ lower not significant, ↑ higher not significant,

‡ associations with FEF_{25-75}, independently of large airways obstruction.

Within the studies presented in this thesis, occupational exposure to pesticides, including the subcategories herbicides and insecticides, was the environmental exposure with the strongest and most consistent association with reduced level of lung function (FEV_1 and FEV_1/FVC), accelerated decline of lung function (FEV_1 and FEV_1/VC) and the prevalence of COPD. Pesticide exposure was not associated with small airways obstruction. A number of studies conducted within the Agricultural Health Study, a large cohort of certified pesticide applicators in Iowa and North Carolina, showed increased prevalence of wheeze, chronic bronchitis and asthma, both in the pesticide applicators (farmers)[1-3], as well as in their spouses[4,5]. Apart from a few small scale cross-sectional studies that found associations between specific types of pesticides and lower levels of FEV_1 and FVC in occupationally exposed farmers from Sri-Lanka[6], South Korea[7] and Costa Rica[8], relatively few studies have focused on the effects of pesticide exposure on lung function[9]. To our knowledge no studies thus far have shown associations between pesticide exposure and the longitudinal decline of lung function. Globally, the agricultural sector employs more than 1.1 billion workers worldwide (about 34% of the global working force)[10] potentially putting a large amount of workers at risk for pesticide exposure. Additionally, people living in agriculture-intensive regions may be at risk for exposure due to pesticide drift[11]. This may be especially relevant in a densely populated country as the Netherlands where large numbers of people live near greenhouses and pesticide-treated farmland. We found that occupational exposure, i.e. handling and spraying of pesticides, affected lung function values. However, little is known about the risks of people living in the vicinity of these pesticide-treated areas in the Netherlands, and such risks were not taken into account during the approval process for pesticides' entry on the Dutch market. Growing concerns about these risks have recently led to a first advisory report of a Committee of the Health Council of the Netherlands, and consequently the initiation of an exposure study among people living in the vicinity of greenhouses and pesticide-treated farmland that will be carried out in 2015 and 2016[12-18]. In order to protect (respiratory) health of appliers and people living nearby, further studies are needed for better understanding of specific chemicals associated with (respiratory) health risks, the underlying biological mechanisms and the exposure-response relationship[9].

Occupational exposure to VGDF was associated with a lower level of FEV_1 and FEV_1/FVC, and obstruction of the large airways, i.e. mild (FEV_1/FVC<70%) and moderate/severe COPD (FEV_1/FVC<70% and FEV_1 percent of predicted<80%). All described associations were adjusted for co-exposure to pesticides. Independently of obstruction of the large airways (FEV_1/FVC≥70%, FEV_1≥80%), VGDF exposure was associated with a lower $FEF_{25-75\%}$, indicating obstruction of the small airways. Specifically exposure to mineral dust and gases and fumes was associated with a lower level of FEV_1 and COPD prevalence, whereas such an association with FEV_1/FVC was less clear (table 1). High exposure to mineral dusts, i.e. aerosols originating from minerals, is common in agricultural workers and gardeners (mineral dusts from the soil), construction workers (fibrous dust from insulation materials, such as asbestos, mineral wool and glass fibers) and concrete placers and tile setters (non-fibrous silica dust) (table 2)[19,20]. Interestingly exposure to mineral dusts, such as coal and silica, has been associated with both restrictive and obstructive effects on lung function[21-23]. Although smoke

exposure has been most consistently shown to be associated with obstructive lung disease, we found a restrictive rather than an obstructive effects of ETS exposure on lung function in subjects carrying the minor allele of *GSTO* SNPs (chapter 6). This may suggest the presence of interstitial abnormalities, which have been shown to be present in about 8% of high resolution CT scans in a cohort of 2,146 smokers[24]. Restrictive patterns were also seen in 8% of subjects with asthma[25]. Additional measures such as total lung capacity (TLC) are needed to support our findings suggesting restrictive effects of ETS on lung function as seen with spirometry[26].

The trend for a negative association between mineral dust exposure and the level of FEF_{25-75} disappeared when we restricted our analysis to subjects without obstruction of the large airways, indicating that the effect on the small airways was secondary to large airway obstruction. A study investigating small airway function in silica dust exposed workers with an FEV_1/FVC>75% and FVC>75% predicted did show a small difference in FEF_{25-75} between low and highly exposed workers, but did primarily show differences in FEF_{75-85}[27], indicating obstruction of the smallest airways[28]. Yet this measure of the end-expiratory flow may be unreliable since it is highly effort dependent. Studies using non-invasive effort-independent techniques such as impulse oscillometry (IOS) or multiple breath nitrogen washout (MBNW), could provide additional insight in the effects of environmental exposures by discerning obstruction of the large and small airways (IOS)[29] and the proximal conducting and acinar airways (MBNW)[30], respectively.

Compared to the larger mineral dust particles, fumes are smaller particles originating from condensation of materials which have been subjected to high temperatures. Welding fume is the most common type of fume construction workers are exposed to. Other examples include fume from coal tar used in built-up roofing and fume from diesel engines[20]. The latter seems relevant for heavy truck drivers and in motor and machinery mechanics (table 2). Examples of toxic gases are carbon monoxide from engine exhaust and hydrogen sulphide produced by bacterial breakdown of organic matter[20].

Interestingly, biological dust was consistently not associated with obstruction of the larger airways, as indicated by FEV_1, FEV_1/FVC, and the prevalence of COPD in our cohorts. In contrast, biological dust was most strongly associated with FEF_{25-75} indicating obstruction of the small airways, independently of obstruction of the larger airways. From our findings we could conclude that biological dust exposure rather affects the small than the larger airways. Biological dust exposure includes fungal and bacterial spores/cells, pollen, viruses, aggregates of these particles and fragments of larger organisms including cotton and wood dust, flour, textile and paper fibers[31], which is reflected by the occupations with high estimated biological dust exposure in the LifeLines sample (table 2).

