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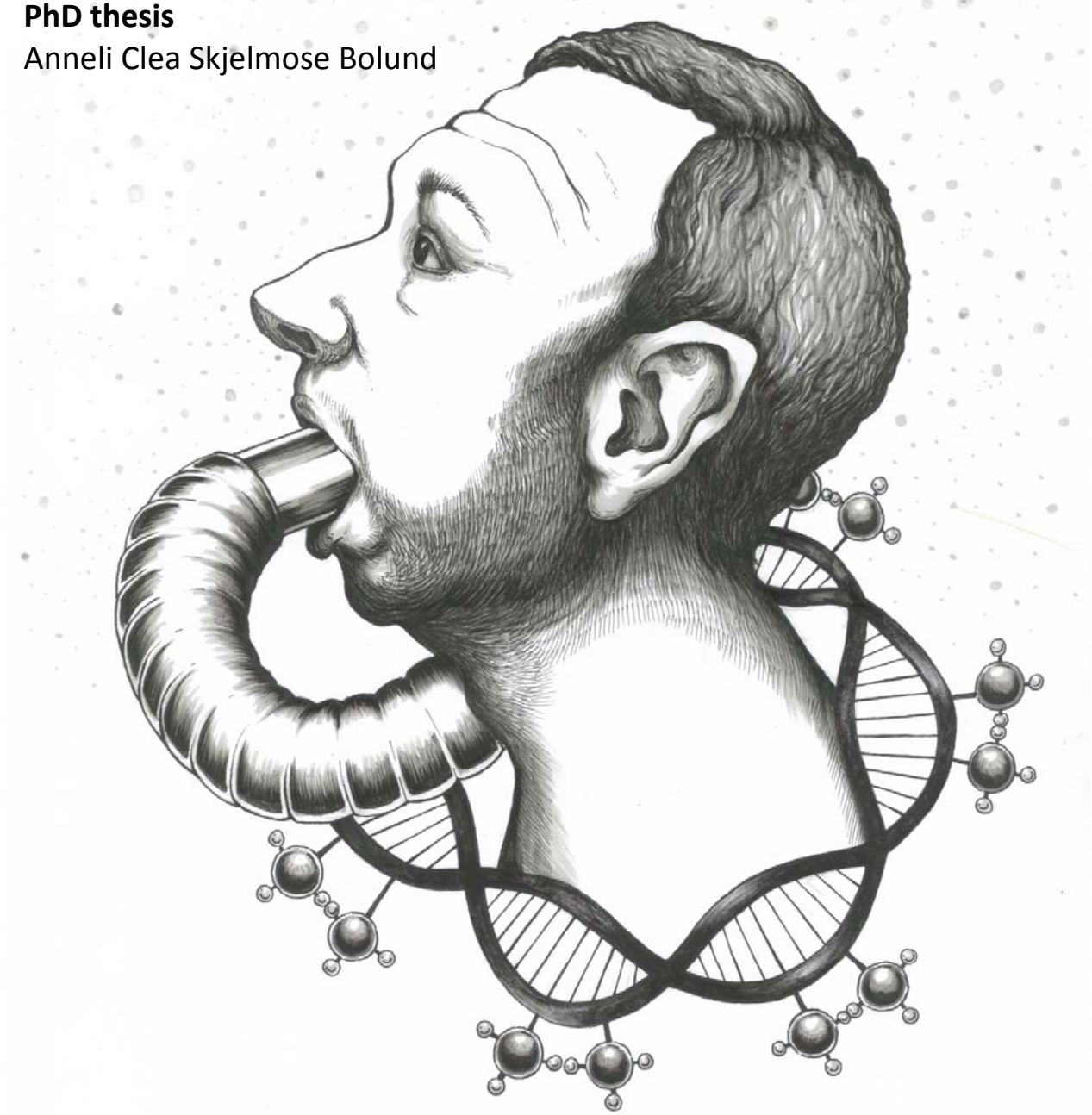
Change in lung function

The impact of organic dust exposure as well as associations with DNA methylation signatures

PhD thesis

Anneli Clea Skjelmose Bolund



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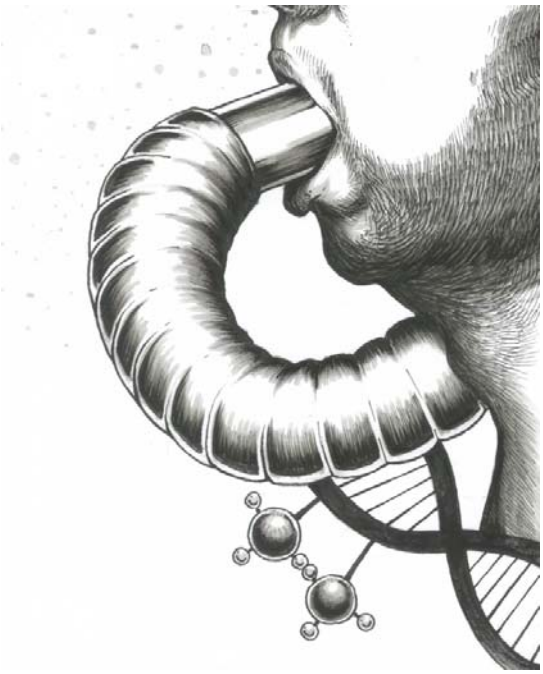

Health
Aarhus University
Department of Public Health
Section for Environment, Occupation and Health

“True wisdom is knowing what you don't know” Confucius, Chinese philosopher, 551-479 BC

To my loving and supportive husband, Morten,
and our fantastic sons, Bertil and Viggo.
I love you to the moon and back.
Let’s go there sometime☺

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Preface

Coming from a background of scientific researchers I have always had an interest in learning more and doing research on my own. As a medical student I heard an interesting presentation on research at the Section for Environment, Occupational and Health at the Department of Public Health, Aarhus University. A follow-up study of young farmers (SUS (Sund Stald/Healthy farm)) was soon to be initiated and they needed a student worker. Out of interest I contacted the section and soon after I was enrolled as a research year student under the main supervision of Professor Torben Sigsgaard and co-supervisor Professor Vivi Schlünssen. During this year (2007-2008) I started working with the SUS-study. I got a hands-on feeling of epidemiological research, participated in planning, contacting and examination of participants, data collection and data cleaning, statistical analysis and writing of scientific work. This led to the possibility of doing a PhD under the main supervision of Professor Vivi Schlünssen after finishing medical school in January 2011.

In April 2011 I started as a PhD-student at the Section for Environment, Occupation and Health on the project concerning the impact of environmental and genetic risk factors on lung function. The focus soon fell on organic dust and longitudinal change in lung function as the section had worked hard to create and investigate two great follow-up studies with detailed exposure measures. A wish for my PhD was to incorporate my (inherited) interest in genetics and collaboration was established with the Danish Twin Register in Odense, making it possible to investigate monozygotic (identical) twins, differences in lung function and blood DNA methylation signatures.

This thesis presents the work performed during my PhD at the section for Environment, Occupation and Health, Aarhus University and the collaboration with the Danish Twin Register, University of Southern Denmark, Odense. It provides an introduction into the field, including a review (Paper I), and an overview of methods and results, as well as a general discussion of the three included original research papers (Paper II-IV). One paper has been published in the peer-review journal *Occupational and Environmental Medicine, British Medical Journal* (Paper II). Two papers have been submitted and are in 2nd round review (Paper I and Paper III) and the last manuscript has recently been submitted (Paper IV).

Enjoy!

October 2016, Anneli Clea Skjelmose Bolund

Supervisors

Main supervisor:

Professor Vivi Schlünssen, MD, PhD

Section for Environment, Occupation and Health

Department of Public Health

Aarhus University, Denmark and

National Research Centre for the Working Environment

Copenhagen, Denmark

Co-supervisors:

Professor Torben Sigsgaard

Section for Environment, Occupation and Health

Department of Public Health

Aarhus University, Denmark

Professor Martin Miller

Institute of Occupational and Environmental Medicine

University of Birmingham, UK

Professor Øyvind Omland

Department of Occupational Medicine

Aalborg University Hospital, Denmark

Professor Anders Børglum

Department of Biomedicine

Aarhus University, Denmark

Evaluation committee

Professor Peter Lange

Section of Social Medicine

Department of Public Health

University of Copenhagen, Denmark

Ass professor Lidwien Smit

Institute for Risk Assessment Science (IRAS)

Utrecht University, the Netherlands

Chairman and moderator of the PhD defence:

Ass professor Bodil Hammer Bech

Section of Epidemiology

Department of Public Health,

Aarhus University, Denmark

Acknowledgements

Working with research during this PhD has been a great personal and professional journey for me and I have learned a lot. This thesis has been created as a joint venture and effort of a large group of people, including supervisors, colleagues, friends and family, whom I would all like to thank.

First of all, I wish to thank my group of five supervisors for giving me this opportunity, and for all you have taught me and contributed with during this process. *Vivi Schlünssen*, for being a great main- and daily supervisor, whom with enthusiasm, determination, and acceptance of my many questions, has guided me through all phases of this project. Thank you for your patience, laughter, and time, despite your own busy schedule. *Torben Sigsgaard*, for initially accepting me as a research year student and getting me started in this interesting field of work. Thank you for always having time for a quick question across the hallway, and for your laughter and happy personality. If it wasn't for you and our mutual interest in genetics, the epigenetic part of this thesis would not have been possible. *Martin Miller*, for your invaluable knowledge and input concerning pulmonary physiology and your many trips to Denmark with time for discussion. Thank you for your persistence and patience with me when trying to explain statistical aspects of the lung function measures and standardisations. I think I get it now. Thank you to *Øyvind Omland* and *Anders Børglum* for your support and advise when I needed it.

The epigenetic part of this thesis was made possible through the collaboration with the Danish Twin Register, University of Southern Denmark, Odense. Thank you for letting me work with your very unique data. Thank you *Lene Christiansen* and *Qihua Tan* for your invaluable expertise, feedback and advice on twin-based research. I would also like to sincerely thank Anna Starnawska for our great teamwork concerning the epigenetic study, and all the time and effort you put into this.

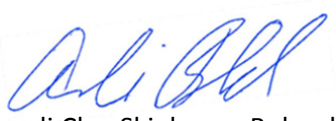
I would also like to express my gratitude to all former and current *colleagues* at Section for Environment Occupation and Health and the *RESPIT group* for always fruitful discussions. A special thank you to *Grethe Elholm*, for our shared office and fun talks, for your energy, and your willingness to answer any question I may have asked. Thank you to *Gitte Juel Holst* for recently showing how it is done and to *Signe Timm* for "hygge" and having your baby the same time as "mine". I really appreciate getting to share this with all of you and that we went through this together.

I greatly appreciate the financial support in form of a PhD-fellowship provided by the Faculty of Health, Aarhus University. Thank you to Danish Ramazzini Centre for the support and affiliation.

A great thank you to all study-subjects that have participated in the studies – you are the reason that any of this was possible.

Last but not least, I would like to thank my family. My father, for showing me that research can be a lifelong professional “hobby” and my sister, for showing me that we can too; my parents, for your endless support, encouragement and love. Thank you for always believing in me. My husband and boys, for reminding me of what is most important in life and for keeping me grounded. You are everything to me.

It has been a great learning experience and I take it with me. If anything, I now know how much I don’t know, which calls for much more research in the future.



October 2016, Anneli Clea Skjelmose Bolund

List of papers

This thesis is based on one review and three original research papers:

Paper I Bolund ACS, Miller MR, Sigsgaard T, Schlünssen V.

The effect of organic dust on long-term change in lung function – a systematic review. *Submitted manuscript in 2nd round review.*

Paper II Bolund ACS, Miller MR, Basinas I, Elholm G, Omland Ø, Sigsgaard T, Schlünssen V. The effect of occupational farming on lung function development in young adults: a 15-year follow-up study. *Occup Environ Med 2015;72:707–713.*

Paper III Bolund ACS, Miller MR, Jacobsen G, Sigsgaard T, Schlünssen V.

New-onset COPD and decline in lung function among wood dust exposed workers – a 6 year follow-up study. *Submitted manuscript in 2nd round review.*

Paper IV Bolund ACS, Starnawska A, Miller MR, Schlünssen V, Backer V, Børglum A, Christensen K, Tan Q, Christiansen L, Sigsgaard T. Lung function discordance in monozygotic twins and associated differences in blood DNA methylation. *Submitted manuscript.*

Figures and tables

Thesis

Summary

Lung function is a predictor of morbidity and mortality in the general population. Organic dust is an occupational exposure that may affect change in lung function negatively. In order to explore if there is a causal relationship between organic dust exposure and excess decline in lung function, emphasis on longitudinal studies is needed and thorough assessments of exposure and outcome are crucial. The aetiology of pulmonary disease and lung function is, however, not only dependant on different external risk factors. Individual genetic variations and the functional state of the genes (epigenetics) are also of importance.

The overall aim of this PhD-thesis was to explore the association between exposure to organic dust and the long-term change in lung function. The included systematic review of previously published literature (Paper I of this thesis), concerning change in lung function and exposure to organic dust, gave some evidence of a causal association between exposure to organic dust and long-term excess decline in lung function. An exposure-response relationship was also indicated, though results were inconsistent.

In our original occupational studies (Paper II and III of this thesis) we explored the association in two occupational follow-up cohorts: The SUS-study, examining 962 young farming students and 172 young controls with a follow-up time of 15 years; and the WOOD-study, examining 1,112 wood workers in the furniture industry and 235 controls with a 6-year follow-up time. Change in lung function was assessed by spirometry at two time points for all individuals and personal exposure assessment was based on dust measurements in the work settings.

The SUS-study found that being a current farmer compared to being an ex-farmer was associated with a negative effect on change in lung function. However, an exposure-response relation between levels of dust exposure and change in lung function was not found in this cohort of young farmers. The WOOD-study found a significant exposure-response relation between wood dust exposure and decline in lung function and increased risk of new-onset COPD for female wood-workers. This was not seen for male wood-workers, possibly due to healthy worker selection bias. However, an increased susceptibility to the adverse effects of organic dust on change in lung function among females was indicated in both studies.

The association between level and change in lung function and genome-wide differential methylation signatures in blood DNA of discordant monozygotic twins was explored (Paper IV of this thesis). The study was based on 169 middle-aged twin pairs with measured lung function at the start and end of an 11 year follow-up period. Several associated differentially methylated CpG-sites in different genes were identified to be associated with level and

change in lung function in this TWIN-study. Interesting enriched pathways with potential importance for lung function were found. These associated genes and enriched pathways were among others involved in oncogenic- and tumour suppression mechanisms, as well as growth and lung tissue repair, which may be implicated in pulmonary physiology.

In conclusion the exposure to organic dust in occupational settings should be kept down in order to avoid adverse health effects on lung function. Furthermore, we need to continue expanding our knowledge of the importance of exposure timing, as well as our understanding of the pathophysiological processes underlying the impaired lung function. This should be possible with the rapid technological developments of large scale genome/epigenome wide association studies that can be combined with epidemiological and functional analyses in the future.

Resumé

Lungefunktion er en prædiktor for sygelighed og dødelighed i den generelle befolkning. Organisk støv er en erhvervsmæssig eksponering, som muligvis kan påvirke ændring i lungefunktion negativt. For at udforske, om der er en årsagssammenhæng mellem organisk støv-eksponering og øget fald i lungefunktion, er der behov for fokus på longitudinelle undersøgelser. Det er ligeledes vigtigt med præcise vurderinger af eksponering og udfald. Ætiologien bag lungesygdom og lungefunktion er dog ikke kun afhængig af forskellige eksterne risikofaktorer. Individuelle genetiske variationer og den funktionelle tilstand af generne (epigenetik) har også betydning.

Det overordnede formål med denne PhD-afhandling var at undersøge sammenhængen mellem udsættelse for organisk støv og ændring i lungefunktion. I den systematiske gennemgang af tidligere publiceret litteratur (Artikel I i denne afhandling), om ændring i lungefunktion og udsættelse for organisk støv, konkluderede vi, at der er nogen evidens for en årsagssammenhæng mellem eksponering for organisk støv og øget fald i lungefunktion. En eksponerings-respons sammenhæng var også indikeret, om end resultaterne var inkonsistente.

I vores originale studier (Artikel II og III i denne afhandling) udforskede vi denne sammenhæng i to erhvervs-kohorter: SUS-studiet, der undersøgte 962 unge landbrugs-studerende og 172 unge kontroller med en follow-up tid på 15 år; og WOOD-studiet, der undersøgte 1.112 træarbejdere i møbelindustrien og 235 kontroller med en 6-års follow-up tid. Ændring i lungefunktion blev vurderet for alle individer ved spirometri målt ved to tidspunkter. Vurdering af personlig eksponering var baseret på støvmålinger i arbejdsmiljøet.

SUS-studiet viste, at dét at være aktuel landmand i forhold til at være ex-landmand havde en negativ effekt på lungefunktionsændringen. Vi fandt ikke en eksponerings-respons sammenhæng mellem niveauer af støveksponering og ændring i lungefunktion i denne kohorte af unge landmænd. WOOD-studiet viste, for kvindelige træ-arbejdere, en klar eksponering-respons sammenhæng mellem træstøvseksponering og lungefunktionsændring samt øget risiko for nyopstået KOL. Dette fandt vi ikke for mandlige træ-arbejdere, muligvis på grund af en selektion af sunde og raske mandlige arbejdere. Begge studier indikerede dog at kvinder har øget følsomhed over for de negative effekter af organisk støv på ændring i lungefunktion.

Sammenhængen mellem lungefunktion og DNA-methyleringsmønstre i blodcellers arvemasse blev undersøgt i enæggede tvillinger (Artikel IV i denne afhandling). Undersøgelsen var baseret på 169 midaldrende tvillingepar med lungefunktion målt i

starten og slutningen af en 11 års follow-up periode. Sammenhæng mellem lungefunktion og adskillige differentielt methylerede CpG-positioner i forskellige gener blev fundet i dette TWIN-studie. Interessante biologiske pathways med potentiel betydning for lungefunktion blev også identificeret. Disse identificerede gener og biologiske pathways var blandt andet involveret i onkogene- og tumorundertrykkende mekanismer, samt vækst og reparation af lungevæv, som alle kan være impliceret i pulmonal fysiologi.

Vores resultater understøtter at man fortsat bør fokusere på at holde eksponering for organisk støv nede i arbejdsmiljøet for at undgå sundhedsskadelige virkninger på lungefunktionen. Desuden er det nødvendigt at vi fortsat udvider vores viden om betydning af timing af eksponering, samt vores forståelse af de patofysiologiske processer bag nedsat lungefunktion. Dette bør være muligt med den rivende teknologiske udvikling der foregår inden for genom/epigenom forskning, hvilket kan kombineres med epidemiologiske og funktionelle analyser i fremtiden.

Abbreviations

BHR	Bronchial Hyper Responsiveness
BMI	Body Mass Index
COPD	Chronic Obstructive Pulmonary Disease
CpG	Cytosine-Phosphate-Guanine
d_{ae}	Aerodynamic diameter of dust particles
DNA	Deoxyribonucleic Acid
DNMT	*De novo* Methyltransferase
DTR	Danish Twin Register
EWAS	Epigenome Wide Association Studies
FDR	False Discovery Rate
FEV_1	Forced Expiratory Volume in the 1^{st} second
FVC	Forced Vital Capacity
GLI	Global Lung Function Initiative
GWAS	Genome Wide Association Studies
LLN	Lower Limit of Normal
LMS	Lambda, Mu, Sigma statistical method
MADT	Middle Aged Danish Twin Study
MZ	Monozygotic
OEL	Occupational Exposure Limit
RNA	Ribonucleic Acid
RTM	Regression To the Mean
SNP	Single Nucleotide Polymorphism
SUS	Sund Stald (Healthy farm)

1. Introduction

Lung function is a predictor of morbidity and mortality in the general population (1–3) and the overall well-being of an individual is linked to the lung function (4). Change in lung function is a slowly proceeding continuous event that happens with age (5,6) and it is possible to detect excessive lung function decline due to its chronic nature. Not until the lung function is quite compromised, highly dependent on the individual, severe symptoms and the feeling of breathlessness appears. However, because of the slow nature of lung function decline, prevention may be possible if lung function is monitored continuously.

The effect of occupational organic dust exposure on level of lung function has been explored extensively in cross-sectional studies and evidence points towards a negative association between exposure to organic dust and level of lung function. However, in order to explore if there is a causal relation between organic dust exposure and excess decline in lung function, emphasis on longitudinal studies is needed and thorough exposure and outcome assessments are crucial. In this thesis it was therefore attempted to investigate the relation between organic dust exposure in farming and wood-working subjects and the long-term change in lung function in two large occupational follow-up studies.

The aetiologies of different diseases, including pulmonary diseases and lung function decline, are not only dependant on different external risk factors. The individual variations of the DNA sequence of genes are basic, but also functional (dys)regulations of the genes are of importance. These functional modifications, referred to as epigenetics (“above” genetics), are defined by underlying genetic mechanisms and triggered by specific environmental factors. Epigenetics is a new approach, teaching us more about the dynamic aspects of regulation of gene activity, and the definition of epigenetics is “any process that influences gene activity without altering the DNA sequence” (7). As a hypothesis-generating part of this thesis we explored the association between change in lung function and epigenetic profiles in monozygotic twins (MZ twins), as MZ twins serve the unique possibility to control for underlying genetic make-up as well as shared environmental exposures.

1.1 Overall aim

The overall aim of this thesis was to explore the association between exposure to organic dust and the change in lung function in a cohort of farmers and a cohort of wood-workers. Furthermore we wished to explore the association between within-twin differences in lung function and epigenetic signatures in monozygotic twins to learn more about the underlying genetic and regulatory aspects of lung function. In the following chapter with detailed background information the specific hypotheses and aims will be stated at the end.

2. Background

This section of the thesis contains the background information on lung function and change in lung function, as well as information on exposure to organic dust and its constituents. A summary of the epidemiological literature concerning change in lung function in association with different occupational exposures and general risk factors will be presented. Furthermore, background information on genetic and epigenetic mechanisms will be given. Finally, the hypotheses and specific aims of the thesis will be stated.

2.1 Lung function

Lung function testing plays an important role in assessment of respiratory health. It is used to diagnose different respiratory diseases, such as Chronic Obstructive Pulmonary Disease (COPD) and Asthma, and to assess normal lung growth and also excess decline in lung function. Lung function increases with age in childhood due to growth and maturation, and then plateaus around 25-30 years of age. Subsequently lung function declines with age in adulthood due to loss of elastic recoil in the lungs and decrease in respiratory muscle strength, among other age-related structural and immunological changes (6,8). Lung function also depends on height as a proxy for chest size and therefore the measurement of standing height needs to be performed accurately (9). Large variability in lung function between individuals, dependent on sex, age, height and ethnicity is evident and the risk of large variability between individual repeated assessments and different assessors is also an issue. Therefore standardisation of measurement manoeuvres is of great importance.

One aspect of lung function testing is to assess the lung capacity of an individual. This is often assessed by spirometry, which measures forced expiratory volume in the first second (FEV_1) and forced vital capacity (FVC). Spirometry is a manoeuvre where full inspiration is followed by maximally forceful and complete expiration (10) into the spirometer, which registers the output. The ratio between the two lung function metrics (FEV_1/FVC) can subsequently be calculated, and is a measure which can help differentiate between obstructive and restrictive pulmonary diseases. The obstructive disease COPD is described in more detail in section **2.1.2**. Restrictive diseases cause a reduction of both FEV_1 and even more so of FVC leading to a normal or increased ratio (FEV_1/FVC). Restriction can result from excessive stiffness in the lungs, pleura or chest wall, which prevents the lungs from fully expanding. Restrictive diseases include interstitial lung diseases, such as pulmonary fibrosis, as well as sarcoidosis and neuromuscular diseases among others. Specific restrictive diseases will not be evaluated in further detail in this thesis.

Spirometry is a non-invasive objective measure, and with trained assessors it is reproducible and sensitive to small changes and is therefore the preferred method to assess lung function.

2.1.1 The GLI 2012 equations

The Global Lung Function Initiative (GLI) was established in 2008 as a European Respiratory Society Task Force (11). The aim was to derive prediction equations for lung function that could be applied globally and that covered the full age-range from 3 to 95 years of age. Several international respiratory societies have subsequently endorsed this effort, and recommend the use of the GLI 2012 equations (newest version of the GLI equations). Previously, prediction equations covered a limited age range, such as childhood, adolescence or adulthood. This led to the use of separate equations for individuals when changing age group from for example adolescence to adulthood, leading to discrepancies and bias of the results (9). With the GLI 2012 reference equations an improved international standard for spirometry reference has been established. They cover the entire age-range in one set of continuous equations and adjusting for the important predictive variables sex, age, height and ethnicity. The equations were based on cross-sectional lung function measurements (spirometry) of 74.187 healthy non-smoking individuals from different countries and ethnicities that had previously been included in different study-populations. The global lung function initiative continues with ongoing data-collection to further improve the reference equations.

The equations were modelled using the LMS (Lambda, Mu, Sigma) method (12) based on the distributional details of the data. Lambda (L: location) is an index of skewness, Mu (M: median) is the median value, and Sigma (S: scatter) is the coefficient of variation, which relates to the between-subject variability. Each component of the distribution was used to derive the best fitting function of each outcome (e.g. FEV_1, FVC, and FEV_1/FVC) as a function of age and height in males and females of different ethnic origin. The prediction equations based on GLI 2012 hence allow modelling of external data based on the expected mean (predicted value), the coefficient of variance, and the skewness of the data. Predicted L, M and S values are calculated using “lookup tables” and are dependent on sex, age, height, and ethnicity. The L, M and S scores are combined to get a z-score (deviation from the predicted in standard deviation (SD)) for an individual observation. Also output of % predicted and lower limit of normal (LLN, 5^{th} percentile of the healthy non-smoking GLI-population) is available. The z-score can also be transformed into the percentile based on the distribution. However, at the extreme values of percentiles (e.g. 1^{st} percentile) the z-score can change significantly (e.g. from -3 to -6 z-scores) without changing the percentile. Therefore, it was chosen to report all results as z-scores in this thesis and the appertaining

articles. The practical utilization of the GLI 2012 equations is described in the Material and Methods section **3.1.1**.

2.1.2 Chronic Obstructive Pulmonary Disease

COPD is a condition characterised by persistent airflow limitation, which results in difficulty of exhaling the air from the lungs. Accompanying features of COPD include chronic bronchitis, emphysema, and/or asthma (13), with loss of lung recoil in damaged lung tissue and narrowing of the airways because of bronchoconstriction and inflammation in the mucous membrane. Breathlessness, chronic cough and/or chronic sputum are the dominant respiratory symptoms of COPD. COPD was estimated to be the 3rd leading cause of mortality in the world in 2010 (14) and is a global health problem with increasing prevalence due to continued exposure to risk factors and an aging population (15). Smoking is considered to be the greatest risk factor for COPD (16), however, occupational exposures to inhalable particles are estimated to account for around 15% of all COPD-cases (17).

Defining COPD in a clear diagnostic manner has been debated during the last decades. The use of a fixed ratio of $FEV_1/FVC < 0.7$, suggested by the Global Initiative for Chronic Obstructive Lung Disease (GOLD), leads to an over diagnosis of COPD in the elderly and an under diagnosis in the younger individuals (15,18). However, GOLD argues that spirometry is only one index used for establishing the clinical diagnosis of COPD, the others being symptoms and risk factors (15). The assessment of COPD severity according to the GOLD criteria has been defined by the grade of severity of post-bronchodilator airflow limitation in subjects with $FEV_1/FVC < 0.7$ as GOLD 1: Mild, $FEV_1 \geq 80\%$ predicted; GOLD 2: Moderate, $50\% \leq FEV_1 < 80\%$ predicted; GOLD 3: Severe $30\% \leq FEV_1 < 50\%$ predicted; and GOLD 4: Very severe, $FEV_1 < 30\%$ predicted; all accompanied by key indicators including symptoms such as chronic cough, chronic sputum, progressive persistent breathlessnes, and risk factors such as smoking and occupational exposures, as well as familiar predisposition (15). According to GOLD these indicators are not diagnostic on their own, but the presence of key indicators supports the diagnosis of COPD, for which spirometry is required (15). This is still a common diagnostic tool used in clinical practice, however, it has been argued that the use of LLN according to the GLI 2012 reference equations accompanied by respiratory symptoms is a more valid way of estimating COPD (18) with less risk of misclassification due to age. The variability of individual lung function measurements around the median is not uniform across all ages (19) but this is standardised for using the z-score approach. The definition used for diagnosis of new-onset COPD in this thesis (WOOD-study, Paper III) was therefore defined by the GLI 2012 equations as $FEV_1/FVC <$ LLN accompanied by symptoms of chronic cough, chronic sputum or breathlessness.

2.2 Organic dust exposure

A major air-borne exposure within different workplaces is organic dust. It is an aggregate of air-suspended particles such as bacteria, moulds and pollens, derived from microbes, animals and plants (20). According to the International Organization for Standardization (ISO 4225:1994), the definition of dust is *"Small solid particles, conventionally taken as those particles below 75 µm in diameter, which settle out under their own weight but which may remain suspended for some time"* (21). A WHO definition summarises dust to be *"solid particles, ranging in size from below 1 µm up to at least 100 µm, which may be or become airborne, depending on their origin, physical characteristics and ambient conditions"* (22). Occupational exposure to organic dust is present in several occupations such as agricultural work and farming (field work, grain storage, and animal confinements), cotton industry, paper work, bakeries, waste work and composting, forestry and woodworking among others. The level of organic dust exposure largely varies between occupations and within occupations but no global overview of occupational dust exposure levels is available. Without careful dust control, work that generates dust can lead to high levels of exposure, in some cases reaching up to hundreds of mg/m^3 (22). Occupational Exposure Limits (OELs) are a key element in the health risk management and are defined based on a combination of dose-response, or exposure-effect assessments (22) for different occupations in different countries, as well as political and economic considerations. Hence, it is not a purely scientifically defined exposure limit. This leads to the limitation, that levels below OEL may still be associated with adverse health effects. The OEL for total organic dust in the working industry in Denmark is set at 3 mg/m^3 (23).

Endotoxins comprise an important part of dust from organic sources. Endotoxins are part of the outer membrane of gram negative bacteria and have well known strong pro-inflammatory properties (24). Endotoxins are ubiquitously present in the environment and are found in the soil, on vegetation, in animals, and in water (25).

Also other correlated particles are present in organic dust, varying between occupations. Hence, different organic dust constituents in farming and grain industry have been shown to be strongly correlated (26,27), such as bacteria, endotoxins, fungal spores, mite units, glucan and ammonia. Similarly, for wood dust exposure, it has been shown that different particles are present in the wood dust, such as endotoxins and low molecular compounds (e.g. terpenes, abietic acid, glycosides and plicatic acid) (28). For simplicity, the collective exposure to organic dust, endotoxins and other particles will be referred to as "organic dust" through the rest of the thesis, unless otherwise specified.

2.2.1 How organic dust can cause pulmonary disease

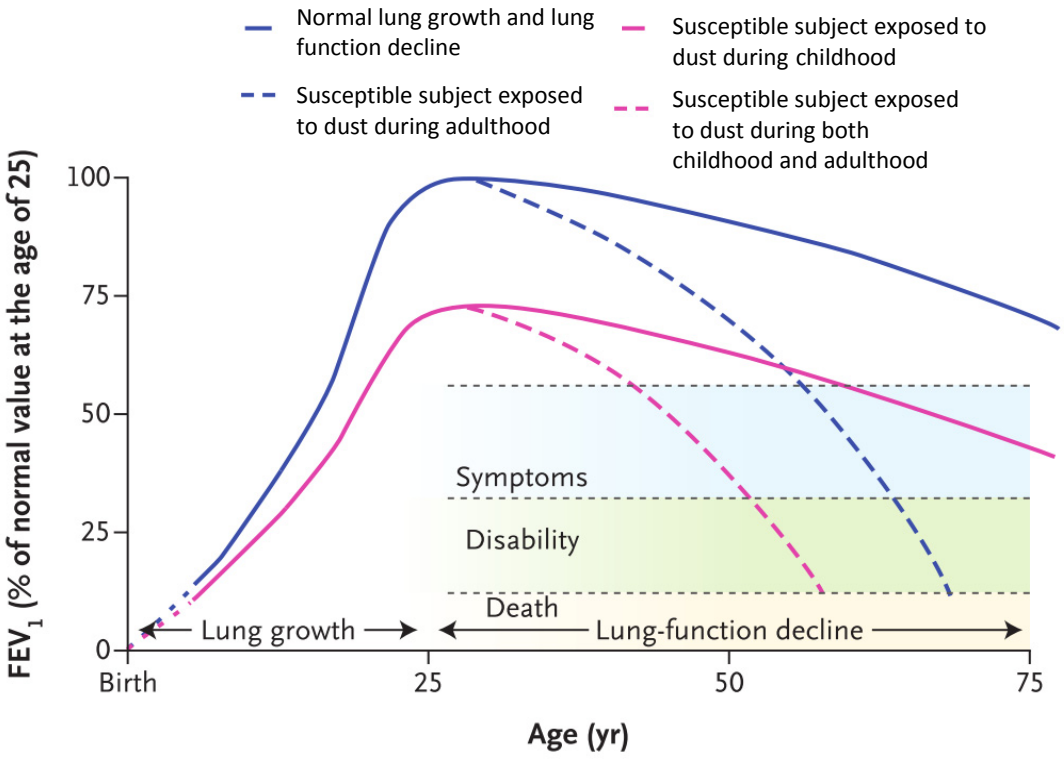
The occupational hazards of organic dust came into focus with the ground breaking work of Bernadino Ramazzini, the father of occupational medicine, in the 17th century (29,30). However, as early as 1555, Olaus Magnus warned about the dangers of inhaling grain dust (31). In the 20th century focus was again turned towards organic dust as a possible risk factor for pulmonary health.

Environmental exposure to organic dust mainly occurs through inhalation, which is the exposure route considered to be of greatest importance concerning pulmonary health. Organic dust particles vary in size (aerodynamic diameter (d_{ae})) and the size of the particle in turn predicts where deposition occurs in the airways and lungs. Further, the smaller particles may present a greater hazard, as they remain airborne longer and are hence more likely to be inhaled (32). Based on the particle size different fractions of dust have been defined. The *inhalable* dust fraction is any particle that enters the body through the nose and/or mouth during breathing ($d_{ae} \leq 100$ µm), the *thoracic* fraction is a sub fraction of the inhalable fraction ($d_{ae} \leq 30$ µm) composed of particles that can penetrate into the tracheo-alveolar region of the lung and the *respirable* fraction is the sub fraction of the inhaled particles ($d_{ae} \leq 10$ µm) that penetrates into the un-ciliated airways and alveolar region of the lung (33). The *thoracic* fraction of dust is considered the most important for the bronchial airways and obstructive disease whereas the *respirable* fraction may be of more importance for lung-parenchymal disease (34) and hence restrictive disorders.

Defence mechanisms for clearing particles from the airways are part of an evolutionary process to protect the individual from viruses, dust, and smoke from cooking as well as other environmental factors. The defence mechanisms include mechanical mechanisms such as nasal filtering, cough and sneeze-reflexes, mucous-ciliary transport in the airways, as well as immunological mechanisms of antibodies, alveolar macrophages, lymphocytes, and enzymes. However, these mechanisms are not always enough to prevent deposition in the airways and lungs and hence fail to prevent possible adverse health effects to occur. Development of chronic diseases takes time and the complex interaction between dose and timing of exposure, other environmental factors, and genetic predisposition (35) determine the individual physiological and immunological response to the exposure of organic dust. How the immunological response in the lung is linked to a possible accelerated loss of lung function is still not fully understood and may also involve factors related to immune regulation, resolution of inflammation, cellular repair, as well as underlying genetic (36) and epigenetic factors. It is, however, biologicaly plausible that exposure to organic dust could have a negative effect on lung function. After inhalation of organic dust, deposition of dust particles and constituents may lead to an inflammatory response in the airways and lungs, which in turn with time, may lead to fibrosis and hence deterioration in lung function in

exposed subjects (37). Figure 2.1 illustrates the hypothetical growth curve of lung function and the possible effects of exposure to dust. However, it is not known to what extend the effect of organic dust exposure is dependent of the timing of exposure, and of age and phase of lung development of the individual.

Figure 2.1 Hypothetical growth curve of lung function and negative effects of exposure to dust at different exposure times. (Adapted from Brusselle (38))



Many cross-sectional studies have assessed the effect of organic dust on level of lung function in different occupational settings such as farming (26,39), grain handling (40,41), wood working (42) and compost handling (43). Castellan et al (1987) (44) were among the first to describe a strong relation between the specific exposure to airborne endotoxin and acute decline in FEV1. However, the dust concentration *per se* was in their study not shown to be associated with an acute change in FEV1.

To explore a possible causal relation between organic dust exposure and long-term change in lung function, emphasis on longitudinal studies is needed as well as thorough exposure and outcome assessments. In the following summary of existing literature on the subject, focus is therefore on longitudinal studies only.

2.3 Risk factors for excess decline in lung function

In the following section the epidemiological literature concerning different risk factors for excess decline in lung function is summarised. A summary of the systematic review concerning exposure to organic dust and the association to change in lung function (Paper I, Appendix I) is first presented.

2.3.1 Organic dust

We performed a systematic review according to the PRISMA criteria (45) exploring the published literature concerning the possible association between exposure to organic dust in different occupational settings and excess decline in lung function in longitudinal studies. In summary (for more detail see Paper I) 1580 potentially relevant publications identified through a literature search were narrowed down to 20 articles that met the inclusion criteria. The most important inclusion criteria, defined prior to the literature search, were based on 1) design: longitudinal studies with minimum follow-up of 1 year, 2) exposure: organic dust in different settings, 3) outcome: longitudinal change in lung function (FEV_1, FVC, FEV_1/FVC), 4) adjustment for smoking, 5) clear exposure gradient and 6) > 50 exposed subjects.

The exposures of the finally included 20 articles were 1) Cotton dust (46–48), 2) Grain dust (49–53), 3) Farm dust (54–61), 4) Paper dust (62), and 5) Wood dust (63–65). The outcome in all studies was change in lung function (FEV_1, FVC, FEV_1/FVC) measured by spirometry at minimum 2 occasions with at least 1 year follow-up time. All studies were adjusted for smoking. Other adjustment factors differed between studies.

For a detailed description of the results of each included study, please see Paper I, Appendix I. Overall, 14 out of the 20 included studies found an association between organic dust exposure and decline in lung function. When comparing exposed subjects with controls, six studies (47,53–56,63) found an association between exposure and decline in lung function. The excess FEV_1 decline in these studies ranged from -12 mL/year to -38 mL/year and the excess FVC decline ranged from -9 mL/year to -34 mL/year. With several years of exposure this could possibly lead to important pulmonary health issues among exposed subjects. When an exposure-response relation was assessed within an exposed population, 12 studies (47–52,54,56,58,61,63,65) found a significant association.

No systematic differences in associations were seen between neither difference in the quality score (based on 6 criteria) of the publications, nor differences in age groups. Higher number of follow-up years did not strengthen the association between organic dust exposure and decline in lung function. The included studies were very heterogenic

concerning study design, exposure and outcome measures, as well as inconsistent in terms of significant findings.

Further, studies not included in the review, due to not fulfilling the inclusion criteria, have explored the associations between exposure to organic dust and negative effects on lung function change. A study by Bünger et al (66) of workers exposed to organic dust from composting plants found that FVC (in percent of predicted) declined significantly for compost workers compared to control subjects during 5 years of follow-up. A new 13-year follow-up of this same cohort showed no greater loss of lung function during the long follow-up period in the compost workers (67). Also hemp workers (68), jute processing workers (69), and pulp mill workers (70) have been longitudinally studied, showing significant associations between exposure to organic dust in these occupations and negative effects on change in lung function.

Despite of the inconsistencies of the study-results, the evidence points towards a causal negative effect of organic dust exposure on long-term change in lung function. Grain dust was the most consistent exposure, as 5 out of 5 studies included in the systematic review found a significant association between grain dust exposure and negative effect on lung function change.

2.3.2 Inorganic dust

Other possible occupational risk factors for change in lung function include inorganic dusts, such as mineral dust (coal, cement, and crystalline silica (e.g. quartz in the granite industry)) and metallic dust (in e.g. stainless steel production and welding (lead, cadmium, chromium, nickel, beryllium and cobalt compounds)) (22). Summarised here are some studies with focus on the effect of these inorganic dust exposures on long-term change in lung function.