Table 2. Occupational categories (ISCO description) with estimated high exposure to biological dust, mineral dust or gases and fumes in the LifeLines sample (as selected in chapter 2 and 3).

n (%)	Biological dust	n (%)	Mineral dust	n (%)	Gases/Fumes
158 (33.8)	Dairy and livestock producers	67 (12.5)	Welders and flame cutters	102 (14.9)	Heavy truck and lorry drivers
92 (19.7)	Carpenters and joiners	64 (11.9)	Agricultural or industrial machinery mechanics and fitters	77 (11.2)	Motor vehicle mechanics and fitters
63 (13.5)	Freight handlers	63 (11.7)	Freight handlers	67 (9.8)	Welders and flame cutters
22 (4.7)	Crop and animal producers (mixed)	50 (9.3)	Gardeners, horticultural and nursery growers	64 (9.3)	Agricultural or industrial machinery mechanics and fitters
22 (4.7)	Bakers and pastry-cooks	48 (8.9)	Building and construction laborers	57 (8.3)	Plumbers and pipe fitters
18 (3.8)	Veterinary assistants	33 (6.1)	Field crop and vegetable growers	41 (6.0)	Painters and related workers
12 (2.6)	Floor layers and tile setters	22 (4.1)	Crop and animal producers (mixed)	31 (4.5)	Butchers, fishmongers and related food preparers
11 (2.4)	Motorized farm and forestry plant operators	22 (4.1)	Construction and maintenance laborers (roads)	22 (3.2)	Earth moving and related plant operators
70 (15.0)	Other	21 (3.9)	Earth moving and related plant operators	21 (3.1)	Ships' engineers
		17 (3.2)	Agricultural and fishery workers (not specified)	20 (2.9)	Printing machine operators
		14 (2.6)	Sculptors, painters and related artists	15 (2.2)	Hand launderers and pressers
		14 (2.6)	Concrete placers, finishers and related workers	14 (2.0)	Sculptors, painters and related artists
		12 (2.2)	Floor layers and tile setters	154 (22.5)	Other
		12 (2.2)	Sheet metal workers		
		11 (2.0)	Motorized farm and forestry plant operators		
		68 (12.6)	Other		
468 (100)	**Total**	**538 (100)**	**Total**	**685 (100)**	**Total**

Contrary to our findings, several studies showed associations between organic dust or endotoxin exposure in farmers and a reduced level of FEV_1, an accelerated decline of FEV_1, and an increased risk for non-atopic asthma and COPD[32,33]. In addition, studies using the same JEM-based exposure estimates have shown associations between high occupational exposure to biological dust and a lower level of FEV_1, as well as higher prevalence of chronic obstructive bronchitis, emphysema and COPD[34-36]. An important difference between studies presented in the literature and our study is that we have adjusted our exposures models for co-exposure, for example our model with biological dust exposure was adjusted for co-exposure to pesticides. It could be that the association between biological dust and lower levels of lung function and an increased prevalence of COPD in other studies were driven by workers in occupations with high biological dust exposure with additionally high exposure to pesticides which was not accounted for. If we had not adjusted our models for co-exposure to pesticides we had found significant cross-sectional associations between high exposure to biological dust and the level of FEV_1 (-81 ml, 95% CI = -124; -38) and FEV_1/FVC (-0.7 %, -1.3; -0.1) in the LifeLines cohort. This would have been in line with the majority of findings published in the literature.

The above mentioned studies found reduced lung function levels and a higher prevalence of COPD associated with biological dust exposure, but not with exposure to mineral dust and gases and fumes[34,36], as we found in our study (table 1). The heterogeneity in findings between studies published in the literature could be due to the heterogeneity of exposure within one exposure category, for example the category mineral dust. Although the job exposure matrix efficiently classifies exposures in a diverse population, the chemical specificity is lacking. Specific exposure composition (i.e. specific chemicals) likely differs between the various occupations grouped to one exposure category (table 2), and additionally may be dependent on the specific tasks, protective equipment and ventilation. These exposure differences may become even larger when comparing studies from different countries.

Although the discrepancies between studies could be due to methodological aspects, an important aspect when considering these heterogeneous findings are differences in personal or other environmental factors, such as age (as proxy for cumulative exposure), personal smoking, genetic susceptibility and the possible interaction between these factors. In our studies we have additionally assessed whether associations of occupational exposures and environmental tobacco smoke with the level of lung function and lung function decline were different for never and even smokers and for males and females. In both the cross-sectional and longitudinal studies presented in this thesis (chapters 2 and 4) we found that the effects of occupational exposures on the large airway function were more pronounced in ever smokers, indicating a synergistic effect between occupational exposure and tobacco smoking. Two US studies showed almost additive effects for combined exposure to VGDF and (heavy) smoking on the risk for COPD, i.e. the odds ratios of VGDF exposure with <10 packyears, heavy smoking (≥10 packyears) without VGDF exposure, and VGDF exposure with heavy smoking (≥10 packyears) were 2.0, 3.7 and 5.9 compared to the reference category (<10 packyears without VGDF exposure) respectively[37,38]. Interestingly, when we assessed associations between VGDF exposure and smoking on the

small airways in chapter 3 we found similar associations in never and ever smokers, indicating that possible synergistic effects of these exposures are mainly relevant in the larger airways.