Coal miners are the most extensively examined group of workers concerning inorganic dust exposure and pulmonary health. Several reviews (71–75) and original research articles (76–79) have concluded that exposure to coal mine dust is a cause of excess decline in lung function. Several studies (80,81) have also concluded that the effect of exposure to coal dust on change in lung function is non-linear, with rapid initial effects in new coal miners in the first year of exposure and less rapid loss of lung function in subsequent years. The initial adverse effects on lung function may be due to inflammation of the small airways in response to new exposure to coal dust (81) with adaptation to the exposure during the following years. However, a few newer studies have also reported lack of convincing evidence of excessive decline in lung function, with exposure to e.g. brown coal dust (82) and coal dust and nitrogen oxide exposure (83). However, this may be due to successful

limitation of individual coal mine dust exposure implemented after finding severe adverse health effects from the exposure.

The effect of exposure in granite workers to quartz and silica on change in lung function has also been extensively debated. However, the evidence is less consistent concerning this exposure. The Vermont granite workers were studied in a 4 year follow-up in 1970-1974 (84) concluding an excessive decline in lung function among granite workers. However, a re-evaluation of Vermont granite workers in an 8 year follow-up study from 1979-1987 (85) concluded that the effects on lung function change were significantly smaller than those estimated previously, and found no indication of accelerated lung function loss during the 8 years of follow-up. This could be partially explained by a non-linear effect with rapid initial declines, as seen for the coal miners; however, an overall slightly accelerated decline would have been expected if this was the case. Other studies have similarly found both significant (86) and non-significant (87) associations between exposure to granite dust and excess decline in lung function. However, a recent systematic review and meta-analysis of respirable quartz dust exposure and airway obstruction (88) as well as a review of occupation and COPD (89) both concluded that overall there is evidence of a significant decline of FEV_1 and FEV_1/FVC and increased risk of COPD related to cumulative respirable quartz (silica) dust exposure. The health hazards of cement dust, which also contains small amounts of quartz, were reviewed by Meo (90) in 2004. It was also here concluded that workers with this exposure had a consistent excess decline in FEV_1 and FVC with prolonged years of service in the cement industry.

Metallic dust has also been studied. Exposure to cobalt dust and change in lung function was studied by Verougstraete et al (91). It was found that cobalt dust exposure negatively influenced change in FEV_1, leading to an excess decline of 64 to 103 mL depending on exposure level during 10 years of follow-up, however only in association with smoking. Cobalt dust exposure was also studied by Rehfisch et al (92) finding a tendency for a dose–response effect between increasing cobalt exposure and annual decline in FEV_1. However, it did not reach statistical significance. The specific exposure to chromium in stainless steel production showed no significant association to change in lung function in a 5 year follow-up study (93).

Despite the inconsistent results, overall there is quite convincing evidence that inorganic dust exposure may also have a negative effect on change in lung function. Coal dust is the most extensively examined inorganic exposure with the most consistent results showing a negative association with change in lung function.

2.3.3 Other exposures

The effect of combined exposures to vapour, dust, gas and fumes (VGDF's) on lung function change has been studied in several population-based studies. Harber et al (94) found that fume exposed workers had a significant reduction in FEV_1 compared with controls. Kauffmann et al (95) found an overall excess decline in FEV_1 for workers exposed to at least one occupational hazard (dust, gas, or heat) compared with controls. Chlorine and sulphur gasses in bleachery workers employed at pulp mills were studied by Mehta et al (96). Declines in both the FEV_1 and FVC were associated with gas exposure. Cross-sectional studies of the effect of biofuel exposure on level of lung function have found consistent results in the developing world, where biofuel is used for domestic purposes, and significant associations between biofuel exposure and level of lung function have been found (97–99). Examining lung function of workers using biofuel in a Danish energy plant found no associations between lung function and the exposure to biofuel (100). However, longitudinal studies of this exposure are lacking.

2.3.4 Individual characteristics

Individual characteristics such as asthma, Bronchial Hyper Responsiveness (BHR), smoking and biological sex also may have an influence on the change in lung function, as well as on susceptibility to adverse effects from different exposures.

2.3.4.1 Asthma and BHR

Asthma is a disease with increasing prevalence and it is estimated that up to 18% of the population in developed countries suffer from the disease (101). Asthma is a disease dominated by inflammation as well as BHR and bronchoconstriction.

Original research (102–105) and several reviews (106,107), evaluating the outcome of asthma in terms of longitudinal change in lung function, have concluded that asthmatics, dependant of their age, have an impaired growth of lung function (children and adolescents) or an accelerated decline in lung function (adults) compared to non-asthmatics. Having BHR *without* being diagnosed with asthma has also been shown to lead to an accelerated decline in lung function (108–110). Though proper prophylactic asthma medication can reduce the associated decline in lung function (111), asthma as well as BHR can lead to severe loss in lung function with time.

2.3.4.2 Smoking

Smoking is inevitably the greatest risk factor for excess decline in lung function (112,113) and development of COPD (16). Smokers suffer an irreversible loss of FEV_1 and FVC and

therefore, smoking is a crucial factor to adjust for. Confounding by smoking could cause great bias when assessing the effect of other exposures on change in lung function. Possible interactions between smoking and dust exposures may also be of importance when trying to assess the effect of dust exposure on long-term change in lung function. Some studies have found significant interactions between smoking and dust exposure on change in lung function (114,115), however, others did not find the association between biological dust and pulmonary function to be modified by smoking (116,117).

2.3.4.3 Biological sex

Biological sex is an important determinant of absolute lung function levels, especially because of the difference in size between males and females. However, other aspects associated to sex, such as hormonal and immunological factors, may also be important to lung function. Sex may therefore be a factor for difference in susceptibility to adverse effects from different exposures. The effect of exposure to smoking on change in lung function has shown differences in susceptibility between the sexes in several studies. Tager et al (112) saw a more rapid decline of FEV_1 as well as an earlier onset of the decline phase among female smokers compared to male smokers. Similarly, Chen et al (118) suggested that cigarette smoking was more detrimental in its effects on lung function in women than in men, as FEV_1 declined more per pack year of smoking among women. However, other studies found no such difference in effect of smoking between sexes (119), or even saw a faster decline among male smokers (120–122). The inconsistencies of the results therefore make it difficult to conclude if either sex is more susceptible to the adverse effects of smoking on change in lung function.

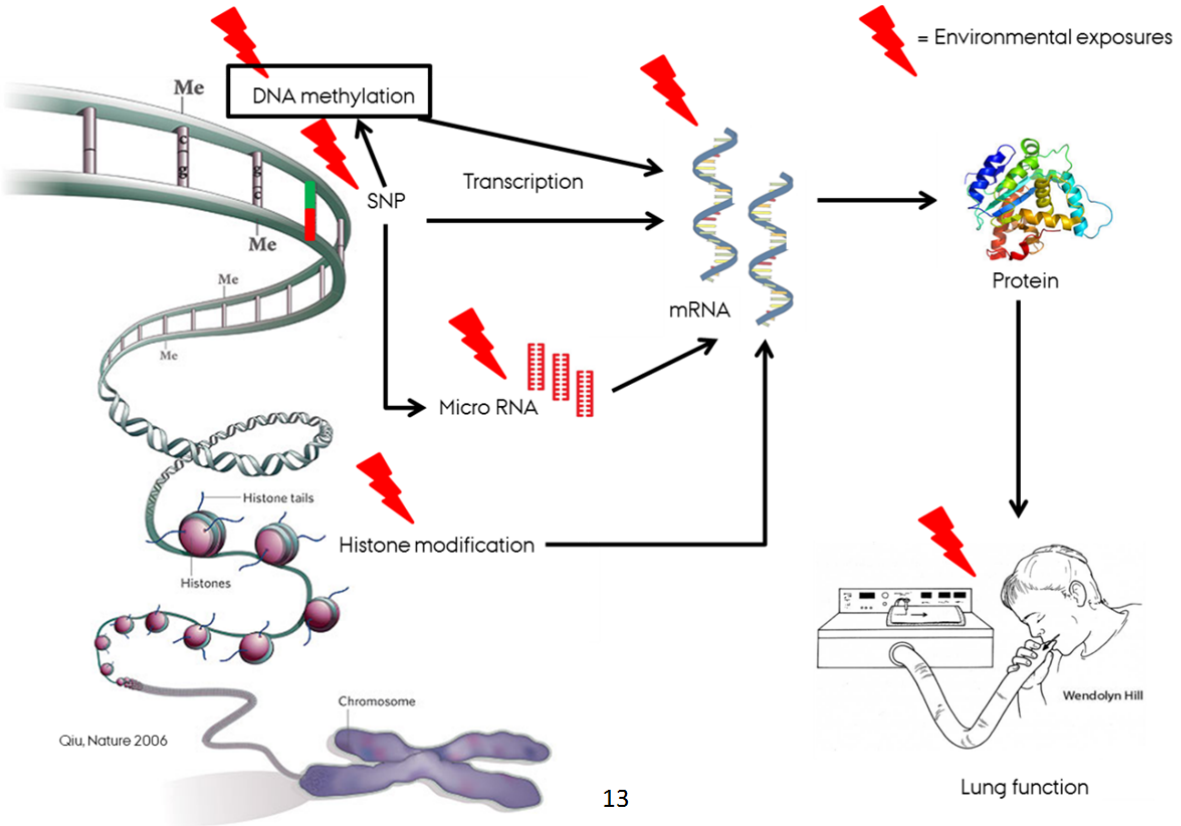
Differences between male and female susceptibility to dust exposure have also been seen in a few studies. In a study by Schlünssen et al (123) (concerning the same population as Paper III in this thesis), female woodworkers exposed to wood dust were shown to have an increased risk of being diagnosed with asthma. Similarly, the same cohort was shown to have greater risk of absolute and relative decline in lung function only among female workers exposed to wood dust (65). A 9-year population-based follow-up study by Sunyer et al (124) revealed a larger decline in FEV_1 among females exposed to high levels of both biological and mineral dust.

Health effects from different exposures are thus also dependant of the individual characteristics such as asthma, BHR and sex. The effect of the underlying genetic and epigenetic background of an individual may therefore also be crucial for the level and change in lung function.

2.4 Genetics and epigenetics

One ground pillar of our very existence is deoxyribonucleic acid (DNA) which consists of a double helix with two strands of phosphodiester linked nucleotides composed of deoxyribose and a base. Four different bases - Adenine (A), Thymine (T), Cytosine (C) and Guanine (G) – make up the base pairs (A-T or C-G) in the double helix structure of DNA. These base pairs make up the genes that code for our phenotypes. More than 99% of all DNA is similar between human individuals (125). However, certain base pairs differ leading to mutations and single nucleotide polymorphisms (SNPs, minor allele frequency < 1%). Functional SNPs may cause changes in gene expression and altered protein structure (126) and in some cases may lead to disease or changes in susceptibility to adverse effects of an environmental exposure. Monozygotic twins, however, are practically genetically identical. Though they are genetically identical they often differ in phenotype. This may be partially explained by the mechanisms of epigenetics, which work "above" the genes and affect gene expression levels and protein synthesis. Epigenetic modifications, which consist of histone modifications, microRNA and DNA methylation influencing gene expression, are affected by different environmental exposures and may be an important link between exposures and the development of pulmonary diseases and lung function (Figure 2.2). For the scope of this thesis the focus will be on DNA methylation only.

Figure 2.2 Environmental exposures and epigenetic changes leading to possible decline in lung function (adapted from Qui (7) and Kim de Jong (127))



2.4.1 DNA methylation

DNA methylation primarily occurs at CpG sites (Cytosine-Phosphate-Guanine) and is carried out by *de novo* methyltransferases (DNMTs) (128). Also maintenance of methylation as well as demethylation is governed by enzymes. DNA methylation tends to be highly prevalent in gene bodies, in which methylation might facilitate transcription, but rare at CpG islands (clusters of un-methylated CpG's) within promoters, in which methylation usually represses gene expression (128). Genome-wide DNA methylation levels change in the genome with age (129). This age-related methylation change is influenced by underlying hereditary, environmental and stochastic factors (130). The epigenetic clock was proposed by Steve Horvath in 2013 (131) describing DNA-methylation-age as functional age-related epigenetic changes at specific sites of the genome. These are common across individuals and are strongly correlated to chronological age, and explain some of the age-related methylation changes.

2.4.2 Monozygotic twins and epigenetics

The use of MZ twins discordant for a trait or disease is becoming a popular and powerful design for Epigenome Wide Association Studies (EWAS). Environmental exposures may lead to differential epigenetic regulation and hence disease status, and using the MZ twin design allows for controlling for individual genetic make-up and some shared environmental factors (132).

Studies comparing MZ twins with dizygotic twins have previously shown a heritability estimate for FEV_1 ranging from 61% to 69% and for FVC from 55% to 63% (133,134), illustrating that a genetic component is very influential on level of lung function. Interestingly, the epigenetic mechanisms may provide further clues to which genes are of importance for lung function and how different phenotypes of MZ twins can originate from the same genotype. In a study by Fraga et al (135) it was found that epigenetic differences increase with age in MZ twin pairs, from hardly any difference in younger MZ twin pairs to significantly different DNA-methylation patterns in older MZ twin pairs.

2.4.3 Lung function and genetics/epigenetics

The MZ twin-design allows for exploration of difference in lung function within twin pairs and the association to DNA methylation. The study of this association is observational and hypothesis generating and the results may reveal plausible genes and biological pathways of importance to lung function.

Previously, Genome Wide Association Studies (GWAS) have identified numerous genetic variants associated with lung function level (136–141) and lung function change (142,143). However, differential DNA methylation may help us understand the mechanisms responsible for change in lung function across lifetime even better.

Also gene-environment interactions have been explored between different genetic variants and different exposures and the effect on level and change in lung function. Several studies have identified gene variants associated with accelerated lung function decline among smokers (144,145), hence illustrating gene-by-smoking interactions. These include genes encoding interleukins and antioxidant enzymes. A study of workers exposed to grain dust (146) found a significant interaction between polymorphisms of genes encoding Tumor Necrosis Factor Alpha (*TNF-α*), a cytokine involved in immune regulation, and the years in the grain industry, influencing the effect of grain exposure on longitudinal lung function decline. Similarly, a study of cotton workers exposed to endotoxin showed that *TNF-α* polymorphisms modify the association between occupational endotoxin exposure and longitudinal lung function decline (147). This illustrates that gene-environment interactions may be of importance for the effects of exposure on lung function.

Smoking is furthermore considered to be one of the strongest environmental modifiers of DNA methylation (148) as well as the greatest predictor of lung function decline (112,113). Several studies (149–151) have explored the effects of smoking on methylation patterns depending on the CpG-site, and a tendency of global hypo-methylation in smokers has been shown in several studies (151–154). Therefore, controlling for smoking is crucial when exploring the association between DNA methylation and lung function to learn more about important genes and pathways possibly driven by factors other than smoking. Stratification by smoking would be another possibility; hence difference in DNA methylation between smokers and non-smokers in association with lung function could be explored, however, amount of smoking may also be relevant.

Only few previous studies have explored the specific association between lung function and DNA methylation. The DNA methylation of nine specified inflammatory genes were explored in association with level of lung function in a study by Lepeule et al (155). Hypo-methylation of the inflammatory genes *CRAT*, *F3* and *TLR2* was shown to be associated with lower level of lung function and hypo-methylation of *IFNγ* and *IL6* was associated with better lung function (155). In a study by Qiu et al (156) and replicated in another cohort, a major finding was that hypo-methylation of *SERPINA1* was negatively associated to lung function. *SERPINA1* is a gene that encodes the acute-phase reactant α1-antitrypsin, which works as an anti-elastase in the lungs. Deficiency of α1-antitrypsin leads to failing maintenance of the structural integrity of the lung and is a well-known monogenic cause of COPD (157). The methylation level of the inflammatory genes *ATP6V1E2*, *FXYD1*, *FUT7*, and *STAT5A* were also

found to be associated with FEV_1 and of the genes *ATP6V1E2*, *FXYD1*, *TRPM2*, and *LRP3* were associated to FEV_1/FVC (156). *Alu* and LINE-1 are repetitive elements that have been studied concerning DNA methylation and association to lung function (158). Hypo-methylation of both *Alu* and LINE-1 was associated to lower cross-sectional FEV_1 level, whereas hypo-methylation of only LINE-1 was associated to faster rate of decline in FEV_1 and FVC. Hypo-methylation of these repetitive elements may increase their activity as retro-transposable sequences, leading to greater genomic instability and more mutations, which in turn may lead to adverse effects on lung function and lung function decline (158).

These findings point to genes possibly causally related to level and change in lung function. As suggested by Lepeule et al (158), the possible mechanism behind a causal relation may be that environmental exposures induce oxidative damage and changes in DNA methylation, which in turn may impact lung function due to altered gene-expression (Figure 2.2). Genes and pathways of possible importance for lung function may be related to regulation of immune and inflammatory system pathways, responses to stress and external stimuli, and coagulation cascades, as hypothesised by Qiu et al (156).

All of these studies were performed on a general population of singletons whereas no study has explored the association between DNA methylation and difference in lung function in MZ twins. This may imply that the results of the methylation studies so far may in part be driven by an underlying genetic component regulating both methylation levels and lung function, whereas studies performed on MZ twins will adjust for the underlying genetic factors and focus on intra-pair methylation differences in association with lung function.

2.5 Hypotheses and aims

The main hypotheses for this thesis were that:

1) Exposure to organic dust is associated to an excess decline in lung function in a dose dependent manner.

2) Sex is an effect modifier of the association between organic dust exposure and lung function decline.

3) Genome-wide DNA methylation signatures may be associated to level and change in lung function.

We aimed to explore the effect of organic dust exposure in farming and woodwork on long-term change in lung function and, in order to explore if sex is an effect modifier of this association, included interactions with sex and/or stratified analyses by sex.

In a study of monozygotic twins we aimed to explore the intra-pair difference in lung function in association with intra-pair DNA methylation differences.

3. Material and methods

This thesis is, in addition to the review (Paper I, Appendix I), based on data from three original studies: a) The SUS-study (Paper II), b) The WOOD-study (Paper III), and c) The TWIN-study (Paper IV), and will be referred to as such throughout the thesis. In this chapter brief descriptions of the three studies are given, including study aims and types, study populations, exposure- and outcome measures, covariates and analytical approaches. For a more detailed description see the corresponding manuscripts (Paper II-IV, Appendix II-IV). Some details are further elaborated in this section. Table 3.1 summarises the details of the four papers.

Table 3.1 Overview of the four papers included in the thesis

	Review - Paper I	SUS-study - Paper II	WOOD-study - Paper III	TWIN-study - Paper IV
Study design	PRISMA criteria for systematic reviews	15 year follow-up study	6 year follow-up study	11 year follow-up study
Study population	Several reviewed studies	Young farming students, female: n=96, male: n=866 Young controls, male: =172 (mean age at baseline = 18.6, min-max: 16.8-24.8)	Middle aged woodworkers in the furniture industry, female: n=185, male: n=927, Control workers, female: n=131, male=104 , (mean age at baseline = 38, min-max: 16.6-64.9)	Older danish MZ-twins n=338 (169 twin pairs) female pairs: n=83, male pairs: n=86, (mean age at baseline = 66, min-max: 56-79)
Exposure	Organic dust exposure in different occupations	Farming exposure. Measurements of organic dust and endotoxin	Wood dust exposure. Measurements of inhalable wood dust	"Within-twin pair difference in cross sectional and longitudinal lung function, zFEV1, zFVC and zFEV1/FVC using GLI2012-equations"
Outcomes	Longitudinal change in lung function FEV1, FVC and FEV1/FVC	Longitudinal change in lung function zFEV1, zFVC and zFEV1/FVC using GLI2012-equations	Longitudinal change in lung function zFEV1, zFVC and zFEV1/FVC using GLI2012-equations	Within-twin pair difference in DNA methylation level at 453,014 genome wide CpG-sites
Covariates	Smoking (inclusion criteria) Other covariates considered in the different studies were age, sex, height, weight, baseline lung function, altitude, atopic status	Smoking, second-hand smoking, farm upbringing, BHR, BMI, sex, atopy, asthma, farm type (age, sex, height and ethnicity used for standardisation of lung function using GLI2012)	Smoking, asthma, weight (age, sex, height and ethnicity used for standardisation of lung function using GLI2012)	Age, sex, smoking, weight, cell composition (age, sex, height and ethnicity also used for standardisation of lung function using GLI2012)
Height used for z-score calculation	-	Baseline height for baseline z-scores Follow-up height for follow-up z-scores	Average of baseline and follow-up height for both baseline and follow-up z-scores	Follow-up height (measured) for both baseline and follow-up z-scores
Statistical methods	-	Multivariable linear regression analyses testing the effect of exposure on change in lung function	Multivariable logistic regression analyses testing the effect of exposure on new-onset COPD Multivariable linear regression analyses testing the effect of exposure on change in lung function	Multivariable linear regression analyses testing the association between within-twin pair difference in lung function and level of DNA methylation

3.1 Lung function assessment

For the three original studies included in this thesis lung function was measured in a similar way and a joint description is stated here.