In our study we found strong interactions between exposure to pesticides and personal smoking, indicating synergistic effects of tobacco smoke and pesticides on the level of lung function. Interactions between smoking and pesticide exposure have been implicated in relation to mortality rates in the US NHANES population, i.e. ever smokers had significantly higher mortality rates compared to never smokers, yet only in subjects with high serum concentrations of organocholorine pesticides (2nd and 3rd tertiles). No associations between ever smoking and mortality were found in subjects with serum concentrations in the lowest tertile[39]. Synergistic effects of pesticide exposure and smoking are biological plausible since both induce free radicals and consequently oxidative stress, and depletion of the antioxidant system by one exposure may increase the susceptibility to the other[40,41].

In chapter 2 we stratified our analysis, and found no consistently significant differences in associations between occupational exposures and the level of lung function between males and females. There were subtle differences suggesting that the associations with exposure to vapors, gases, dusts and fumes are somewhat more pronounced in males, whereas the associations with pesticides seem somewhat more pronounced in females. Based on the aggregated type of exposure assessment used in our studies (JEM-based) it is difficult to assess gender differences, since it has been shown that occupational exposure patterns differ between males and females, even when they have the same occupation[42]. Females seem to be more susceptible to effects of tobacco smoke on lung function level given the same exposure history[43,44], and it is conceivable that females are also more susceptible to occupational exposures than males. In relation to lung cancer risk, females were more susceptible to the effects of air pollution (Cadmium and Nickel) in areas close to coal-fired power stations, which was suggested to be due to differences in metabolism of toxicants under the influence of estrogen levels[45]. Other explanations for the increased susceptibility of females compared to males include gender specific genetic susceptibility and a relatively higher exposure dose locally in the airways due to their smaller size in females. Finally, differential effects of environmental exposures on DNA methylation patterns in males and females should be considered, potentially mediated by sex hormones[46]. Further studies with specific exposure assessment, i.e. specific chemicals and doses, are needed to determine whether there are gender differences in susceptibility to occupational exposures.

Interactions between smoking and occupational exposures are of importance for determining the optimal strategy and effects of preventive strategies. Smoking cessation will likely be the most effective strategy. Yet the available evidence shows that occupational exposures, whether or not in synergy with other exposures such as (environmental tobacco) smoking, are important contributors to the global burden of COPD. Interestingly also in COPD patients occupational exposure to VGDF was associated with an increased risk for having an FEV_1 below 30% of predicted, independently of

packyears smoked[47]. Moreover, VGDF exposure was associated with a larger prevalence of respiratory[48] and COPD[47] related work inactivity in COPD patients. This may advocate the need for screening and monitoring programs for early detection of reduced and accelerated decline in lung function in the occupational setting. Preventive strategies may become increasingly important since the health-care costs associated with COPD are expected to rise along with the rising COPD prevalence. For example in the Dutch situation it is expected that 70% more people will have COPD in 2032, which will cause health-care associated costs to rise to 1.4 billion euro in 2032, more than triple the amount of 2007[49].

Genetic susceptibility

Despite the successes of GWA studies in identifying novel loci associated with COPD, the newly identified genetic variants have so far only explained a small proportion of the genetic contribution to this complex disease. Explanations for this "missing heritability" are amongst others gene-environment interactions, rare genetic variants and epigenetic mechanisms[50]. Thus far, genome-wide association studies aiming to find novel susceptibility genes associated with COPD have disregarded environmental factors that may underlie the development of this disease. Hancock et al (2012) were one of the first showing that genome-wide gene-environment interaction studies, in their case with personal smoking (ever smoker or packyears), yields novel loci associated with the level of lung function (FEV_1, FEV_1/FVC) that would be missed when only focusing on direct genetic effects. It is likely that genetic susceptibility is also of importance for occupational exposures and environmental tobacco smoke exposure.

Therefore, the second aim of this PhD project was to assess whether there is inter-individual difference in genetic susceptibility to the effects of occupational exposures and environmental tobacco smoke exposure on the level of lung function. With the studies presented in this thesis we are one of the first assessing gene-by-environmental exposure interactions in a genome-wide hypothesis free manner, and identified several genetic variants that affected individual susceptibility to effects of environmental exposures in relation to the level of FEV_1 (table 3).

Most identified genes have not been identified in previous genome-wide association studies assessing the direct link between genetic variants and lung function levels or COPD prevalence. Based on their biological function they are plausible candidates involved in exposure mediated disease development (table 3). In order to get additional insight in the biological plausibility of this genes and potential pathways involved in disease development we have performed pathway and gene expression analysis. In chapter 7 we have performed a pathway analysis based on p-values obtained from the SNP-level interaction analysis with environmental tobacco smoke exposure, using i-GSEA-4-GWAS, an online tool based on gene-set enrichment analysis[51]. This analysis revealed three pathways, i.e. the apoptosis, P38 MAPK and TNF pathways, which are all plausible biological pathways involved in environmental tobacco smoking mediated impaired lung function. This approach may yield additional insight in the interactions between exposure and genetic variation because it takes all genes in a pathway into account, instead of focusing only on the genome-wide significant

hits[51]. In chapter 8 we extended our findings from GWI analysis to gene expression analysis and found that two of the SNPs in interaction with mineral dust and gases and fumes exposure were cis-eQTLs in lung tissue. This suggests that these SNPs may affect individual susceptibility to occupational exposures by altering gene expression levels. The other SNPs identified in the GWI study were no cis-eQTLs in lung tissue but may affect individual susceptibility to occupational exposures via other mechanisms, i.e. changed protein structure, altered miRNA levels or methylation. Determining the functional mechanisms of the identified genetic variants will be an important focus in future studies.

Table 3. Genes and pathways identified in this thesis that interact with environmental exposures on the level of FEV_1.

Environmental Exposures		Outcome	Identified gene	Identified pathway	Pathway/Function
Occupational	VGDF	.	.	.	.
	Biological dust	FEV_1	*PCDH9*	.	cell adhesion, calcium ion binding
	Mineral dust	FEV_1	*ZMAT4*	.	DNA binding and zinc ion binding[a]
			OLIG3	.	DNA binding, RNA polymerase II transcription co-repressor activity, protein dimerization activity[a]
			GALNT13	.	mucin type O-Glycan biosynthesis
	Gases/Fumes	FEV_1	*PDE4D*	.	signal transduction (beta-adrenergic receptor via cAMP and PKA signaling)
			ODZ2	.	cell cell adhesion (neuronal)
			TMEM176A	.	regulation of dendritic cell differentiation
	Pesticides	FEV_1	*NOS1*	.	NOS signaling, apoptosis
	Herbicides	.	.	.	.
	Insecticides	.	.	.	.
ETS	*In utero*	FEV_1	‡*GSTO1/2*	.	oxidative stress, detoxification
	Daily	FEV_1	‡*GSTO1/2*	.	oxidative stress, detoxification
		FEV_1	*ACTLBL2*	.	ATP binding[a]
		FEV_1	*ZFHX4*	.	sequence-specific DNA binding transcription factor[a]
		FEV_1		Apoptosis	apoptosis
		FEV_1		P38 MAPK	cellular responses to cytokines and stress
		FEV_1		TNF	induces various signaling pathways (apoptosis, cell survival, inflammation and immune response)
	Workplace	FEV_1	‡*GSTO1/2*	.	oxidative stress, detoxification

‡Candidate gene approach, no significant interaction on the level of FEV_1/FVC.

[a]Proposed Molecular/cellular function by the Gene Ontology Database (http://www.geneontology.org/).

FUTURE PERSPECTIVES

Epidemiological studies

In this thesis we have shown that environmental exposures independently or in interaction with personal smoking are associated with the level of lung function, the decline of lung function and the prevalence of COPD. Moreover, the GWI studies presented in this thesis have yielded several novel loci, adding to those already known to be associated with lung function or the development of COPD directly. However, several important issues remain to be studied.

First, associations between environmental exposures and lung function level and the prevalence of COPD were studied in cross-sectional settings. In chapter 4 we studied associations with the longitudinal decline of lung function, yet this study was somewhat limited by the fact that occupational exposure was ascertained at the last visit. Prospective longitudinal studies are needed to assess associations between environmental exposures and the development of COPD. Within the prospective LifeLines cohort study such data will be available in the near future.

Second, the genome-wide interaction studies presented in this thesis have focused on the level of FEV_1 as proxy for the level of lung function. People with a lower level of lung function are more prone to experience respiratory symptoms and limitations in exercise capacity, and are at increased the risk to develop COPD later in life. However, in order to fully unravel the pathways leading to the development of COPD, future studies should focus on other phenotypes as well. These studies could start with assessing associations with the level of FEV_1/FVC, which is a better indicator of airway obstruction than the level of FEV_1. Additionally gene-by-exposure interactions should be studied in association with COPD prevalence. COPD could be spirometry defined COPD, i.e. using FEV_1/FVC<70% and severity stages according to the level of FEV_1 or the lower limit of normal (LLN). The latter is considered less likely to result in misclassification of COPD in subjects of young or older age. However, it may miss subjects with mild airflow obstruction that are still at increased risk for COPD-related hospitalization and all-cause mortality compared to subjects with normal lung function[52]. Potentially more sensitive criteria could be used to define COPD, including symptoms and risk for exacerbations as proposed in the new Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria[53]. Moreover, studies assessing associations with sub-phenotypes of COPD (i.e. chronic bronchitis, small airways disease and emphysema) may give additional insight in specific pathways underlying development of the different COPD phenotypes and may lead to the development of specific therapeutic targets. More detailed information on COPD sub-phenotypes may be acquired for instance by adding multiple nitrogen breath washout[30], impulse oscillometry[29] or low-dose CT scans[54] to the characterization of individuals participating in LifeLines.

Finally to answer the question why and how never smokers develop COPD, associations between genes, exposures, and their interactions on lung function levels and COPD prevalence should be studied in a subgroup including only never

smokers. Concordantly with studies in smokers, even more valuable will be studies in longitudinal settings focusing on never smoking individuals that develop COPD. GWA or GWI studies in these settings will teach us whether the same or different pathways underlie smoking and non-smoking COPD. If different pathways underlie smoking and non-smoking COPD this may have consequences for both prevention and treatment of this non-smoking 'phenotype', i.e. different underlying pathways could imply different options for therapeutics and different responses to existing therapeutics.

Post-GWAS

With the studies presented in this thesis we are one of the first assessing genome-wide interactions with environmental exposures such as occupational exposures and environmental tobacco smoke exposure in relation to lung function level and identified several novel loci. An important future challenge of these studies will be to understand the functional consequences of these loci, for example does a SNP change protein structure or gene-expression? Going from an identified and replicated SNP to a functional meaning of this newly identified variant is important to fully unravel the pathways underlying both smoking and non-smoking COPD, and to be able to translate findings into clinical benefits, such as biomarkers, drug targets, screening and prevention strategies[55]. There are several options to go from identified SNPs to functional mechanisms. *Expression analysis* assesses whether a SNP is associated with expression of a gene nearby (cis-eQTL) or further away (trans-eQTL). In chapter 8 of this thesis we have assessed whether identified SNPs were cis-eQTLs in lung tissue, which gave us additional insight in potential pathways underlying the observed associations. Other options for post-GWAS analysis include re-sequencing, experimental models and epigenetic mechanisms. *Re-sequencing or fine mapping* may be used to capture the causal SNP that is in LD with the SNP associated with the outcome under study. In addition, fine mapping, or deep sequencing in genetically isolated populations may also be used to identify rare variants associated with COPD, thereby potentially explaining some of the "missing heritability" of the disease. *Epigenetic mechanisms*, such as methylation, histone modification and micro RNAs contribute to gene regulation, and may mediate associations between SNPs and disease found in GWA or GWI studies[55, 56]. Once there is substantial suggestive evidence for a gene involved in the disease (development), *experimental models* such as knock-out or over-expression of a gene in animals or cells may yield better understanding of biological pathways leading to disease.