For each participant lung function was assessed at both baseline and follow-up by measuring the two lung function indices FEV_1 and FVC using a spirometer. The highest obtained values for FEV_1 and FVC with good quality from three attempts were accepted for further analyses and for calculating the ratio FEV_1/FVC. In order to calculate z-scores, data were applied to the GLI 2012 equations (11) providing $zFEV_1$, zFVC and $zFEV_1$/FVC for both baseline and follow-up measures (see section **2.1.1** (The GLI 2012 equations) and section **3.1.1** (Using GLI 2012 reference equations)). Subsequently change in z-score during the follow-up period was calculated.

3.1.1 Using GLI 2012 reference equations

Lung function is expressed as z-scores using the GLI 2012 equations in all included studies in this thesis. Therefore, a joint description of the practical use of the equations is stated here.

To apply the GLI 2012 equations practically, the external datasets consisting of individuals with known sex, age, height, ethnicity and lung function measures (FEV_1, FVC and FEV_1/FVC) were loaded to the GLI2012_DataConversion.EXE program (159,160) for both baseline and follow-up examinations. The program converts the data into results for each individual provided as z-scores, % of predicted, and percentiles and also supplies the lower limit of normal (LLN) compared to the GLI 2012 reference population. The calculations can also be done by hand by using the lookup tables to retrieve the sex-, age-, height-, and ethnicity-specific values for L, M, and S and through the equations calculate the z-score for the individual.

The focus of this thesis was to look at change in lung function, so the change in z-scores (Δz-score) for an individual was calculated as the follow-up (FUP) z-score subtracted the baseline (BL) z-score:

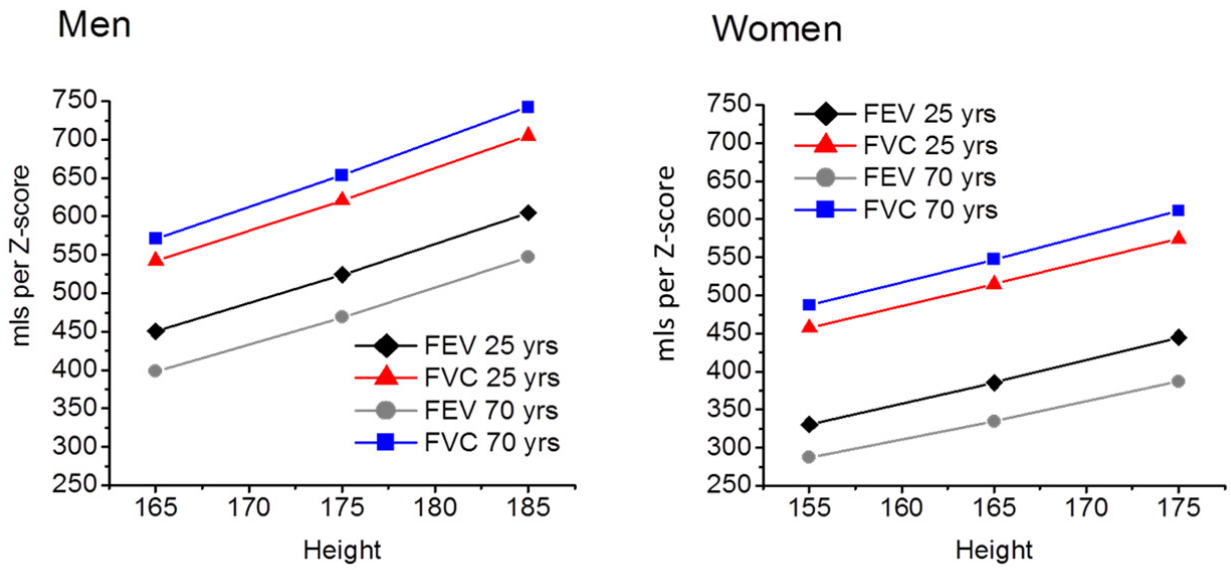
$$\Delta z\text{-}score = z\text{-}score_{FUP} - z\text{-}score_{BL}$$

The interpretation of the Δz-score is complex. It is dependent on the age group and hence, the stage of lung function development. In general, a decline in z-score means that the subject has not achieved the expected lung function in population terms at follow-up based on their baseline value. For young subjects, in the phase of lung development, this can still mean that they have had an increase in absolute lung function (in litres) during the follow-up period, but that the increase was not as large as expected from the reference population – hence the z-score change becomes negative (larger z-score at baseline than at follow-up).

For older subjects, who are in a phase of lung function decline, a negative Δz-score means that they have decreased more than expected from the reference population. Contrary, an increase in z-score means that the subject has achieved better lung function than expected from the reference population. However, dependant on age, it can still be both an absolute increase or decline in lung function (in litres).

In order to calculate what one z-score change is equivalent to in absolute lung function it must be evaluated for a standard person of a specific sex, and for a specific age and height (and ethnicity) as illustrated in Figure 3.1. For a 25 year old man, with a height of 175 cm, a 1 unit z-score change is equivalent to approximately 510 mL in FEV_1 and 650 mL in FVC.

Figure 3.1 The difference in absolute lung function equivalent to 1 z-score dependent on sex, age (25 and 70 years) and height.



3.1.2 Smoking

Smoking is an important possible confounder for the effects of other exposures on lung function and was included as covariates in all analyses in all three studies. Smoking was expressed as pack years (equal to 20 cigarettes/day x years) (see Appendix V for calculation of cigarette equivalents). Smokers were further divided into categorical variables based on amounts of smoking and smoking status, and smokers who quit smoking less than 2 years prior to assessment were defined as current smokers at the assessment time.

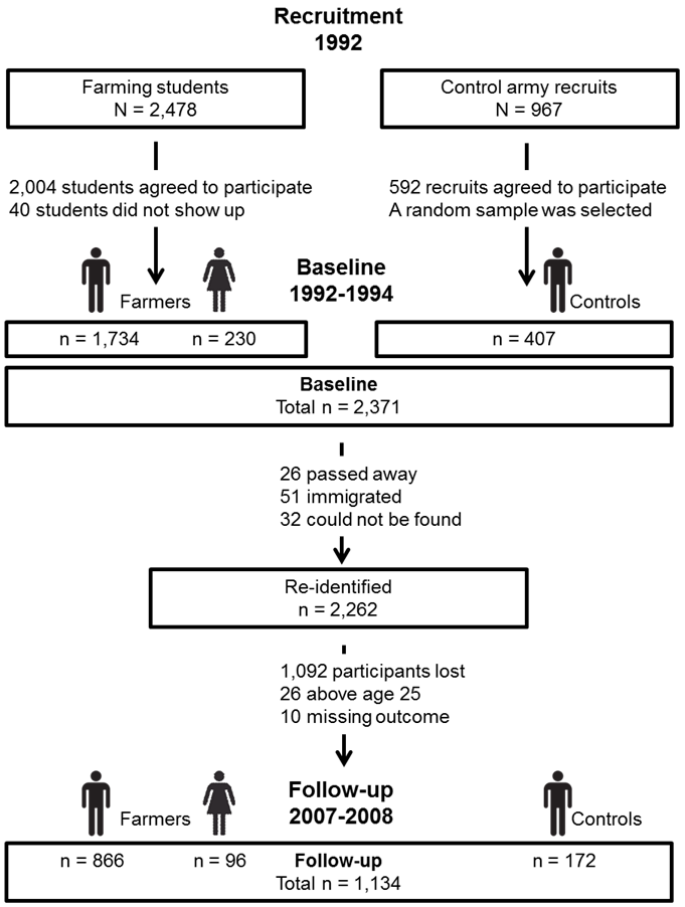
3.2 The SUS-study (Paper II)

The SUS-study (Sund Stald (Healthy Farm)) is a 15 year follow-up study of young farming students initiated with a baseline study in 1992-1994 and followed up in 2007-2008. The study was established with the intention to study the farming specific occupational risk factors for different respiratory disorders, including change in lung function among young farming students (161) which was the aim of this study.

3.2.1 The SUS-study population

The original baseline study consisted of 1,734 young male and 230 young female farming students. Male army draftees were invited to participate as controls. A random sample of young male draftees was chosen leading to 407 controls. Figure 3.2 shows an overview of the study population.

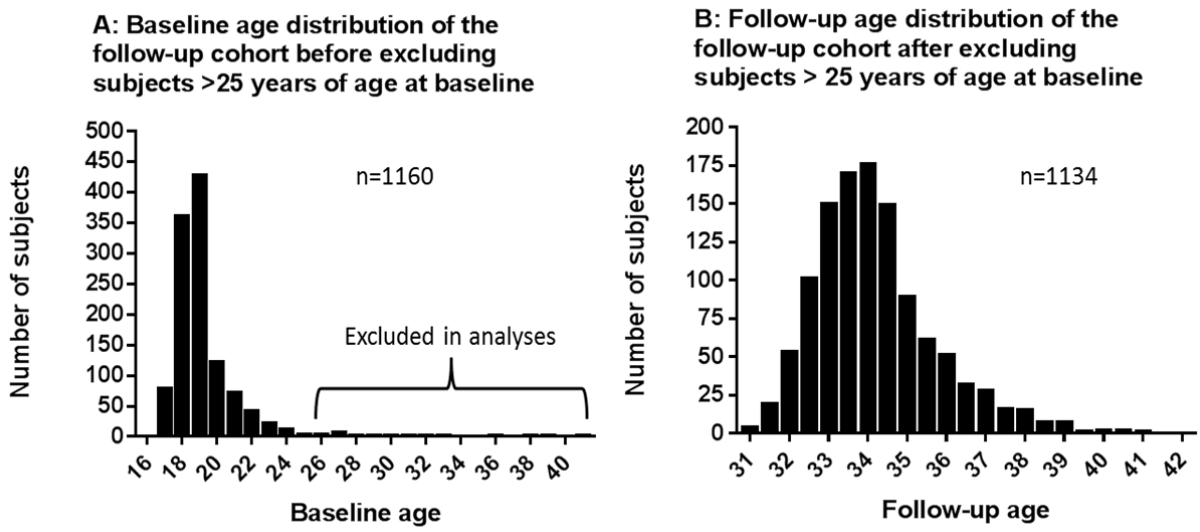
Figure 3.2 **Overview of the SUS-study population from recruitment to baseline and follow-up with full valid lung function measures, questionnaire data and age < 25 years at baseline**



In the original study-protocol the intension was to study only male farmers to ensure an appropriate matching with the male army draftees that were invited to participate as controls. However, the male farming students insisted that their female colleagues should also be included in the study, otherwise they would refuse to participate. Hence both sexes were represented among farmers, whereas only males were represented in the control population.

In 2007-2008, the follow-up of the cohort was conducted with the participation of 1170 subjects (51.7%). The final follow-up group for this study (n=1134) was comprised of 866 male farmers, 96 female farmers and 172 male controls with valid lung function measurements, questionnaire data and an age < 25 years at baseline. This age criterion was selected because the focus of the study was effect of farming exposure on lung function development during young adulthood. It also allowed us to create the best match between exposed and controls. Participants above 25 years of age at baseline (male farmers n=15, female farmers n=11, controls n=0) were therefore excluded. Figure 3.3 shows the age distribution of the full follow-up cohort at baseline and follow-up.

Figure 3.3 Age distribution at baseline (A) and follow-up (B) of the SUS-cohort



3.2.2 Methods used in the SUS-study

Participants answered questionnaires and underwent examinations at both baseline and follow-up using nearly the same methodology (161), and exposure was assigned to each individual based on the work of Ioannis Basinas et. al. (162) in 2008-2009. These methods will be presented in brief in the following section.

3.2.2.1 Measures in the SUS-study

Lung function was assessed as described in section **3.1-3.1.1**. Height was measured at baseline and follow-up and weight at follow-up, from which body mass index (BMI) at follow-up was calculated as weight in kg/height in m^2. Z-scores were calculated using baseline height and follow-up height for the two time points.

Bronchial hyper-responsiveness (BHR), expressed as provocation dose leading to a 20% drop in FEV_1 (PD_{20}), was recorded with a calibrated DeVilbiss No. 40 nebulizer (Devilbiss Healthcare, LLC, Johnstown, PA, USA) (161) connected to an "artificial hand", which uses compressed air to produce a pressure pulse similar to that created by a hand, but with less variability and better reproducibility (163). The provocation agents used were histamine at baseline and methacholine at follow-up.

The participants underwent skin prick tests (SPT) at both baseline and follow-up using a panel of 9 common inhalant allergens (grass, mugwort, horse, dog, cat, two house dust mites (*Dermatophagoides pteronyssinus* (*DermP*) and *Dermathophagoides farinae* (*DermF*)), and two moulds (*Alternaria alternata* and *Cladosporium herbarum*)). Each individual was assigned an atopic status. Positive atopic status was defined as at least one positive reaction of ≥ 3 mm in mean of the longest diameter and the orthogonal diameter to any of the 9 allergens, provided the control (saline) was negative. Furthermore blood samples and exhaled breath condensate were collected from each participant at both baseline and follow-up but this material was not used in this thesis.

3.2.2.2 Questionnaires used in the SUS-study

Participants answered questionnaires dealing with general health issues, respiratory symptoms, asthma, allergy and smoking habits at baseline and follow-up. For all participants with occupational farming experience the type of farm, the duration and type of work was recorded in a farming-specific occupational questionnaire for all employments since the age of 15 years, assuming possible full-time work from this age.

3.2.2.3 Exposure assignment in the SUS-study

The exposure was assessed as an overall cumulative exposure estimate for inhalable dust and endotoxin during the follow-up period and was estimated for each participant based on personal exposure measurements (n=507) performed on a random sample of the SUS-population (n=327 farmers) in 2008–2009 by Basinas et al (162). Sampling was performed using a conductive plastic inhalable conical sampler (CIS; JS Holdings, Stevenage, UK) mounted with a 37 mm glass-fibre filter (Whatman International Ltd, Maidstone, UK) placed near the breathing zone of the participant and connected to a portable pump (AirChek

XR5000, SKC Inc., Eighty Four, PA, USA) working at a flow rate of 3.5 L/min (162). Dust concentration was estimated by pre- and post-weighing the filter to determine the amount of dust and giving a total sample weight concentration (mg/m^3). Endotoxin concentration in extracts from the filter was estimated with the kinetic chromogenic Limulus Amoebocyte Lysate assay as described previously by Spaan et. al. (164) giving a total endotoxin concentration (EU/m^3).

Dust and endotoxin concentrations for pig, cattle and field farming were estimated as mean time-weighted averages (TWA, 8 h) over an 8 hour work day. The TWA shows average daily exposure of a worker to occupational dust and endotoxin and takes into account task-based exposure estimates throughout a working day. The TWA of dust was determined by product of the total sample weight concentration (mg/m^3) of the dust sampler and the quotient between the 8 hours standard and the total sampling time. Similarly was done for TWA of endotoxin (EU/m^3). The mean TWA dust estimates were 2.48 mg/m^3, 0.56 mg/m^3 and 0.59 mg/m^3 for pig, cattle and field work, respectively. For endotoxin the TWA estimates were 1,107 EU/m^3, 198.2 EU/m^3 and 49.4 EU/m^3, respectively.

For every participant, the total number of work years, standardised to a 40 h work week, in pig, cattle and field farming were calculated using information from the occupational questionnaires. Cumulated exposures were calculated as the sum of the products of the TWA dust and endotoxin concentrations and the total work years during the follow-up period (sum of each employment period) for each type of work (for an example of this calculation see Appendix VI).

3.3 The WOOD-study (Paper III)

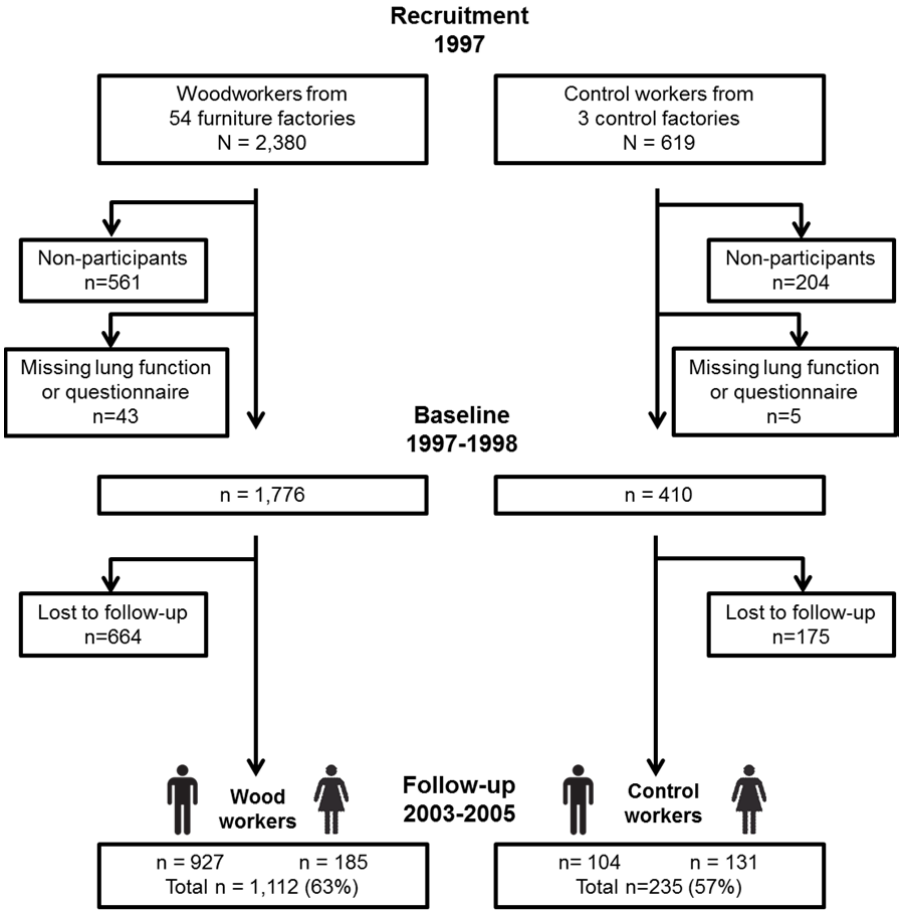
The WOOD-study is a 6 year follow-up study of a cohort of wood workers in the Danish furniture industry established with the objective to study the impact of wood dust exposure on pulmonary health effects. It was initiated with a baseline study in 1997-1998 (123,165) and followed up in 2003-2005 (65). The aim of this part of the study was to investigate the association between exposure to inhalable wood dust and new-onset COPD as well as long-term change in lung function.

3.3.1 The WOOD-study population

The study population was established in 1997-1998 with visits to 54 furniture factories with more than 4 employees, out of 86 identified in Viborg County, Denmark (123,165). The population consisted of workers in wood processing, product assembly and storage units. Three factories producing refrigerators and hearing aids were invited to serve as a control population. Valid lung function tests, personal characteristics (sex, age, height and ethnicity) and questionnaire data were available for 1,776 woodworkers and 410 controls at baseline.

In 2003-2005 a follow-up was conducted (65). This resulted in a participation of 1,112 woodworkers (63%) and 235 controls (57%) with valid lung function measurements at both baseline and follow-up. The study population is presented in a flow chart in Figure 3.4.

Figure 3.4 Overview of the WOOD-study population from recruitment to baseline and follow-up with full valid lung function and questionnaire data



3.3.2 Methods used in the WOOD-study

Participants answered questionnaires and underwent examinations at both baseline and follow-up using the same methodology, and exposure was assigned to each individual based on area dust measurements performed at baseline (123,165) and follow-up (65). These methods will be presented in the following.

3.3.2.1 Measures in the WOOD-study

Lung function was assessed as described in section **3.1-3.1.1**. Height and weight was measured at baseline and follow-up, from which body mass index (BMI) was calculated as weight in kg/height in m^2, as well as change in BMI during the follow-up period. The average of the baseline and follow-up height for each participant was used to calculate lung function z-scores at both baseline and follow-up. This was done in order to minimise any possible errors related to measuring height and was done under the assumption that this group of workers (average age at baseline = 39 years) was not expected to change in height during the 6 years of follow-up. Blood samples were collected from a subpopulation of the participants at follow-up but this material was not used in this thesis.

The definition of newly developed cases of COPD (new-onset COPD) during the follow-up period was defined as FEV_1/FVC falling below the Lower Limit of Normal (LLN) at follow-up, having previously been above the LLN at baseline, accompanied by symptoms of chronic cough or chronic sputum or breathlessness at follow-up, independent of symptoms at baseline.

3.3.2.2 Exposure assignment in the WOOD-study

The exposure assessments for this study were based on work done previously. Passive dust monitors were used to measure exposure to wood dust at both baseline (123,165) and follow-up (65). Sampling was performed on transparent sticky foils and light extinction was measured before and after sampling. The difference in light extinction between the measurements was a measure of dust-covered foil area and was converted into equivalent inhalable dust levels by linear regression models based on calibration measurements (123). As described by Schlünssen et al. (166) an internal job exposure matrix (JEM) was constructed at baseline (12 groups, 2,217 measurements, 1,581 individuals) and at follow-up (7 groups, 1,355 measurements, 1,044 individuals) based on factory size and work task. The groups were identified in a random effect analysis, where grouping by task and factory size achieved the greatest contrast between groups and therefore this approach was chosen for further analyses.

Participants answered questionnaires concerning information on respiratory health, smoking habits, and occupational history including work tasks.

Wood dust exposure was evaluated in two ways; as that occurring at baseline and that occurring during the follow-up period. The level of wood dust exposure at each time point was evaluated from the equivalent JEM at baseline and follow-up. Individual cumulative inhalable wood dust exposure during the follow-up period was calculated as the sum of the exposure for the first half of the period (the product of the level of exposure at baseline and years in the industry during 1997-2000), and the exposure for the second half of the follow-

up period (the product of the follow-up level of exposure and years in the industry during 2001-2004). If wood workers ceased to work in the furniture industry during the follow-up period they would only get the cumulative exposure corresponding to the years and months they worked in the industry. For analyses, exposed wood workers were compared with control workers, who, in all analyses, were evaluated as non-exposed.

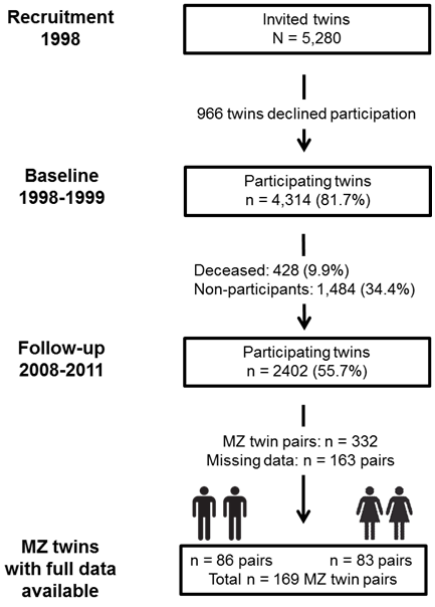
3.4 The TWIN-study (Paper IV)

The TWIN-study is a study on a sub-population from the Danish Twin Register (DTR) (167) of MZ twin pairs from the Middle Aged Danish Twin Study (MADT-study) (168). This cohort forms an 11 year follow-up study of twins with the aim of studying different health problems, including pulmonary health and lung function, assessed both cross-sectionally and longitudinally. The aim of our hypothesis generating study was to investigate twin pair discordance for level and change in lung function and differences in DNA-methylation signatures, with the unique possibility in the MZ twin design of controlling for the underlying genetic make-up and shared environment.

3.4.1 The TWIN-study population

The study population in this sub-study was comprised of 169 intact MZ twin pairs (86 male pairs and 83 female pairs) seen at both baseline and follow-up, with a mean age at follow-up of 66 years (range: 56-79). The baseline assessment took place in 1998-1999 and the follow-up examinations were conducted on average 11 years later in 2008-2011 (169). An overview of the TWIN-study population can be seen in Figure 3.5.

Figure 3.5 Overview of the TWIN-study population from recruitment to baseline and follow-up with full data available



3.4.2 Methods used in the TWIN-study

Participants answered questionnaires and underwent examinations at both baseline and follow-up using the same methodology. These methods will be presented in the following.

3.4.2.1 Measures in the TWIN-study

Lung function was assessed as described in section **3.1-3.1.1**. Height and weight were self-reported at baseline and measured by lay interviewers at follow-up. Body Mass Index (BMI) (and change in BMI) was calculated from these data as weight in kg/height in m^2. In the TWIN-study the objectively measured follow-up height for each participant was used to calculate lung function z-scores at both baseline and follow-up. This was done in order to minimise any possible errors related to self-reported height (baseline assessment) and was done under the assumption that this group of twins (average age at baseline = 55 years) was not expected to change in height during the 11 years of follow-up. Two different portable spirometry devices were used for the TWIN-study, the Micro DL at baseline and the "Easy One" at follow-up, under the assumption that the measurements were comparable for the two devices.

Intra-pair (IP) differences in z-score for level and change in lung function were calculated as the absolute difference in e.g. zFEV1 between the "superior" twin (the twin with higher zFEV1) and the "inferior" twin (the twin with lower zFEV1):

$$\Delta zFEV1_{IP} = zFEV1_{superior} - zFEV1_{inferior}$$

3.4.2.2 DNA methylation in the TWIN-study

In brief, genomic DNA was extracted from leukocytes in the buffy-coat, bisulfide converted and analysed using the Infinium HumanMethylation450 BeadChips (Illumina) array. After quality control 453.014 good quality probes (CpG-sites) remained for Epigenome Wide Association Study (EWAS) analyses. β-values (the proportion of methylation) for each probe were logit transformed giving an M-value=$\log_2(\beta/1-\beta)$. Blood cell types were counted in the same blood as that analysed for DNA methylation, giving the distribution of subtypes (monocytes, lymphocytes, basophils, neutrophils, and eosinophils). These distributions were used to adjust for individual differences in blood cell composition from which genomic DNA was extracted.

3.5 Statistical analyses

The lung function outcomes of interest were standardised for sex, age, height and ethnicity using the GLI 2012 equations in all included studies as described in section **3.1-3.1.1**.

For the SUS-study and the WOOD-study analysis methods were very similar and are here described together. Multivariable regression models were applied to assess the association between organic dust exposure and the lung function outcomes. In both the SUS-study and the WOOD-study *a priori* selected confounders with plausible effect on lung function change (linear regression) and new-onset COPD (logistic regression in the WOOD-study) were included. In the WOOD-study all analyses were stratified by sex, as effect modification by sex was evident. Furthermore, all analyses were conducted in two ways in the WOOD-study; as 1) follow-up analyses with dust exposure level assessed at baseline, and 2) analyses with accumulated dust exposure during the follow-up period, as the SUS-study. In the SUS-study interactions between exposures and the variables sex and smoking, as well as between BHR and place of up-bringing, were explored. The level of significance was defined as $p<0.05$. Statistical analyses were carried out using Stata version 12 and 14 (StataCorp. 2011 and 2015. Stata Statistical Software: Release 12 and 14. College Station, TX: StataCorp LP).

For the TWIN-study EWAS analyses for within-pair differences for both *cross-sectional* and *longitudinal* lung function z-scores were performed. The intra-pair differences (IP) in DNA-methylation level (M-value) for each probe were calculated as the "superior" minus the "inferior" twin based on the explanatory variable (e.g. $\Delta zFEV1_{ip}$). The same was done for all other included variables for each twin pair. Associations between intra-pair DNA-methylation difference and both the *cross-sectional* and the *longitudinal* intra-pair lung function difference were investigated using linear regression models. The models were adjusted for sex, age, BMI/BMI-change during the follow-up period, ever smoking history (total pack-years/smoking pack-years during the follow-up period), smoking status at follow-up, as well as blood cell composition difference within each twin pair. Results with a p-value $< 10^{-6}$ as well as the corresponding false-discovery rate (FDR)–adjusted P-value < 0.05 were reported as significant. In order to explore if associated genes were overrepresented in specific pathways, pathway enrichment analyses were performed with the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) (170) against the gene regions included in the 450K DNA-methylation array. Associated probes from EWAS with a p-value $< 10^{-5}$ were used for pathway enrichment analyses. Benjamini-Hochberg (BH) correction method (171) was used to correct for multiple testing for the enrichment analyses. All analyses were performed in R (http://www.R-project.org/) and STATA14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Figures for this thesis were produced using GraphPad Prism version 7 (GraphPad Software, La Jolla, California, USA) and Microsoft PowerPoint® 2010.

4. Results

This chapter summarises the main results for each study presented in the thesis. Further details on the study populations and the complete results for the original articles (Paper II-IV) are presented in the corresponding manuscripts in the Appendices II-IV.

4.1 Main results from the SUS-study (Paper II)

The young SUS-cohort (median age at baseline 18.7 years) of farming students and controls had a follow-up time of 15 years. It consisted of 962 original farming students and 172 controls. In absolute values (litres) the average of FEV_1 decreased slightly (-0.1 L) whereas the average of FVC increased (+0.2 L) during the follow-up period, illustrating the phase of lung development and the plateau phase. When assessing the standardised change in lung function (Δz-scores), both farmers and controls had an increase in $\Delta zFEV_1$ and ΔzFVC during the follow-up period, with only smoking farmers having a significantly smaller increase in $\Delta zFEV_1$ compared to non-smoking farmers. $\Delta zFEV_1$/FVC decreased for all groups during the follow-up period.

When comparing change in lung function z-scores for current- and ex-farmers with that of controls in adjusted multivariable linear regression analyses, no significant differences were seen. However, within farmer analyses, comparing current farmers with ex-farmers, showed that current farmers had a significantly worse effect on $\Delta zFEV_1$ (-0.12, p=0.006) and on $\Delta zFEV_1$/FVC (-0.15, p=0.009) than ex-farmers (Table 4.1).

Table 4.1 Regression analyses of change in lung function z-scores in models including (A) current- and ex-farmers compared with controls and (B) current farmers compared with ex-farmers (controls excluded).

Regression models	$\Delta zFEV_1$	ΔzFVC	ΔzRatio
Exposure status	β-coef. (95% CI)	β-coef. (95% CI)	β-coef. (95% CI)
A) Control	0	0	0
Ex-farmer	0.05 (-0.07 to 0.16)	-0.05 (-0.16 to 0.06)	0.14 (-0.01 to 0.29)
Current farmer	-0.07 (-0.19 to 0.05)	-0.09 (-0.21 to 0.03)	-0.01 (-0.17 to 0.15)
B) Ex-farmers	0	0	0
Current farmers	**-0.12 (-0.21 to -0.03)**	-0.04 (-0.13 to 0.04)	**-0.15 (-0.26 to -0.04)**

Bold shows significance $p < 0.05$. Mutually adjusted for smoking, second hand smoking, being raised on a farm, baseline BHR, and follow-up BMI

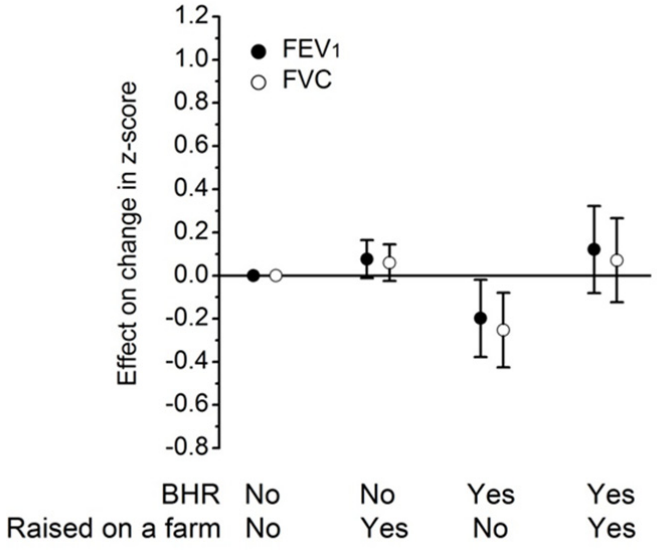
The negative effect of current farming compared to ex-farming on Δz-scores of lung function was stronger among females, illustrated by a significant interaction between sex and

current farming in model B (Table 4.1). This indicated that females were more susceptible to the adverse effects of current farming on change in lung function.

No significant exposure-response relations were found between exposure and change in Δz-scores of lung function when assessing farming exposure as either accumulated quartiles of dust or endotoxin, or as farming type (only cattle, only pig, or mixed farmers).

In general smoking, BHR and BMI were associated with a negative effect on the Δz-scores of lung function, whereas farm upbringing was associated with a protective effect and hence larger positive Δz-scores. An interaction term between BHR and farm upbringing was subsequently found. This illustrated, that not being raised on a farm for those with BHR was associated with a negative effect on the $\Delta zFEV_1$ and ΔzFVC, whereas the negative effect of BHR was attenuated for those raised on a farm (Figure 4.1).

Figure 4.1 **Mean estimates of the effect, with 95% CI, on $\Delta zFEV_1$ and ΔzFVC during the follow-up period according to the status of baseline Bronchial Hyper Responsiveness (BHR) and being raised on a farm for farmers and controls combined (n=1,058)**



Adjusted for farming status, smoking, second-hand smoking, and BMI.
Reference: Participants with no BHR and no farm-upbringing (n=500).

In summary we found no significant differences in Δz-scores of lung function between farmers and controls and neither an exposure-response relation between level of dust or endotoxin and change in lung function. Current farming exposure was, however, associated with a negative effect on change in lung function compared to ex-farming, with females being more susceptible to this adverse effect. Furthermore, farm upbringing attenuated the negative effect of BHR on change in lung function.

4.2 Main results from the WOOD-study (Paper III)

The WOOD-cohort of woodworkers and controls (median age at baseline 38 years) had a follow-up time of 6 years. It consisted of 1,112 woodworkers and 235 controls. In absolute values the average of both FEV_1 and of FVC decreased slightly during the follow-up period for both groups, illustrating the phase of lung decline with increasing age. Preliminary explorative analyses showed that sex was an effect modifier of the association between wood dust exposure and change in lung function and therefore stratification by sex was performed for all analyses. When assessing the standardised change in lung function (Δz-scores), female woodworkers and male controls tended to decrease in both $\Delta zFEV_1$ and ΔzFVC during the follow-up period, whereas female controls and male woodworkers did not change in z-score. However, male woodworkers started at a lower z-score level at baseline. Being a smoker was associated with a greater decline in especially $\Delta zFEV_1$ compared with non-smokers. Similarly, new-onset COPD was seen primarily among smokers, showing that smoking is the main risk factor for both decline in lung function and for developing COPD. Among females only, there were a significantly higher number of subjects with new-onset COPD among smoking woodworkers compared to smoking controls.

When exploring an exposure-response relation between wood dust exposure during the follow-up period and new-onset COPD in adjusted logistic regression (Table 4.2) it was found that, for women in the highest exposed group, the adjusted OR (95% CI) of developing COPD (NB: only 10 cases) was 12.00 (1.3-111.0) and a significant test for trend with increasing exposure level was found (p=0.017) (NB: smokers only, due to no cases among female non-smokers). The same tendency was seen for logistic regression of the effect of baseline dust exposure in women. For males no such exposure-response relation was found, however smoking was the greatest predictor of new-onset COPD in males (Table 4.2). Asthma was also a significant predictor of new-onset COPD in males in logistic regression of the effect of exposure level at baseline.

Multivariable linear regression analyses comparing change in lung function z-scores for woodworkers with that of controls, revealed significant differences for females. Female woodworkers with increasing levels of dust exposure had significantly worse effect on $\Delta zFEV_1$ (-0.32 (-0.56 to -0.08), p=0.009, for 3rd quartile of exposure) than female controls (Table 4.3) with a test for trend of -0.08 (-0.14 to -0.03, p=0.005) with increasing exposures. Among males, primarily smoking predicted a negative effect on $\Delta zFEV_1$. A positive association was seen between level of wood dust exposure at baseline and change in lung function during the follow-up period among males, possibly indicating a strong healthy workers selection effect.

Table 4.2 Logistic regression of new-onset COPD during the follow-up period, comparing woodworkers with controls stratified by sex

	New-onset COPD[$]	
	Female, OR^ (95% CI)	**Male, OR^ (95% CI)**
Exposure during the follow-up period	n=141 (smokers only)!	n=900
Controls: 0 mg/m3*year	1 (ref.)	1 (ref.)
Low exp.: >0 & ≤3.75 mg/m3*year	5.57 (0.6-52.2)+	0.82 (0.3-2.7)
High exp.: >3.75 up to 7.55 mg/m3*year	**12.00 (1.3-111.0)+**	0.72 (0.2-2.4)
Follow-up smoking[#]	-	**7.05 (2.4-20.76)**
Asthma at baseline	0.99 (0.1-10.3)	2.74 (0.8-9.8)
Weight change during the follow-up period	0.97 (0.9-1.1)	0.98 (0.9-1.04)

Bold shows significance p<0.05. Cases vary due to missing values.
[$] New-onset COPD: follow-up FEV1/FVC<LLN + symptoms if at baseline not FEV1/FVC<LLN
^ Adjusted Odds Ratio
[#] Current smoker during the follow-up period (yes/no)
+ Test for trend, OR (95% CI): 3.1 (1.2-7.8), p=0.017

Table 4.3 Multivariable linear regression of change in lung function z-score for FEV1 during the follow-up period, comparing different levels of exposure in woodworkers with the controls

	ΔzFEV1	
	Female, β-coef. (95% CI)	**Male, β-coef. (95% CI)**
Exposure during the follow-up period, n=number	n=265	n=911
Control: 0 mg/m3*year	0 (ref.)	0 (ref.)
1st quart exp.: >0 to ≤2.97 mg/m3*year	0.02 (-0.18 to 0.23)+	0.09 (-0.03 to 0.21)
2nd quart exp.: >2.97 to ≤3.75 mg/m3*year	-0.05 (-0.26 to 0.17)+	0.03 (-0.10 to 0.15)
3rd quart exp.: >3.75 to ≤4.71 mg/m3*year	**-0.32 (-0.56 to -0.08)+**	0.05 (-0.07 to 0.17)
4th quart exp.: >4.71 up to 7.55 mg/m3*year	**-0.31 (-0.62 to -0.001)+**	0.08 (-0.04 to 0.20)
Ex-smoker	**0.25 (0.04 to 0.46)**	0.005 (-0.08 to 0.09)
Smoker low (≤6 pack years)	-0.13 (-0.33 to 0.07)	**-0.11 (-0.20 to -0.02)**
Smoker high (>6 pack years)	**-0.44 (-0.66 to -0.23)**	**-0.20 (-0.30 to -0.11)**
Asthma at baseline	0.07 (-0.22 to 0.36)	-0.08 (-0.22 to 0.06)
Weight change during the follow-up period	**-0.01 (-0.02 to -0.002)**	**-0.02 (-0.03 to -0.016)**

Bold shows significance p<0.05. Cases vary due to missing values.
+ Test for trend, E (95% CI): -0.08 (-0.14 to -0.03), p=0.005

The difference between sexes in effect of wood dust exposure on change in lung function was supported by a significant interaction term between wood dust exposure and sex in the full non-stratified analysis, suggesting effect modification by sex. Furthermore, more female woodworkers were situated in the lower exposure categories whereas more male

woodworkers were in the higher exposure categories. Females were also more negatively affected by smoking than men with larger effects of heavy smoking on change in lung function for females in linear regression analyses (Table 4.3).