Epigenetics

An important focus of future studies will be on the role of epigenetic ("above" genetic) mechanisms in COPD. Epigenetic mechanisms are crucial for normal development of multi-cellular eukaryotic organisms; it allows cells to develop into differential cell types by altering gene expression without changing the nucleotide sequence[57]. Epigenetic mechanisms, including histone modifications, micro RNA and DNA methylation, are affected by environmental exposures and may be an important link between these exposures and the development of complex airway diseases[58,59] (figure 1). DNA methylation, i.e. binding of a methyl group to a cytosine base adjacent to a guanine base (CpG site), has probably been

most extensively studied thus far. Many CpG sites are found in regulatory regions of genes and active demethylation of these sites is needed to allow gene transcription. Platforms have recently become available for high throughput DNA methylation profiling of CpG sites. Genome-wide methylation patterns were shown to be associated with smoking status and time since quitting[60-62], as well as with the presence and severity of COPD[63]. Other epigenetic mechanism include histone modifications (acetylation and methylation) that affects the accessibility of the DNA for transcription, and non-coding RNAs such as micro RNAs (miRNA) that regulate gene expression post-transcriptionally through degradation of gene transcripts or inhibition of protein translation. Cigarette smoke has been associated with both histone modifications[64] and altered expression of miRNA[65]. Moreover, histone-4 acetylation was increased at the NF-kB binding site of *interleukin 8* in COPD patients compared to non-smokers[66] and differential miRNA expression was seen in the lungs tissue of COPD patients compared to healthy smokers[67]. Unraveling the role of epigenetic mechanisms in mediating associations between the environment and gene expression and their role in the development of COPD will be an important focus of further studies. Findings from these studies may improve our understanding of biological pathways underlying COPD development and may provide new targets for screening as well as therapeutic interventions[58].

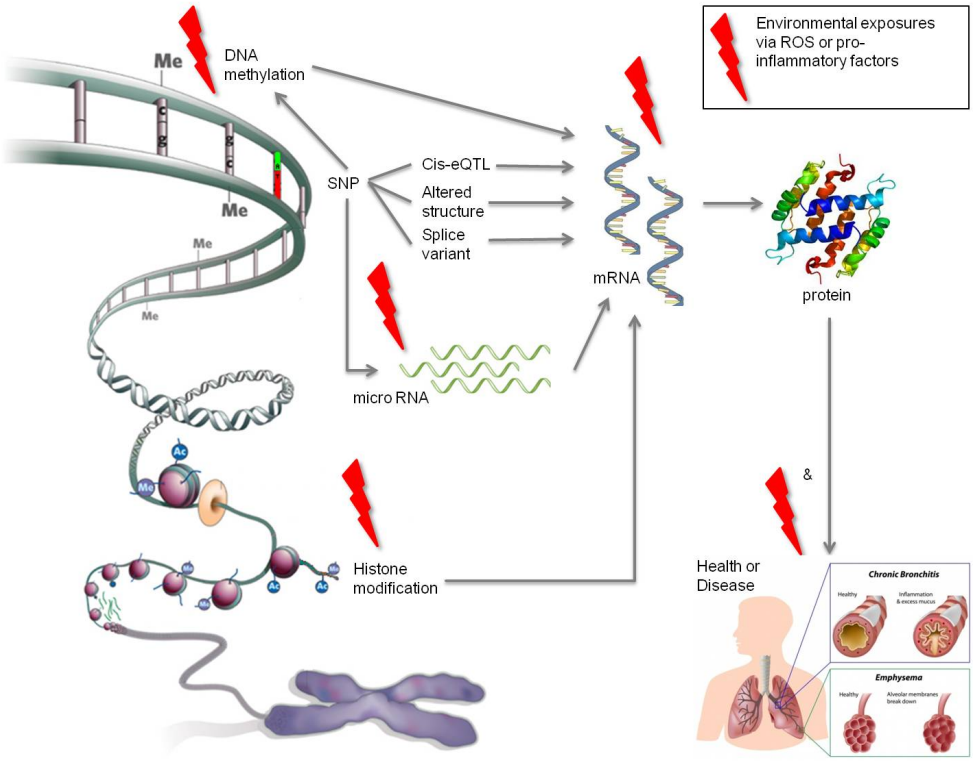

Figure 1. Genetic and epigenetic mechanisms: potential targets for mediating the association between genes, environmental exposures and disease. (picture modified from Qui, 2006[68]).

Focusing on DNA methylation, thus far most studies have focused on a small part of the puzzle, i.e. they either investigated associations between exposures and methylation levels, associations between methylation levels and disease prevalence, associations between methylation and gene expression levels, or associations between gene expression levels and disease (figure 2a). An important next step will be to perform mediation analysis studying associations between environment and disease via epigenetic mechanisms, such as DNA methylation (figure 2b). Another important and challenging focus of future studies will be on integrating genotype (SNP), epigenetic (methylation) and expression data in order to explain potential associations between environmental exposures and disease development. For example the role of SNPs in altering the potential for methylation or the expression of miRNAs (figure 2b – dashed lines). Additionally DNA methylation may affect the association between genetic variants and disease, as was shown for a genetic variant and DNA methylation levels of the *interleukin-4 receptor* gene in relation to asthma risk at age 18 years[69]. Integrating and analyzing genotype, methylation, gene expression and phenotype data will be an important challenge in future studies and will require new approaches such as network analysis as well as large computational power, especially for genome-wide data.