In summary, we found significant differences in incidence of new-onset COPD and Δz-scores of lung function between female woodworkers and controls, and exposure-response relations with increasing wood dust exposure among females. This suggests that females may be more susceptible to the adverse effect of organic wood dust on lung function than males. However, smoking was the strongest predictor of adverse pulmonary health effects for both sexes.

4.3 Main results from the TWIN-study (Paper IV)

In the older cohort of MADT MZ twins (median age at baseline 55 years) the follow-up period was on average 11 years (min-max: 9.6-13.4 years). It consisted of 83 female and 86 male MZ twin pairs. Absolute FEV_1 and FVC (Litres), as well as FEV_1/FVC declined during the follow-up period as expected for this age-group. Standardised Δz-scores tended to increase in both ΔzFEV_1 and especially ΔzFVC, illustrating less absolute decline than expected, whereas ΔzFEV_1/FVC declined during the follow-up period, illustrating that the zFEV_1/FVC was higher than expected at baseline but lower at follow-up. Median intra-pair differences in lung function were the same for male and female twin pairs. However, the variance of the intra-pair differences tended to be higher in male twin pairs, possibly due to the greater discordance in smoking history within male twin pairs (mean difference of 14 pack years vs. 8 pack years for women ($p<0.05$) (smoking twin pairs)).

EWAS analyses for intra-pair ($_{IP}$) difference in level of lung function at follow-up showed several associated differentially methylated CpG-sites (Table 4.4). EWAS for intra-pair difference in change in lung function during the follow-up period showed fewer differentially methylated CpG-sites for this association (Table 4.5). Results are shown for log-transformed intra-pair differences to avoid inflation of the results due to otherwise extremely skewed distributions.

Table 4.4 EWAS-analyses for intra-pair difference in level of lung function at follow-up (p-value < 10^{-5})

Lung function measure	Probe	Estimate	P-value	Chromosome	Bp (hg19)	Proximal gene	CGI feature	Methylation* of "inferior" twin
Log-ΔzFEV1_{IP}	cg04261072	-0.054	4.33E-06	13	79977499	RBM26	Body - shelf	Hyper
	cg10196163	-0.062	7.62E-06	14	105560628	NA	IGR - shore	Hyper
	cg12552820	-0.033	2.69E-06	1	2231925	SKI	Body - shore	Hyper
	cg13971574	-0.037	9.65E-06	2	39102874	MORN2	TSS1500 - island	Hyper
	cg18582260	-0.079	3.99E-06	13	25085301	PARP4	5'UTR - shore	Hyper
	cg20552903	-0.038	5.81E-06	6	33289678	DAXX	Body - shore	Hyper
	cg23759053	-0.136	5.99E-06	7	34173999	BMPER	Body - open sea	Hyper
	cg23840275	-0.045	2.62E-06	13	20969493	NA	IGR - shore	Hyper
	cg27180671	-0.050	7.51E-06	17	65527566	PITPNC1	Body - open sea	Hyper
Log-ΔzFVC$_{IP}$	cg00008488	-0.0935	8.23E-06	5	175199915	NA	IGR - shore	Hyper
	cg00306721	-0.0797	9.54E-06	11	62477480	BSCL2	TSS1500 - island	Hyper
	cg01028379	0.0617	2.84E-06	7	4798471	FOXK1	Body - shore	Hypo
	cg02071292	-0.2366	**7.14E-08**^{FDR}	12	75785097	GLIPR1L2	1stExon - island	Hyper
	cg07311024	-0.1553	1.96E-06	12	75785089	GLIPR1L2	1stExon - island	Hyper
	cg15909232	-0.1167	5.08E-06	7	156235420	NA	IGR - open sea	Hyper
	cg15942481	-0.1366	8.77E-06	12	75785230	GLIPR1L2	Body - island	Hyper
	cg17154159	0.0832	6.02E-06	6	160401562	IGF2R	Body - open sea	Hypo
	cg22089890	-0.0391	4.20E-06	17	48708077	NA	IGR - shelf	Hyper
	cg25249300	-0.0660	**1.36E-07**	2	54483341	TSPYL6	1stExon - island	Hyper
ΔzFEV1/FVC$_{IP}$	cg00347643	0.1747	7.88E-06	7	75957202	YWHAG	3'UTR – shore	Hypo
	cg00995220	0.1333	**2.13E-07**	6	56259582	NA	IGR - open sea	Hypo
	cg04697953	0.0849	4.43E-06	2	179299404	PRKRA	Body - open sea	Hypo
	cg06244016	0.1917	2.17E-06	6	151186511	MTHFD1L	TSS200 – shore	Hypo
	cg07219303	0.1575	3.92E-06	4	100140905	ADH6	TSS1500 - open sea	Hypo
	cg11980944	0.0863	8.55E-06	1	205399731	NA	IGR - open sea	Hypo
	cg13107302	0.0995	5.69E-06	17	75237970	NA	IGR - open sea	Hypo
	cg13912599	0.1229	2.66E-06	1	150959380	ANXA9	Body - open sea	Hypo
	cg18221862	-0.1038	4.81E-06	2	193059230	TMEFF2	1stExon – island	Hyper
	cg18537205	-0.1205	9.86E-06	10	114575091	VTI1A	Body - open sea	Hyper
	cg19529957	0.1367	5.50E-06	7	4198590	SDK1	Body - open sea	Hypo

Estimate: Intra-pair difference in M-value (logit transformed beta). Probes were annotated with the most proximal gene, genomic position Bp (hg19) and CpG island context (CGI-feature). TSS200: region spanning from Transcription start site (TSS) to 200 bp upstream of TSS; TSS1500: region spanning from - 200 to - 1500 bp upstream of TSS; 5'UTR: 5' untranslated region; IGR: intergenic region; FDR: False discovery rate<0.05; *Relative DNA methylation of "inferior" twin compared to "superior" twin; Hyper: Hyper-methylation; Hypo: Hypo-methylation. Bold indicates significance p<1 x 10^{-6}.

Table 4.5 EWAS-analyses for intra-pair difference in change in lung function during the follow-up period (p-value < 10^{-5})

Lung function measure	Probe	Estimate	P-value	Chromosome	Bp (hg19)	Proximal gene	CGI feature	Methylation* of "inferior" twin
Log-ΔzFEV1-change$_{IP}$	cg19484381	-0.055	1.55E-06	6	28890673	TRIM27	Body - shore	Hyper
	cg27261494	-0.081	5.77E-06	6	74104097	DDX43	TSS200 - shore	Hyper
Log-ΔzFVC-change$_{IP}$	cg12796186	-0.066	3.28E-06	1	10458599	PGD	TSS1500 - island	Hyper
	cg14514174	-0.044	6.39E-06	9	99181512	ZNF367	TSS1500 - island	Hyper
ΔzFEV1/FVC-change$_{IP}$	cg00552805	0.0551	9.84E-06	7	44119858	POLM	Body - shore	Hypo
	cg06375580	0.0449	9.25E-06	12	42538820	GXYLT1	TSS200 - island	Hypo
	cg12733656	0.0396	8.82E-06	7	6388695	C7orf70	TSS200 - island	Hypo

Estimate: Intra-pair difference in M-value (logit transformed beta)
Probes were annotated with the most proximal gene, genomic position Bp (hg19) and CpG island context (CGI-feature).
TSS200: region spanning from Transcription start site (TSS) to 200 bp upstream of TSS;
TSS1500: region spanning from - 200 to - 1500 bp upstream of TSS; 5'UTR: 5' untranslated region; IGR: intergenic region
*Relative DNA methylation of "inferior" twin compared to "superior" twin; Hyper: Hyper-methylation; Hypo: Hypo-methylation.

The regression analysis for intra-pair difference in DNA methylation in association with intra-pair difference in level of zFEV1 at follow-up (log-ΔzFEV1$_{IP}$) identified nine CpG sites with p-value < 10^{-5}. No CpG-sites reached genome wide significance of a p-value < 10^{-6} in this analysis. Analysis of intra-pair difference in zFVC (log-ΔzFVC$_{IP}$) found ten CpG sites with p-value < 10^{-5}, with the most significant probe identified as cg02071292 annotated to GLI (glioma) pathogenesis-related 1 like 2 (*GLIPR1L2*) with p-value = 7.14 x 10^{-8} (FDR adjusted p-value = 0.03). Two other probes (cg07311024 p-value = 1.96 x 10^{-6}, and cg15942481 p-value = 8.77 x 10^{-6}) in this analysis were also annotated to *GLIPR1L2* (Table 4.4).

Analysis of intra-pair difference in change in zFEV1 (log-ΔzFEV1-change$_{IP}$) found only two CpG sites with p-value < 10^{-5}. The probe with the lowest p-value (1.55 x 10^{-6}) was cg19484381 mapping to Tripartite motif containing 27 (*TRIM27*). Intra-pair difference in change in zFVC (log-ΔzFVC-change$_{IP}$) also identified two associated probes. The probe with the lowest p-value (3.28 x 10^{-6}) was cg12796186 mapping to Phosphogluconate dehydrogenase (*PGD*) (Table 4.5).

In general, the highlighted findings in both cross-sectional and longitudinal models showed higher DNA methylation for the "inferior" twin for zFEV1 and zFVC, whereas zFEV1/FVC showed lower DNA methylation for the "inferior" twin in identified probes (Table 4.4 and 4.5). The reason for this difference is unknown.

Pathway enrichment analyses, exploring if associated genes from EWAS analyses were overrepresented in specific pathways, were performed on all EWAS results with p-value < 10^{-5}. Cross-sectional EWAS results showed more enriched pathways (Table 4.6) than longitudinal EWAS (see table 6, Paper IV, Appendix IV). Intra-pair difference in level of zFEV1 identified several enriched pathways from both Gene Ontology, and Pathway Commons (Table 4.6). Several other pathways driven by the same genes reached statistical significance (12 pathways with BH-adj p-value < 0.05) (see supplementary table S1 in attachment to paper IV). Enrichment signal in all pathways for zFEV1 was driven by *SKI* ('Sloan-Kettering Institute') proto-oncogene in combination with either BMP binding endothelial regulator (*BMPER*) or death domain associated protein (*DAXX*).

Table 4.6 Pathway enrichment analyses for Gene Ontology (GO), KEGG and Pathway Commons (PC) (BH-corrected p-value < 0.1) based on significant findings from EWAS for level of lung function

Lung function measure	Data base	Pathway name	#Genes	Genes	Statistics
Log-ΔzFEV1_{IP}	GO	Negative regulation of BMP signaling pathway	2	*BMPER SKI*	C=32; O=2; E=0.01; R=142.86; rawP=7.87e-05; **adjP=0.0114**
		Ubiquitin protein ligase binding	2	*DAXX SKI*	C=147; O=2; E=0.05; R=38.69; rawP=0.0010; **adjP=0.0090**
		Promyelocytic leukaemia protein (PML) body	2	*DAXX SKI*	C=72; O=2; E=0.02; R=87.37; rawP=0.0002; **adjP=0.0052**
	PC	Transforming growth factor beta (TGF-β) receptor *(TGFBR)*	2	*DAXX SKI*	C=125; O=2; E=0.05; R=43.92; rawP=0.0009; **adjP=0.0450**
Log-ΔzFVC$_{IP}$	No significant pathway enrichment results				
ΔzFEV1/FVC$_{IP}$	GO	Protein homodimerization activity	3	*MTHFD1L ANXA9 PRKRA*	C=554; O=3; E=0.27; R=11.00; rawP=0.0018; **adjP=0.0324**
	KEGG	Metabolic pathways	2	*ADH6 MTHFD1L*	C=1093; O=2; E=0.46; R=4.39; rawP=0.0720; adjP=0.0720

C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category;
E: the expected number in the category; R: ratio of enrichment; rawP: p value from hypergeometric test;
adjP: p-value adjusted by the multiple test adjustment (BH). Bold shows significance p<0.05.

Of greatest interest from this study was the fact that three seemingly significant probes for intra-pair difference in level of zFVC were annotated to *GLIPR1L2*. This gene has been shown to have a wide expression profile with the highest levels in testis and lower levels in lung, and other tissues (172), which was also confirmed by the GTEx portal containing RNA-seq based expression profiles in 53 tissues from 544 post-mortem donors (173,174). However, *GLIPR1L2*, situated on chromosome 12, is part of the CAP superfamily, and is expressed in the immune tissues and involved in a variety of physiological processes. These include innate immunity, inhibition of ion channels and proteases, and interaction with immunoglobulin proteins, as well as tumour-suppressor and pro-oncogenic genes in different tissues (172,175). The *GLIPR1L2* is interestingly also a p53 target gene encoding functional p53 response elements that induce tumour-suppression (172).

Interesting enriched pathways were also identified, especially for intra-pair difference in level of zFEV1. ”Negative regulation of BMP signalling pathway” involving the genes *BMPER* and *SKI* is involved in malignant tumour growth and metastasis (176,177), as well as angiogenesis (178). “TGFBR”-pathway involving the genes *DAXX* and *SKI* plays an important role for normal lung morphogenesis and hence lung function (179). TGF-β is involved in normal lung tissue repair in adults through its pro-fibrotic effects, however over-expression of TGF-β is associated with different lung diseases, including lung fibrosis (179,180). It is indeed plausible that the TGFBR-pathway is of importance for lung function, as regulation of the activity of TGF-β can be governed through the expression of the TGF-β receptors (179).

In summary, it was found that DNA methylation signatures are associated to level and change in lung function in MZ twin pairs, identifying several CpG-sites and biological pathways of possible importance for lung function. Specifically oncogenic- and tumour suppressor-related genes (*GLIPR1L2*, *BMPER*, *SKI*, and *DAXX*), as well as TGF-β-receptor related genes could be involved in level and change in lung function.

5. General discussion

Respiratory health is important for the well-being, comorbidity risk and mortality of an individual, as well as for the economic and social burden of the society. As the average age of the world's population is increasing the prevalence of COPD also increases (14,15). Lung function level and decline seems to be influenced by several occupational and environmental factors as well as genetic/epigenetic predisposition. This makes it challenging to study effects of different specific exposures and factors on lung function. Through this PhD-thesis I have tried to elucidate different risk factors with possible association to change in lung function.

In this section the main findings and methodological considerations of the SUS-study (Paper II) and the WOOD-study (Paper III) are first presented, followed by the TWIN-study (Paper IV). Issues regarding design, confounding, potential bias, and generalizability will be discussed.

5.1 Main findings of the SUS-study and the WOOD-study

In both the SUS-study and the WOOD-study we explored the association between exposure to organic dust in occupational settings and change in lung function. Overall in the SUS-study (Paper II), we found no differences in change in lung function between exposed and controls nor any exposure-response relation between levels of organic dust exposure and lung function change. However, continued farming was associated with a negative effect on lung function change compared to those who quit farming during the follow-up period, with a greater effect among female farmers (current vs. ex-farmers). Likewise, the WOOD-study (Paper III) found evidence of greater susceptibility among female wood workers, showing both a higher incidence of new-onset COPD (among smokers only) and a greater decline in lung function with increasing levels of wood dust exposure. This was not seen among males. On the contrary, male wood workers seemed to be protected against adverse effects on change in lung function with increasing levels of dust exposure, most likely illustrating a strong healthy workers selection effect. The strongest predictor for excess decline in lung function was, however, smoking in both studies.

5.1.1 Comparison with previous literature

The levels of dust and endotoxin exposure found in the SUS-study (162) are similar to that found in other farming environments in Denmark (181) and the Netherlands (182) using similar sampling and analytical methods, as well as similar farming methods. However, 47% of the total dust measurements in the SUS-study exceeded the 3 mg/m^3 Danish Occupational Exposure Limit (OEL) for total organic dust (162). Even more of the endotoxin measurements (93%) exceeded the recommended health-based exposure limit of 90 EU/m^3 defined by the Health Council of the

Netherlands (183). Thus, it seems that the young Danish farmers in the SUS-study were exposed to dust and endotoxin levels that may well be associated to adverse health effects.

The dust exposure levels in the WOOD-study did not exceed the 3 mg/m^3 Danish OEL for total organic dust (65,165), however, came close to the 1 mg/m^3 Danish OEL for inhalable wood dust (23). Exposure levels were also lower than that found in other studies of the dry wood industry in Europe (184,185). This suggests that the Danish furniture industry lies in the low end of the wood dust exposure distribution in Europe. Adverse health effects in the WOOD-study should hence be less likely, though some evidence of adverse health effects were found for women in the WOOD-study. Furthermore, the OEL is an exposure level based only partly on health based dose-response relations. Also political and economic aspects are included in the definition of OEL.

Previous studies in the farming and wood industry on the effect of organic dust exposure and change in lung function have shown inconsistent results (The REVIEW, Paper I), however, overall pointing towards a negative association. A significant difference in lung function decline for farmers compared to controls has been seen in a few studies (54–56), whereas other studies (58,59) found no such association. In a study by Mauny et al (60) they found no difference in change in lung function between different methods of fodder drying (modern vs. traditional). This was despite the hypothesis that traditional fodder drying farmers would be exposed to higher levels of organic dust than modern barn-drying farmers, as previously shown (186). However, they did not measure the actual levels of dust exposure. A significant exposure-response relation between level of dust exposure and change in lung function was found in two studies among farmers (54,61) in contrast to our negative finding of no exposure-response relation between neither level of dust nor level of endotoxin exposure and change in lung function in the SUS-study (Paper II).

Exposure to wood dust was studied by Noertjojo et al (63) showing a significantly greater annual decline in FEV_1 and FVC among sawmill workers compared with controls. This study also found a significant exposure-response relation between wood dust and decline in FVC. However, sawmill dust exposure is known to be associated with higher levels of endotoxin and mould exposure than dry wood exposure (187). In a study by Glindmeyer et al (64) no association was found between inhalable wood dust exposure and adverse effects on lung function change. However, in a sub analysis respirable residual particulate matter was found to be associated with decline in FEV_1 and FVC in an exposure-response manner in subgroups of the study, indicating possible differences in potency of different elements and fractions of dust. We found a significant exposure-response relation between accumulated wood dust exposure and decline in FEV_1 and FVC for female wood workers only (Paper III). This is despite low levels of wood dust exposure overall and female wood workers having lower exposure levels than male wood workers. The same was true for the exposure levels among female farmers and male farmers. Thus, despite lower exposure levels, females seem more susceptible to the adverse effects of both wood-dust and current farming exposure on change in lung function in our two cohorts.

5.2 Methodological considerations of the SUS-study and the WOOD-study

5.2.1 Design

Both the SUS-study and the WOOD-study were designed as longitudinal follow-up studies with long term follow-up times (15 and 6 years respectively). This was in order to ensure reliable assessment of lung function decline over time and to achieve robust estimates of the effects of dust exposure, as well as to infer causality, for which the cross-sectional design is inadequate.

5.2.1.1 Lung function

All lung function measurements were performed according to guidelines (10) and underwent quality control. To assess the quality of the spirometry measurements the curves were inspected. Reasons for rejection of measurements included bad quality in terms of zero flow error (flow prior to the expiration), inspiration or coughing in the phase of expiration, a slow rise time due to lack of forced expiration, or premature termination of the expiration. From three spirometry attempts the maximum values of FEV_1 and FVC from accepted measurements for each subject were used in the subsequent analysis. The same few assessors performed spirometry at baseline and follow-up in both the SUS-study and the WOOD-study, using the same spirometer for all measurements. This is believed to ensure good internal consistency and reproducibility.

We used the GLI 2012 equations to standardise lung function measures. This was based on a methodological consideration that arose from the problems of assessing lung function change in young subjects (SUS-study). Some of these young subjects may still be in the phase of lung development, and hence have increasing absolute lung function (litres) during the follow-up period. When examining these subjects once at the age of 17-25 years and again at the age of 32-40 years it is not known when, and to what level, these subjects peak in absolute lung function. Therefore, it is difficult to estimate the absolute change in lung function during the follow-up period. Some individuals may have had a great increase in lung function followed by a great decline, and hence no large change in absolute lung function over time, whereas others may have had a small increase followed by the same great decline in lung function and hence a large change in absolute lung function over time. Although these two types of individuals in theory have the same great decline in lung function (from their peak lung function level) the decline would only be detected in the latter. To evade this issue we chose to use the z-score approach. Believing that an individual will follow the lung function distribution of the reference population of same sex, age, height and ethnicity, we avoid taking the absolute values into account, as well as the peak level of lung function of the individual. Furthermore, we avoid discontinuities from using separate age-dependant equations (as used previously (9,188)), when individuals move from one set of equations to the next, e.g. <18 years of age to >18 years of age, with the risk of introducing bias.

An issue with using the GLI 2012 equations in comparison to our studies portraying longitudinal change in lung function is that the reference equations are based on cross-sectional data, which may lead to discrepancies. It is well known that, due to cohort effects, longitudinal change in lung function differs from that estimated from cross sectional prediction equations (5,189). However, this is an issue influencing the z-score change of both the exposed subjects and the controls in our studies. And as these are the groups being compared (exposed vs. controls – opposed to only one group being compared to the cross-sectional reference population of GLI 2012), it should not be a problem in assessing the adverse effect of dust exposure on change in lung function. However, the estimated size of the effect calculated as Litres based on the z-score change may be influenced somewhat by the discrepancies arising from longitudinal data being compared to cross-sectional data. In both the SUS-study and the WOOD-study we did sensitivity analyses, where the outcome of absolute change in lung function was standardised by dividing by height in m^3 (e.g. FEV_1/m^3). This did not change the associations found.

Defining new-onset COPD using Lower Limit of Normal (LLN) from GLI 2012 equations in the WOOD-study was decided due to it being a more valid approach than using a fixed ratio of $FEV_1/FVC < 0.7$. The use of a fixed ratio has been shown to lead to an over diagnosis of COPD in the elderly and an under diagnosis in the younger individuals (15,18) as the variability of individual measurements around the median is not uniform across all ages (9,19). However, it has been shown that the subgroup classified as normal using the LLN method but obstructed using the GOLD criteria have an increased risk of mortality (190). For this subgroup, interpreting lung function data using fixed thresholds may still be of clinical relevance to define patients at risk of early respiratory death. However, when estimating COPD based on the fixed ratio there is a great risk of misdiagnosing cardiac disease as COPD (191) leading to incorrect treatment. Furthermore, in a new study by Vaz Fragoso et al (192) the z-score approach was more clinically meaningful when evaluating FEV_1 over time than liters and percent predicted (or alternatively in L/m^3), as z-score accounted for the effect of aging and was more frequently associated with multiple cardiopulmonary predictors. Therefore, using the z-score approach for change in lung function and LLN to define COPD seems to be the most correct and most clinically meaningfull approach.

A controversial issue concerning whether or not to adjust for the baseline value of lung function in regression analyses estimating the effect on change in lung function over time has been highly debated. The effect of correlated errors between lung function level and lung function change on the regression coeficients may lead to bias due to the “horse racing effect” (193). This effect implies that loss of lung function in adults is negativly associated with attained level of lung function, also explained as, the lower one’s lung function the more one loses (194). This is due to loss in lung function being correlated with the loss prior to the first measurement and therefore also with the first measurement itself (194). However, many studies choose to adjust for the baseline lung function value despite of this issue. We decided not to adjust for the baseline value in our studies to avoid possible overadjustment.

The problem of regression to the mean (RTM) in longitudinal studies, due to random error, a non-systematic variation in the observed values around a true mean in a subject or a cohort (195), must also be considered. The issue arrises from relatively high (or relatively low) observations being more likely to be followed by less extreme observations nearer the subject's true mean. The practical problem caused by RTM is the need to distinguish a real change from this expected change due to the natural variation (195). We have tried to account for this issue by reducing the variability of lung function measures by repeating the lung function measurements at least three times in order to ensure the best possible effort of spirometry of an individual at each assessment time.

5.2.1.2 Exposure

The exposure assessments in both the SUS-study and the WOOD-study are considered to be of high quality. We used a group based approach based on internal job exposure matrices (JEMs) defined from dust measurements in the respective cohorts. JEMs and occupational questionnaires, with the time spent in different jobs and job-tasks, were used to estimate the exposure of each individual. Variability in exposure must, however, be taken into consideration. Within-worker day-to-day variability was large, especially in the SUS-study of farmers (162), but also in the WOOD-study (196). Between-worker variability was also considerable in both studies, though lower than the within-worker variability. Dust exposure variability appears with changing work tasks during a work day leading to systematic differences, as well as random effects due to behavioural differences (197). The levels of dust and endotoxin are also greatly dependent on farm-type, e.g. field, cattle, swine, and poultry (162) and in the SUS-study we calculated an average accumulated exposure of all employments during the follow-up period to assess the overall exposure. Some work tasks are characterised by seasonal work, e.g. field work within farming exposure, which increases the variability. We attempted to account for this in the SUS-study, where different seasonal measurements were performed and an average calculated. The approach used to estimate dust exposures in both the SUS-study and the WOOD-study was hence group-based estimates taking different variance components into account.

In an attempt to ensure exposure estimates *before* the outcome of interest, we included linear regression analyses in the WOOD-study correlating the level of dust exposure *at baseline* with change in lung function during the follow-up period. Using accumulated exposures during the follow-up period, as applied in the SUS-study, could be a source of bias, as the order of exposure and outcome cannot be specified. At what point during the follow-up period did the lung function decline actually happen? If this was at the beginning of the occupational exposure – as seen in studies of new coal miners (80,81), where rapid initial effects were seen in the first year of exposure – then the accumulated dust exposure may not be the best exposure measure to asses. Furthermore, affected individuals selectively leaving the industry may be a source for healthy worker selection bias (see section **5.5** Sources of potential bias for all three studies). However, we

expect the decline in lung function to be a continuous change over time. Furthermore, an excess decline in lung function must be profound in order to affect the individual sufficiently and hence lead to a healthy worker selection bias. Though, other associated pulmonary symptoms may also be part of a selection out of the industry. We furthermore, saw that the level of wood-dust exposure at baseline was strongly correlated to the accumulated dust exposure during the follow-up period in the WOOD-study (Paper III). Both exposure assessment methods hence lead to similar associations with change in lung function. To our knowledge no other study has compared the exposure strategies of baseline level of dust exposure versus assessments of accumulated dust exposure during the follow-up period.

Due to correlations between different exposures in the work settings (e.g. bacteria, endotoxins, fungal spores and mite units) it is difficult to state exactly which exposures in the industry in question are accountable for the adverse effects on lung function change. In the SUS-study we saw no exposure-response relation neither between accumulated dust nor accumulated endotoxin levels and change in lung function. However, the current farmer status was, as expected, associated with higher accumulated dust levels, whereas the ex-farmer status had lower accumulated dust levels. Though, when assessing only current farmers, no exposure-response relation was seen with increasing levels of accumulated exposure. The same was true for ex-farmers. Hence the negative effect of current farming on excess decline in lung function may represent a more acute effect.

5.2.1.3 Age

In the SUS-study we excluded participants >25 years of age at baseline because age is an important determinant for lung function change, and we were especially interested in the possible adverse effects of dust exposure during the last phase of lung development in young adults. Furthermore, the restriction ensured an age-fitted comparison to the controls, who were all <25 years of age. The young farmers of the SUS-study were farming students at baseline and were therefore only entering the farming industry as professionals. This restricts the number of years in the industry to the follow-up period, though work at farms prior to baseline was also frequent. However, if the total accumulated exposure is the important metric of dust exposure, it may need more years of follow-up to ensure overall accumulated exposures that cause detectable decrements in lung function in this young cohort.

In the WOOD-study the age distribution of the cohort spanned a wider age-range from 16 to 65 years at baseline. This leaves for more years with work in the furniture industry prior to baseline for the older segment of the cohort. The accumulated exposure during the work life may have affected the baseline level of lung function. However, exposed workers did not start at lower z-score values of lung function than controls (Paper III).

We saw no great difference in the size of effect of dust exposure on change in lung function in the two studies (for the compared groups with effect), though comparison is difficult due to different dust types, different levels of exposure, and different follow-up years for the two populations of differing age. In the review (Paper I) we also did not see any difference in effect of dust exposure on change in lung function when comparing different age groups, though the comparison between studies should be done with caution, due to differing material and methods, as well as statistical approaches.

5.2.1.4 Other included confounders and interactions

Other parameters plausible to be influential on change in lung function were included in the models to adjust for possible confounding. Interaction terms were introduced in the models to assess possible effect modification.

In the SUS-study follow-up BMI was negatively associated to change in lung function. Due to lack of weight information at baseline, the change in weight or BMI was not available in the SUS-study. In the WOOD-study weight-gain during the follow-up period was associated to excess decline in lung function. In a study of steel workers a strong relation between weight-gain and longitudinal decline in lung function was also seen (198). This illustrates that adjustment for weight-gain or BMI-change is important when analysing the effect of an exposure on change in lung function, especially if it is expected that weight-gain is differentially distributed in the compared groups. It could be hypothesised that the work tasks in the exposed groups are physically demanding, leading to lower risk of weight gain among the exposed than among the controls, although other factors such as lifestyle and eating habits are also important. If adjustment for weight-gain was not performed this could lead to an underestimation of the effect of dust exposure, due to the controls decreasing in lung function due to weight-gain. Thus, the decrements in the exposed subjects in comparison would seem smaller. This may be an issue in the SUS-study, as we did not have the opportunity to correct for change in weight or BMI during the follow-up period. However, follow-up BMI of farmers and controls was comparable, though the variability was greater among farmers with larger maximal values (BMI up to 50).

Baseline asthma was a significant predictor for decline in lung function in the WOOD-study, though only among males. In the SUS-study baseline asthma and Bronchial Hyper Responsiveness (BHR) was also negatively associated with change in lung function. However, having asthma or BHR at follow-up was even more strongly associated with impaired lung function during the follow-up period than the baseline asthma and BHR status, perhaps depicting an acute effect on lung function at follow-up. Similarly, it cannot be ruled out that the negative effect of being a current farmer at follow-up in the SUS-study is illustrating an acute effect of exposure.

Smoking was assessed for all participants, both as smoking status at the assessment time and as accumulated pack years smoked overall and during the follow-up period. Current smokers were defined if they were smokers within the last two years prior to assessment. This is a quite conservative estimate. However, the more acute and partly reversible effects of smoking exposure, opposed to the chronic persistent effects, have been shown to affect lung function for some time after smoking cessation (120,199,200).

The tendency of a greater unadjusted effect of smoking on lung function change in the exposed workers found in both the SUS-study and the WOOD-study, could illustrate an effect modification by dust exposure on the effect of smoking on change in lung function. However, no significant interactions between smoking and dust exposure were found in adjusted regression analyses in either study. The reason for the lack of unadjusted effect of smoking among the controls may be that there were too few participants to detect a true effect on change in lung function in the control groups, whereas the size of the estimates point toward a possible difference in lung function change between smokers and non-smokers, also among controls.

Height is a surprisingly complex measure to acquire correctly. It also has a great influence in the prediction equations. A 1% overestimation in height introduces bias that ranges from +1% up to +40% (for short older females) in lung function predictions (9) depending on which prediction equation is used. In the SUS-study, where subjects were young and some still growing, we used baseline and follow-up height for standardising lung function at the two time points, respectively. In the WOOD-study, we evaluated that using an average of the two height measures from baseline and follow-up for standardisation of lung function at the two time points would help adjust for the errors related to measuring height. According to the age distribution in the WOOD-study, with no expectance of change in height during the follow-up period, we therefore chose this approach for standardisation of lung function in the WOOD-study.

In the SUS-study we found effect modification by farm upbringing on the negative association between BHR and lung function. Being raised on a farm was in itself a protective factor for lung function change, whereas BHR in itself was associated to an excess decline in lung function. However, among those not raised on a farm, BHR had a negative effect on change in lung function, whereas, among those raised on a farm, change in lung function was not negatively affected by BHR. No significant difference in prevalence of BHR at baseline was seen between those raised on a farm and those not – neither among farmers nor among controls. Thus, it seems that farm upbringing attenuates the negative effect of BHR on lung function. Previous studies have similarly found adverse effects of BHR on cross-sectional level and longitudinal change in lung function (108–110,201). One study reported that high occupational endotoxin exposure was a risk factor for BHR (202), which is in contrast to our finding of no significant difference in BHR prevalence between farmers and controls. However, within farmers, the highest quartile of endotoxin exposure in the SUS-study had a twofold increased risk of having BHR (OR (95% CI): 2.0 (1.16-3.57)) compared to

the lowest quartile (203), supporting previous findings. The protective effect of farm upbringing on the adverse effect of BHR on lung function has, to our knowledge, not been seen before. This effect may, however, be equivalent to the protective effect of farm up-bringing on asthma and allergy shown in several studies (204–208). This protective effect may be due to environmental modulation of inflammatory responses during a childhood with exposure to farming.

A limitation to the SUS-study is the lack of female controls. However, the lung function change of the female farmers was standardised using the GLI 2012 equations, giving a z-score change in comparison with the female reference population of same age, height and ethnicity. One problem that may still be present, though, is that the error between longitudinal and cross-sectional equations may be dependent on sex. However, the estimated effects did not change significantly when all women were excluded from analyses in the SUS-study.

5.3 Main findings of the TWIN-study

The TWIN-study illustrated that several differentially methylated CpG-sites within twin pairs were associated to level and change in lung function. Many associated CpG-sites were identified for level of zFEV1, zFVC, and zFEV1/FVC, whereas fewer were identified for change in lung function. A major finding was the association between DNA methylation of three CpG-sites in *GLIPR1L2* and intra pair difference in level of zFVC. Though the three probes were in close proximity to each other within the gene and hence might not be independently methylated, the finding of three probes within the same gene makes it less likely to have been found by chance (type I error, false positive). Also enriched pathways were identified, especially for level of zFEV1, with plausible biological importance for lung function, including oncogenic- and tumour suppressor-related pathways, as well as the TGF-β-receptor pathway.

5.3.1 Comparison with previous literature

Only few studies have explored the association between DNA methylation and level and change in lung function, and none has looked at this association in MZ twins. DNA methylation of the inflammatory genes *CRAT*, *F3* and *TLR2*, as well as *IFNγ* and *IL6* were found to be associated with lung function level (155). This illustrates the possible importance of activation and inactivation of inflammatory genes for level of lung function. Seven of the nine genes assessed in that study were represented with CpG-sites in the 450 BeadChips used in our TWIN-study. However, no overlap in significant genes was found with our study. Qiu et al (156) found that hypo-methylation of a CpG-site in *SERPINA1* (among 348 other significant CpG-sites in different genes) was negatively associated to level of lung function. This finding was replicated in another cohort. Other top associations for CpG sites found by Qiu et al were in *ATP6V1E2*, *FXYD1*, *FUT7*, *STAT5A*, *TRPM2*, and

LRP3 (156) with no overlap to our study, despite the fact that 6 out of the 7 most significant genes were represented in the 450 BeadChips used in our study. Also DNA methylation of the repetitive elements *Alu* and LINE-1 in association with lung function level and change has been studied (158) showing significant associations. Alu and LINE-1 were, however, not included in our study using the 450 BeadChips. With these previous studies being performed on a general population and not on twin samples, the results may be driven by an underlying genetic component. In our study, using the MZ twin design, the genetic factors for both lung function and DNA methylation are accounted for. Also shared environmental factors from in utero, birth, upbringing and during life will be partly adjusted for using the MZ twin design. Therefore, the results should be explained by other environmental factors that may influence DNA methylation and lung function in the present TWIN-study and not by the underlying genetic component.

5.4 Methodological considerations of the TWIN-study

5.4.1 Design

The TWIN-study was based on a sub-population of the Middle aged Danish Twin study from the Danish Twin Register. The association between intra pair difference in DNA methylation and cross sectional lung function or longitudinal change in lung function during an 11 year follow-up period was assessed. This was done in order to identify different genes and pathways of possible importance for both level of lung function and the continuous decline in long function with age.

5.4.1.1 Lung function

Lung function measurements were performed according to guidelines (2) and underwent quality control similar to that of the SUS-study and the WOOD-study. In the TWIN-study multiple interviewers performed examinations in the participant's homes at baseline and at five study centres at follow-up using different spirometry devices at each assessment time. All assessors underwent proper training in methods of spirometry, however, multiple assessors as well as devices lead to greater variation and lower reproducibility and hence a risk of lower validity of lung function tests, which was a weakness in the TWIN-study.

Issues of using the GLI 2012 equations for standardisation of lung function and the discrepancies arising from longitudinal data being compared to cross-sectional data are also present in the TWIN-study. However, with the MZ twin design, comparing intra pair differences in z-score of lung function change, it should not be a problem, as the comparison is internal in the cohort.

In the TWIN-study log-transformation was applied to independent lung function variables with extremely skewed distributions (intra pair differences of zFEV1, zFVC, zFEV1-change and zFVC-change) to avoid inflation of the Epigenome Wide Association Study (EWAS) results. As log-

transformation of skewed independent variables tends to scale down and thus reduce the weight of the variables in statistical testing this was not done for models with independent variables having only slightly skewed distributions. Despite the log-transformation reducing the weight of the more extreme variables (high twin discordance) we found statistical significant results. It would also have been interesting to look at the associations only among the 50% most lung function discordant twin pairs, in order to see if other probes and genes reached statistical significance in such models. However, due to lack of power (too few twin pairs and too low discordance) this was not possible.

5.4.1.2 DNA Methylation

In the TWIN-study peripheral blood was used for studying DNA methylation signatures associated with lung function. This approach was chosen as blood samples represent an easily accessible source of cells, which can be collected at multiple research centres, at low cost and without the need of complex equipment. It has been debated, though, if blood cells can be used as a surrogate to study aberrant methylation patterns associated with disease in other tissues, e.g. the lungs. An early component of lung function decline is inflammation and infiltration of the lung tissue with leukocytes (155). Furthermore, a “spill-over” of cytokines from the lung tissue to the systemic blood circulation has been suggested, as inflammatory cytokines are elevated in circulating blood of COPD patients (209). Inflammatory processes in the lungs may therefore be associated to DNA methylation levels in circulating immune cells. The effects of smoking, inhaled through the lungs, also leaves differentially methylated marks on blood methylome, providing evidence of the use of blood cell DNA methylation patterns as biomarkers of smoking exposure (154). Similarly, a new study by Baglietto et al (210) showed that DNA methylation changes measured in blood samples preceding cancer diagnosis are associated with smoking and lung cancer risk, further supporting the use of blood cells as a surrogate for physiological processes in the lungs.

In order to control for technical variation, blood DNA methylation-data were normalised by correcting for intensity-related dye biases from different chemistry and design of probes, as well as by removing technical artefacts between samples on different arrays. The obtained β-values (the proportion of DNA-methylation) were further logit transformed giving an M-value=$\log_2(\beta/1-\beta)$ for each probe. This transformation makes it difficult to estimate the effect sizes found in the regression analyses. This is further complicated by the lung function measures being standardised as z-scores as well as the intra pair differences in lung function z-scores being log-transformed. Therefore, the results from the TWIN-study are hypothesis generating, stating only significant associations between blood DNA methylation and lung function level and change, with the relative “size” of DNA methylation presented as hypo- or hyper-methylation for the “inferior” twin compared to the “superior” twin.

Other environmental effects than smoking may also be important for DNA methylation studies when performed in adult individuals, whose lifestyle choices (such as level of exercise, diet, alcohol use, and medication) potentially would influence DNA methylation levels at multiple sites in the genome. For example, if DNA methylation levels are influenced by medication taken for any disease in association with the outcome, it may affect the associations found between differentially methylated loci and the disorder of interest.

Also stochastic methylation at an early developmental stage, leaving marks in the blood and other tissues, may be of importance. These methylation marks may, by chance, have negative functional consequences in the lungs, and could hence be identified as associated to lung function in the EWAS.

It is difficult to infer causality in this study. First of all, it should be noted that it was only possible to correlate longitudinal lung function change with DNA methylation status at follow-up, as blood samples from the participants in the TWIN-study were only collected at follow-up. Secondly, it is not possible to determine whether DNA methylation signatures are a cause or a consequence of the changes in lung function. However, the possible mechanism behind a causal relation may be that environmental exposures induce oxidative damage and changes in DNA methylation, which may in turn impact lung function due to altered gene-expression, as also suggested by Lepeule et al (158). Still, although significant associations have been found between DNA methylation and lung function, direction of causality cannot be inferred.