Within the LifeLines cohort study whole-genome methylation data will be available in a subsample of 2,000 subjects, which will allow assessment of DNA methylation, SNPs and their interactions with environmental exposures such as ETS, occupational exposure and ambient air pollution in relation to COPD development. This may contribute to early identification of groups at increased risk to develop COPD and the discovery of novel biological mechanisms underlying disease development, with the ultimate goal to open new possibilities for targeted interventions to prevent the development of this burdensome disease[70].

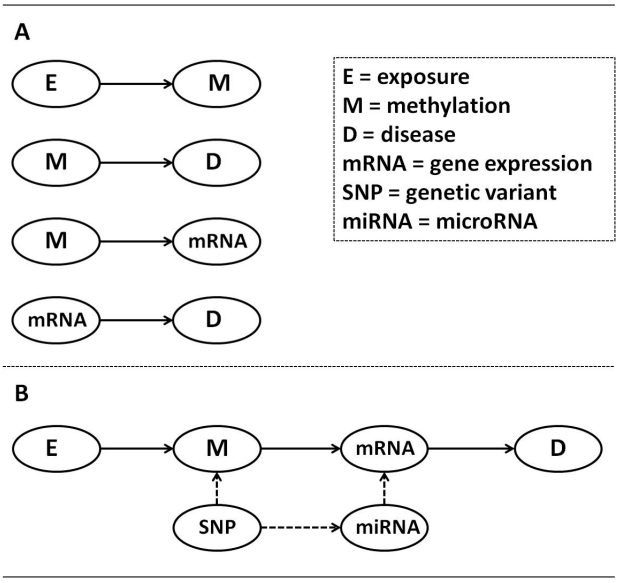

Figure 2. Investigated associations thus far (A). The important and challenging focus of future studies will be on the effect of environmental exposures on disease via epigenetic mechanisms (B) and the role of (disease) associated SNPs, i.e. inducing a methylation site or altering the expression of miRNAs (B – dashed lines).

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11

Samenvatting

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SAMENVATTING

COPD (Chronic Obstructive Pulmonary Disease of chronisch obstructieve longziekte) is een chronische vernauwing van de luchtwegen en/of aantasting van het elastische longweefsel al dan niet gepaard gaand met chronisch opgeven van slijm. COPD wordt veroorzaakt door een abnormale reactie op de inademing van schadelijke stoffen, zoals tabaksrook. De luchtwegvernauwing is permanent aanwezig, is grotendeels onomkeerbaar, en heeft in de meeste gevallen een progressief verloop.

De inademing van schadelijke deeltjes leidt tot ontsteking, structurele verandering en slijmproductie in de centrale luchtwegen (chronische bronchitis), ontsteking en structurele veranderingen in de perifere luchtwegen (bronchiolitis, kleine luchtweg ziekte), en verlies van longweefsel en elastische retractiekracht van het longweefsel (emfyseem). Deze drie kenmerken van COPD kunnen samen en in verschillende ernst voorkomen.

Roken wordt gezien als de belangrijkste risicofactor voor de ontwikkeling van COPD, echter 25-45% van alle COPD patiënten heeft nooit gerookt. Andere risicofactoren voor COPD in de Westerse wereld zijn passief roken (meeroken), blootstelling aan verschillende werkgerelateerde stoffen (stoffen, gassen en dampen) en luchtvervuiling (industrie en verkeer). Ook speelt genetische gevoeligheid een belangrijke rol. Slechts een deel van alle rokers ontwikkelt uiteindelijk COPD, dit wordt grotendeels bepaald door individuele verschillen in genetische gevoeligheid. Genetische gevoeligheid speelt zeer waarschijnlijk ook een rol bij de reactie op blootstelling aan andere risicofactoren zoals passief roken en blootstelling aan werkgerelateerde stoffen. Het is belangrijk te onderzoeken welke factoren naast actief roken geassocieerd zijn met de ontwikkeling van COPD, en welke biologische mechanismen hier aan ten grondslag liggen.

In dit proefschrift onderzochten we of passief roken en werkgerelateerde blootstelling geassocieerd zijn met het longfunctie niveau en met de prevalentie van COPD, en welke genetische varianten een rol spelen bij de individuele gevoeligheid voor de effecten van deze blootstellingen in relatie tot longfunctieniveau.

In hoofdstuk 2 lieten we zien dat werkgerelateerde blootstelling aan gassen, dampen, mineraal stof en pesticiden geassocieerd is met een lagere longfunctie en een hogere prevalentie van COPD. Met name de effecten van blootstelling aan pesticiden op longfunctieniveau waren van klinisch relevante grootte. Deze effecten waren sterker bij rokers dan bij niet rokers, dit suggereert een synergistisch effect: het effect van de twee blootstellingen is samen groter dan de som van elk van de blootstellingen afzonderlijk. We vonden geen verschillen in effecten tussen mannen en vrouwen.

In hoofdstuk 3 onderzochten we specifiek de effecten van blootstelling aan werkgerelateerde stoffen op de kleine luchtwegen en vonden dat blootstelling aan biologisch stof, gassen en dampen geassocieerd is met obstructie van de kleine luchtwegen. Deze effecten waren onafhankelijk van obstructie van de grote luchtwegen (zoals beschreven in hoofdstuk 2). In tegenstelling tot de effecten op de grote luchtwegen (hoofdstuk 2) waren de effecten op de kleine

luchtwegen niet verschillend bij rokers en niet rokers. In tegenstelling tot de grote luchtwegen, vond we geen associatie tussen blootstelling aan pesticiden en obstructie van de kleine luchtwegen.

In hoofdstuk 4 onderzochten we de effecten van blootstelling aan werkgerelateerde stoffen op de afname van longfunctie over de tijd. Afname van longfunctie over de tijd treedt bij iedereen op en hoort bij normale veroudering. Echter, bij een versnelde afname van longfunctie is er een verhoogd risico op de ontwikkeling van respiratoire klachten (kortademigheid) en uiteindelijk de ontwikkeling van COPD. In onze studie vonden we dat werkgerelateerde blootstelling aan pesticiden is geassocieerd met een versnelde afname van longfunctie. Deze associaties waren sterker bij rokers dan bij niet-rokers, wat wederom een synergistisch effect van actief roken en blootstelling aan pesticiden suggereert.

In hoofdstuk 5 onderzochten we de risicofactoren voor chronische mucus (slijm) productie (CMH) bij individuen met en zonder COPD. We vonden verschillende risicofactoren voor CMH bij individuen met en individuen zonder COPD. Bij individuen met COPD zagen we dat een hoger risico op CMH was geassocieerd met een sterkere rookgeschiedenis (langer en meer roken) en blootstelling aan passief roken. Bij individuen zonder COPD zagen we dat een hoger risico op CMH was geassocieerd met het mannelijk geslacht, hogere BMI, sterkere rookgeschiedenis, op dit moment actief roken, en werkgerelateerde blootstelling aan mineraal stof, gassen en dampen.

In hoofdstuk 6 onderzochten we de effecten van roken tijdens de zwangerschap (blootstelling *in utero*) en blootstelling aan passief roken tijdens volwassen leeftijd in relatie tot longfunctieniveau op volwassen leeftijd. Zowel blootstelling *in utero* als op volwassen leeftijd was geassocieerd met een lagere longfunctie op volwassen leeftijd, deze effecten waren sterker in niet-rokers dan in rokers. Het belangrijkste doel van deze studie was om te onderzoeken of genetische variatie in de genen *Gluthatione-S-Transferases Omega (GSTO) 1* and *2* de gevoeligheid voor de effecten van passsief roken beïnvloeden. Deze *GSTO* genen spelen een belangrijke rol in oxidatieve stress reacties en de detoxificatie van schadelijke stoffen, en zijn daarom plausibele kandidaten voor onderzoek naar genetische gevoeligheid voor schadelijke blootstellingen zoals passief roken. In onze studie vonden we dat dragers van de minder voorkomende genetische variant een hoger longfunctieniveau hadden vergeleken met niet-dragers, echter alleen als hun moeder rookte tijdens de zwangerschap. Dragers van deze variant hadden echter een lagere longfunctie vergeleken met niet-dragers bij passief roken op volwassen leeftijd. We vonden dus dat genetische variatie in biologisch plausibele genen de gevoeligheid voor de effecten van factoren zoals passief roken beïnvloeden, maar de effecten kunnen anders zijn in verschillende levensfasen.

In tegenstelling tot hoofdstuk 6 waar we kandidaat genen hebben onderzocht, gebruikten we in hoofdstuk 7 een hypothese vrije methode met als doel nieuwe genen en biologische mechanismen te vinden die de individuele gevoeligheid voor het effect van blootstelling aan passief roken beïnvloeden. We onderzochten eerst 10.817 individuen in de LifeLines studie en verifieerden onze bevindingen in 1.276 individuen in de Zwitserse SAPALDIA studie. We vonden 2

nieuwe genen, *actin, beta-like 2* (*ACTBL2*) en *zinc finger homeobox 4 (ZFHX4)* ,die in beide studies (LifeLines en SAPALDIA) geassocieerd waren met longfunctie niveau bij individuen die één uur per dag of meer werden blootgesteld aan passief roken. Deze genen zijn niet eerder in verband gebracht met passief roken, longfunctie of COPD. Naast de specifieke genen onderzochten we mogelijk onderliggende biologische mechanismen (pathways). We vonden drie biologische mechanismen die, in onze dataset, vaker naar boven kwamen dan verwacht op basis van kans. Dit waren de zogenaamde apoptosis, p38 MAPK and TNF pathways. Deze mechanismen zijn eerder in verband gebracht met COPD pathologie en kunnen mogelijk een rol spelen de gevoeligheid voor passief roken in relatie tot longfunctie.

In hoofdstuk 8 onderzochten we, wederom gebruikmakend van een hypothese vrije methode, de genetische gevoeligheid voor de effecten van werkgerelateerde blootstelling aan biologisch stof, mineraal stof en gassen en dampen, allen in relatie tot longfunctie niveau. We onderzochten 12.400 individuen in de LifeLines studie en verifieerden onze bevindingen in 1.436 individuen in de Vlagtwedde-Vlaardingen studie. We vonden 7 genetische varianten die geassocieerd waren met longfunctie niveau in de individuen met blootstelling aan één van de drie onderzochte stoffen (biologisch stof, mineraal stof of gassen en dampen). Enkele gevonden genetische varianten zijn biologische gezien mogelijk relevant voor longfunctie niveau, zoals *GALNT13* and *PCDH9*. In een additionele analyse keken we naar de effecten van deze 7 genetische varianten op de expressie van dichtbij liggende genen. We vonden dat 2 van de 7 varianten geassocieerd waren met genexpressie van *TMEM176A* and *PDE4D.* Effecten op genexpressie geven een indicatie voor de onderliggende biologische mechanismen waarbij deze genetische varianten individuele genetische gevoeligheid kunnen beïnvloeden, bijvoorbeeld door te zorgen voor een lager of hoger eiwitniveau.

Tenslotte onderzochten we in hoofdstuk 9 de genetische gevoeligheid voor de effecten van werkgerelateerde blootstelling aan pesticiden in relatie tot longfunctie, wederom gebruikmakend van een hypothese vrije methode. We onderzochten 12.400 individuen in de LifeLines studie en 1.436 individuen in de Vlagtwedde-Vlaardingen studie. Daarna meta-analyseerden we de effecten uit beide studies (de afzonderlijke effecten samenbrengen tot één effectschatting). We vonden 4 genetische varianten in 3 verschillende genen. De meest interessante variant lag in het gen *nitric oxide synthase 1* (*NOS1*). Dragers van de minder voorkomende genetische variant waren meer gevoelig voor de effecten van pesticide blootstelling wanneer er gekeken werd in relatie tot longfunctie niveau. Dit gen is eerder beschreven als gevoeligheidsgen voor pesticide blootstelling als risicofactor voor de ziekte van Parkinson, en in relatie tot het ontstekingsproces en de ziekteprogressie die wordt waargenomen bij mensen met COPD. Daarom is dit *NOS1* gen een geschikte kandidaat voor verder onderzoek naar pesticide gevoeligheid in relatie tot longfunctie niveau en de ontwikkeling van COPD.

Concluderend, de verschillende studies die zijn beschreven in dit proefschrift laten zien dat passief roken en beroepsblootstelling geassocieerd zijn met een lagere longfunctie en een hogere prevalentie van COPD. Daarnaast identificeerden we verschillende factoren die mogelijk een rol spelen bij de individuele gevoeligheid voor de effecten van deze blootstellingen, zoals actief roken en genetische varianten in nog niet eerder gevonden genen. Verder

onderzoek zal zich moeten richten op de biologische functie van de nieuw gevonden genetische varianten, en de onderliggende biologische mechanismen via welke deze genetische varianten uiteindelijk kunnen leiden tot de ontwikkeling van COPD. Tenslotte kunnen interventies gericht op het voorkomen van blootstelling aan tabaksrook (meeroken) en werkgerelateerde stoffen bijdragen aan betere gezondheid van de longen en uiteindelijk leiden tot een lagere COPD prevalentie.

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wetenschap, en over hoe om te gaan met de mogelijkheden en de valkuilen die we op ons pad tegenkwamen. Verder waren de vrijdagmiddagborrels, de etentjes en de wintersport, samen met Frank, Miriam, Mark en Pieter, natuurlijk een aangename afwisseling.

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Familie van Dijk, altijd erg attent en volop interesse in wat ik doe, vooral in het begin vaak de vraag "heb je al iets nieuws ontdekt?". Die vraag is de laatste tijd iets minder gekomen. Misschien is het duidelijk geworden dat in de wetenschap de stapjes vaak, vooral voor de buitenwereld, relatief klein zijn en niet zo snel gaan. Verder zorgt de immer aanwezige gezelligheid altijd voor de nodige ontspanning. Ook tijdens de prachtige reis naar Israël en Jordanië was het altijd relaxt, gezellig en waren de grappen flauw; het was fantastisch.

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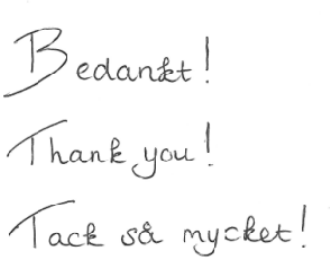

CURRICULUM VITAE

About the author

Kim de Jong was born on 25 September 1986 in Joure, the Netherlands. She graduated from secondary school at the OSG Sevenwolden in Heerenveen in 2004. In September 2004 she started her study Life Science and Technology at the University of Groningen. Her Bachelor research project was performed in Dr. C.M. Gordijn's human chronobiology group and entitled "The effect of artificial dawn and light on the quality of awakening". She obtained her bachelors degree, with specialization Molecular Physiology and Pharmacology, in 2007.

In September 2007 she started her Master studies Energy and Environmental Sciences at the Institute for Energy and Environment (IVEM) at the University of Groningen. Her Master research project entitled "Green neighbourhood environments in relation to neighbourhood satisfaction, physical activity, BMI,vitality and perceived physical and mental health status" was supervised by associate Prof. M. Albin and Prof. J. Björk and carried out at the department of Occupational and Environmental Medicine (OEM) at the university of Lund, Sweden. After this internship she was awarded with a stipend to facilitate the writing of two scientific papers for international publication based on the thesis (publications in *Environ Health*, 2011; *Health Place*, 2012). In January and February 2010 she was a research assistant in a project assessing the epidemiology of snakebite accidents in Nicaragua, Central America using Geo Information Systems (published by Hanson et al., *PLoS Negl Trop Dis*, 2010).

In September 2010 she started her PhD under supervision of Prof. H.M. Boezen, Prof. D.S. Postma and Dr. J.M. Vonk at the department for Epidemiology, unit for Chronic Airway Diseases at the UMCG in Groningen. The project was focused on interactions between genes and the environment in relation to COPD and related phenotypes. In the first part of her project she investigated the associations of environmental tobacco smoke and job exposures with the level and decline of lung function as well as the prevalence of airway obstruction. In further studies she focused on gene-by-environment interactions mainly using genome-wide approaches. The manuscript of this thesis was submitted to the reading committee in March 2014 and will be defended at September 22nd 2014.

During her PhD project she finished her training and obtained an official registration as Epidemiologist A by the Netherlands Epidemiological Society. In April 2014 she started as post-doc on the project "Genes and Exposures underlying COPD onset", which is focused on COPD in non-smokers and coordinated by Prof. H.M. Boezen.

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susceptibility
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pesticides
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gases fumes
small airways
COPD
DNA
gene environment interaction
SNP
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tobacco smoking
airflow obstruction
non-smoking
occupational exposure
GWIS
lung function
environment
lung function decline
genes
biological dust