We acknowledge that, as 453,014 tests (number of probes) were performed for each outcome measure in EWAS analyses, the significant cut off to reach genome-wide significance according to the conservative Bonferroni correction for multiple testing would have to be 0.05/453,014 = 1.1 x 10^{-7}. However, for EWAS it should be noted that not all 453,014 tests are independent, due to correlation of DNA methylation in the genome (e.g. along CpG Islands). This should allow for lowering the requirement for genome-wide significance. Therefore, we presented results with a p-value < 10^{-6} as being statistically significant, and included findings with p-value < 10^{-5} in tables and in pathway enrichment analyses.

5.4.1.3 Included confounders

All analyses were adjusted for sex, age, BMI, smoking history (total pack-years), smoking status at follow-up, as well as blood cell composition difference within each twin pair. BMI-change during the follow-up period (instead of cross-sectional BMI) was additionally adjusted for in the longitudinal models, as were smoking pack-years during the follow-up period (instead of total pack-years).

There is a possible risk of over-adjustment for sex and age in the TWIN-study, as sex and age are included in the calculation of z-scores for lung function to standardise the measures. However, we

decided to further adjust for sex and age in all the EWAS models as DNA methylation also varies significantly between the sexes (211) and with age (130).

A strong point of the TWIN-study is the adjustment for cell count differences assessed by flow-cytometry of the same blood samples as DNA methylation data was generated from. The usual approach for correction of blood composition is using an estimation of blood cell composition based on the DNA methylation data (212), which is less precise than using the actual flow-cytometry profiles.

A further strength in the TWIN-study is adjustment for smoking status and smoking history of each individual, which is crucial for both lung function and for DNA methylation patterns. Hence, the DNA methylation signatures associated with lung function reported in this study should not be driven by the effect of smoking but rather by other possible environmental and stochastic effects. However, we cannot be sure of complete adjustment for smoking effects on the blood-methylome with the type of smoking information available for this study. Though we did not find any overlap with previous findings of differentially methylated loci associated with smoking (213).

Height was objectively measured only at follow-up in the TWIN-study. According to the TWIN-study age distribution, with no expectance of change in height during the follow-up period, and to minimise any possible errors related to self-reported height (baseline assessment, which might reflect younger maximum height), standardisations of lung function for both baseline and follow-up values were performed using follow-up height.

5.5 Sources of potential bias in the SUS-study, WOOD-study and TWIN-study

All information on occupational exposure was partly based on questionnaire data in the SUS-study and the WOOD-study, in combination with dust measurements to assess the exposure. Smoking was in all three studies based on questionnaires. This may be a source of potential information bias. If the self-reporting of occupational exposure (e.g. time spent in the barn) is higher among subjects with decline in lung function, this will lead to an overestimation of the association between organic dust exposure and change in lung function. Furthermore, smoking may be prone to underreporting, leading to an overestimation of the effect of smoking on change in lung function. However, any information bias is thought to be of non-differential type, as there is no reason to believe that the level of reporting bias should be different between compared groups. Furthermore, an excess decline in lung function should not lead to a differential self-report of exposure.

In occupational studies a common potential bias is caused by healthy workers selection effect, which is a phenomenon that covers different possible scenarios; healthy individuals are selected into occupations with high demands to physical capability (healthy hire selection effect); healthy workers, that can withstand the negative health effects of exposure, stay in occupations and in work tasks with higher exposure; “unhealthy” or more sensitive workers, that cannot withstand the

negative effects of exposure, tend to drop out of the occupation or choose tasks with lower exposure (214). This phenomenon tends to underestimate the true risk associated with the exposure, as it may mask negative health effects caused by otherwise harmful workplace exposures. Furthermore, in longitudinal studies a major concern is participation bias and the attrition during the follow-up period (215,216). Comparison of participants and non-participants at follow-up can, however, shed light on possible participation bias. Previous research has demonstrated these issues by showing that the average decline in lung function over the first year in grain workers was associated with duration of follow-up, and that grain workers who left their jobs early in their employment suffered greater annual loss in lung function than workers who stayed in the industry (217). Healthy worker selection effect has also been seen in several other occupations, such as the granite industry (87) and among farmers (218). However, a protective effect found of farming on asthma could not be explained by a healthy worker selection into farming by Eduard et al (219), illustrating that effects of exposure (positive and negative) may still be possible to detect, despite healthy selection effects.

The selection methods used for the three cohorts in this thesis may, to some extent, be a source of potential selection bias. For the SUS-study young farmers in Denmark in their first year of farming school were invited. There may be a selection of healthy subjects into the farming schools, as the work-tasks in the farming industry are well-known to be physically demanding. However, the fact that the young SUS-study participants were entering the farming industry results in less risk of selection out of the farming industry prior to baseline. During the follow-up period, there may have been some selection out of the industry as a few significant differences in baseline characteristics were seen between current and ex farmers at follow-up (Supplementary Table S2, Paper II). For instance, current farmers were less likely to have had asthma at baseline, tended to smoke less and were more likely to have been raised on a farm.

Due to a participation rate at follow-up of only 51.7% there may also be some participation problems in the SUS-study. Comparing participants and non-participant at follow-up showed some differences in baseline characteristics (Supplementary Table S1, Paper II). Participants were less likely to have been smokers at baseline. Participating male farmers were more likely to have been raised on a farm, had less asthma at baseline and had significantly better z-scores of lung function at baseline compared to non-participating male farmers. This illustrates a healthy participation selection in the SUS-study, possibly underestimating the true effect of organic dust exposure on change in lung function.

The WOOD-study cohort consisted of wood workers in Denmark, for which healthy hire selection may also be a source of potential bias. We did not compare current and ex-wood workers in this study. However this was done in previous analyses of the same cohort (65), showing that wood workers still employed in the wood industry at follow-up had a larger absolute decline in lung function than wood workers who had left the wood industry. However, other differences between

ex- and current wood workers and hence possible healthy worker selection bias will be further elaborated in future analyses. Healthy worker selection bias may be the reason for the positive exposure-response relation found between level of wood dust exposure at baseline and change in lung function during the follow-up period among male wood workers. Thus, healthier individuals who can withstand the exposure may tend to work with the highest exposed tasks.

In the WOOD-study there was also an evident participation selection. Non-participants were younger, tended to smoke more and had significantly worse z-scores of lung function at baseline compared to participants, significantly so for male wood-workers (Supplementary Table S1, Paper III). This illustrates a self selection of healthier subjects at follow-up. Controls were chosen from three factories without organic dust exposure. However, male controls in the WOOD-study were shown to have lower lung function at baseline than expected from the reference equations (low z-scores) possibly depicting an adverse effect from some other types of exposure prior to baseline assessment. This may be part of the explanation of the positive association between organic dust exposure and change in lung function among male wood workers compared to controls. The seemingly increased susceptibility among female wood-workers may partly be explained by the evident selection of healthy male wood workers. However, a tendency of a healthy participant selection among women was also seen, though not reaching statistical significance possibly due to lack of power due to too few female subjects.

The TWIN-cohort consisted of older MZ twins. A source of potential bias in this study is that approximately 10% of the original baseline cohort was deceased at follow-up, which led to the risk of participants having better lung health as they were still alive and participating at follow-up. As a consequence intact MZ twin pairs included in the cohort may have high concordance in lung function. However, if any such bias was present it would tend to underestimate possible associations with DNA methylation due to low variance of the studied trait.

5.6 External validity and generalizability

Occupational studies from different countries are difficult to compare due to differences in dust exposure levels, influences of temperature, humidity, and different working methods. However, we consider our results to be representative for young Danish farmers and Danish wood workers, as the cohorts were selected from large geographical areas in Denmark, covering the farming schools and the wood factories well, with quite large sample sizes. Furthermore, workers in other countries in the temperate zones with similar dust exposure levels and working methods as in Denmark are thought to be comparable to our study populations.

An issue in the WOOD-study was the low incidence of new-onset COPD during the follow-up period. This limited the power to study the association between wood dust exposure and new-onset COPD. The risk of random error hence was present, and the point estimates of the association were not

precise and had wide confidence intervals, e.g. for the logistic regression of new-onset COPD in female wood workers (NB: only 10 cases) comparing the highest with the lowest dust exposed group, OR (95% CI): 12.00 (1.3-111.0). Therefore these results need to be confirmed by further studies.

As for generalizability of the TWIN-study to other populations it must be emphasised that this unique cohort consisted of Caucasian elderly MZ-twins with the unique opportunity to control for underlying genetic background and shared environment. How DNA methylation in younger cohorts and of other ethnicities would be associated with lung function still remains to be explored. Validation is further needed in order to ensure strength and relevance of our results.

5.7 Clinical relevance

In the SUS-study and the WOOD-study we attempted to quantify the effect size of organic dust exposure on absolute change in lung function calculated from z-score for a standard person. This is complicated by the complex nature of lung function and the complexity of the equations. However, in the SUS-study the effect of the current farmer status on change in FEV_1 during the follow-up period (standardised for a male aged 25 with the height 175 cm (1 z-score=515.5mL)) would equate to -0.12 x 515.5 mL = -62 mL (95%CI -108 to -15.5 mL) over 15 years of follow-up compared to the ex-farmer status. In the WOOD-study the effect of exposure to 3rd quartile wood dust on change in FEV_1 for a female (standardised for a female aged 40 years and with height 167 cm (1 z-score=390 mL)) would equate to -0.32 x 390 mL = -125 mL (95%CI -203 to -35 mL) over 6 years compared to a control. It must be kept in mind that these excess declines are in addition to the age-dependent decline in lung function expected among all individuals, equivalent to that of the healthy reference population. Accumulated during a work life, the effect of organic dust exposure could account for an additional loss in lung function reaching up to 625 mL in FEV_1 in a 30 year work period. Though smoking affects the decrease on an even larger scale, the effect of organic dust exposure could still cause an important clinical reduction in lung function with time.

The findings of the TWIN-study illustrate that blood DNA methylation signatures are associated to the level and change in lung function, pointing to genes possibly related to the traits. Furthermore, the identification of enriched biological pathways could help lead to better understanding of pulmonary physiology in the future.

6. Conclusions

In the studies included in this PhD-thesis we have investigated the association between exposure to organic dust and change in lung function using an epidemiological approach in two occupational cohorts, as well as through a systematic review. Furthermore, the association between DNA methylation signatures and level and change in lung function in MZ twin pairs was explored with a hypothesis generating approach. The main conclusions of this thesis in relation to the aims are the following:

1. Organic dust and change in lung function

a. The SUS-study (Paper II) illustrated within farmer differences in lung function change, showing that current farmers had a significantly negative effect on lung function change during the follow-up period compared to ex-farmers. However, no significant differences between exposed and controls were seen and no exposure-response relations between increasing levels of dust or endotoxin and change in lung function were found.

b. The WOOD-study (Paper III) showed significant differences in incidence of new-onset COPD and change in lung function between female woodworkers and controls and a dose-response relation with increasing levels of wood-dust exposure for females only. A positive association between level of wood dust exposure and change in lung function among males was found, possibly due to healthy worker selection effect among male wood workers.

2. Sex as an effect modifier

a. Effect modification by sex was found in the SUS-study, where a significant interaction between sex and current farming was present in association with change in lung function. This indicated that female farmers were more susceptible to the adverse effects of current farming than male farmers.

b. The sex-stratified results of the WOOD-study indicated that there was significant effect modification by sex on the effect of wood dust exposure on change in lung function. This was supported by a significant interaction between wood dust exposure and sex in non-stratified analyses.

In summary, the SUS-study and the WOOD-study provided some evidence of a negative effect of organic dust exposure on change in lung function with females being more susceptible, though an exposure-response relation could only be found among females in the WOOD-study. Also the

review (Paper I) pointed toward a negative association between organic dust exposure and long-term change in lung function, though the results were inconsistent.

3. DNA methylation signatures and lung function

- **a.** In the TWIN-study EWAS analyses for intra-pair differences in level and change of lung function identified several associated differentially methylated CpG-sites annotated to different genes of possible importance for lung function. An interesting finding was three seemingly associated probes in *GLIPR1L2*, a gene involved in a variety of physiological processes, including tumour-suppression.
- **b.** Interesting enriched pathways were also identified in the TWIN-study. These were among others involved in oncogenic- and tumour suppression as well as growth and lung tissue repair, and the signals in all pathways were primarily driven by the same few important genes.

In summary the blood DNA methylation level of specific CpG-sites and hence possible regulation of the involved genes and biological pathways may be of importance for general pulmonary physiology and for lung function decline.

7. Implications and future perspectives

The conclusions from the review (Paper I) as well as the findings of the SUS-study (Paper II) and the WOOD-study (Paper III) overall give some evidence of a negative association between organic dust exposure and long-term change in lung function, with females being more susceptible. An evident problem in the SUS-study and the WOOD-study, as well as the reviewed studies, is the possible effect of healthy worker selection bias, possibly leading to an underestimation of the adverse effects of organic dust on change in lung function. Hence, the adverse effect of organic dust may be stronger than that illustrated from these studies. Further elaboration of this selection bias as well as further follow-up of the present cohorts may be able to shed light on this. Future comparison between farmers and wood workers staying in their occupation with those that drop out of the industry could help understand this issue in greater detail.

Solutions to avoid possible adverse effects on lung function from organic dust exposure could be to further reduce dust exposure in occupational settings. This could be implemented by creating occupational exposure limits that are even lower than today, as well as engineering work places with improved environmental conditions and lower exposure. Furthermore, the use of personal protective equipment, such as masks, could help reduce inhalable dust exposure, in particular when performing work tasks with known high levels of dust. However, the use of protective gear is limited due to the discomfort associated with this use, and is regulated to a maximum use of 2 hours per day for e.g. filter masks. The importance of reduction of other risk factors, in particular smoking, is emphasised by smoking being the most significant risk factor for COPD and accelerated lung function decline in our cohorts. However, emphasis on possible protective effects from organic dust exposure should also be remembered. This includes the protective effect of farm upbringing on the adverse effect of BHR on lung function, and the protective effect of farm up-bringing and organic dust exposure on asthma and allergy and the possible positive modulation of inflammatory responses.

These concepts lead us to the relevance of epigenetics and its importance for pulmonary physiology. As the first step we have tried to elucidate possibly important associations between blood cell DNA methylation and level and change in lung function. The objective was to identify genes and pathways of possible importance to lung function. Though the interpretation of obtained results is not straight forward, we believe that we are expanding our knowledge and understanding of the pathophysiological processes underlying impaired lung function. Still, it may only provide a small piece of the puzzle. The TWIN-study confirms that, even in blood, it is possible to identify differentially methylated sites that associate with level and change in lung function. These findings should be replicated in independent cohorts to confirm validity of the results. In future studies, methylation samples should ideally be collected at both baseline and follow-up, in order to compare DNA methylation signatures and changes in these in association with lung function.

Differentially methylated loci over time should help us understand causal associations with level and change in lung function in more detail.

Furthermore, gene-environment interactions would be of great interest to study. Blood-samples are available for the SUS-study and for part of the WOOD-study. These could be used for analyses of possible interactions between organic dust exposure and differences in the genome/epigenome and the association with level and change in lung function.

For the mechanisms of DNA methylation to be useful in the future we need to understand the role of regulation and function of genes and biological pathways for lung disease in more detail. It may become possible to modify these mechanisms therapeutically to reduce the burden of lung disease among patients (220). Whole-Genome Bisulphite Sequencing (WGBS) can provide a comprehensive view of methylation patterns at single-base resolution across the full genome (221). However, it remains a great challenge to interpret and functionally test the findings due to the complexity of the genetic and epigenetic mechanisms. Still, the dramatic technological development should provide us with new possibilities for genome/epigenome analyses and functional editing in the near future.

8. References

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9. Appendices

Appendix I Paper I – The REVIEW

Appendix II Paper II – The SUS-study

Appendix III Paper III – The WOOD-study

Appendix IV Paper IV – The TWIN-study

Appendix V Calculation of cigarette equivalents

Appendix VI Calculation of cumulative exposure in the SUS-study

Appendix I

Paper I: The REVIEW

The effect of organic dust on long-term change in lung function – a systematic review. *(Submitted manuscript in 2nd round review).*

Bolund ACS, Miller MR, Sigsgaard T, Schlünssen V.

Appendix I

Paper I: The REVIEW

The effect of organic dust exposure on long-term change in lung function – a systematic review

(Submitted manuscript in 2nd round review).

Author of correspondence:

MD Anneli Clea Skjelmose Bolund,

Department of Public Health, Section of Environment Occupation & Health, Danish Ramazzini Centre,

University of Aarhus, Bartholins Allé 2, DK-8000 Aarhus C, Denmark

abol@ph.au.dk Tel.:+45 87 16 80 19 Mobile: +45 21 25 54 47

Anneli C S Bolund[a], Martin R Miller[b], Torben Sigsgaard[a], and Vivi Schlünssen[a, c].

[a] Department of Public Health, Section of Environment Occupation and Health, Danish Ramazzini Centre, University of Aarhus, 8000 Aarhus C, Denmark.

[b] Institute of Occupational and Environmental Medicine, University of Birmingham, UK.

[c] National Research Centre for the Working Environment, Copenhagen

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"Take home" message

This systematic review finds some evidence of a causal association between exposure to organic dust and change in lung function.

ABSTRACT

Background

Lung function is a predictor of morbidity and mortality and the chronic nature of lung function decline allows for preventive initiatives. Pro-inflammatory constituents of organic dust are considered a cause of compromised respiratory health. The aim of this systematic review was to reveal the impact of organic dust exposure on long-term change in lung function.

Methods

The literature search was performed according to the PRISMA criteria. Predefined criteria concerned study-design: longitudinal, ≥1 year follow-up, ≥50 exposed; exposure-measures: organic dust, measured or estimated, in different occupational settings; and outcome-measures: change in lung function measured by spirometry. Based on these criteria, 1580 potentially relevant publications were narrowed down to 20 included publications. Quality was evaluated based on 6 criteria (maximum score 6).

Results

Overall, 14 studies found an association between exposure to organic dust and long-term change in lung function. Grain dust exposure showed the most consistent negative associations to change in lung function. Exposure to wood dust, farming and cotton dust were less consistent. The significant effect size of exposure on FEV_1 ranged from -12 to -38 mL/year excess loss compared to controls. 12 studies revealed a significant exposure–response relation between organic dust and change in lung function.

Conclusion

The results were inconsistent across varying study design and different exposure measures and outcomes. However, we conclude that there is some evidence of a causal association between exposure to organic dust and long-term excess decline in lung function.

INTRODUCTION

The definition of several chronic pulmonary diseases, such as asthma and COPD, includes reference to lung function and they are associated with accelerated lung function decline. These diseases are a leading cause of morbidity and mortality and are a global health problem with increasing prevalence, leading to a substantial economic and social burden worldwide (1,2). Lung function on its own is also a substantial predictor of morbidity, mortality (3–5), and cognitive and physical functioning (6) in the general population. Change in lung function over time is therefore of interest and because of the slow evolution and chronic nature of lung function decline it presents opportunities for prevention (7).

Organic dust – an aggregate of particles from plants, animals and microbes suspended in the air (8), containing bacteria, molds, pollens and chemicals– is a well-established major air pollutant within different workplaces. The mechanisms of lung damage from organic dust work partly through one of its constituents, endotoxin. Endotoxin is a building stone of the outer membrane of Gram negative bacteria and is considered to be a cause of respiratory disease among workers exposed to organic dust due to its strong potency in comparison with other pro-inflammatory microbial constituents of organic dust (9). The association between exposure to endotoxin and acute decline in lung function was first described by Castellan et al (1987)(10). They found a strong relation between airborne endotoxin exposure and mean percentage acute decline in forced expiratory volume in the 1st second (FEV_1), whereas the dust concentration *per se* was not correlated with an acute change in FEV_1 (10).

Occupational exposure to organic dust and a possible association to respiratory disease and level of lung function has been investigated in several studies for different occupations, especially cotton work, wood work, farming and grain work. There is increasing interest in exposures in utero and early in life and the impact this has on the developing lung (11). Cross-sectional studies cannot uncover the sequence of events — whether exposure occurred before, after or during the onset of the disease outcome and is not well suited to infer causality (12). In this systematic review we therefore aimed to examine the effect of exposure to organic dust in different occupational settings on longitudinal change in lung function and to clarify where future research contributions are needed. To our knowledge no other systematic review concerning lung function change in relation to exposure to organic dust has been conducted.

METHODS

Search strategy

The literature search was performed according to the PRISMA criteria, a recommended method for systematic reviews (13). The search strategy, inclusion and exclusion criteria were developed among the authors. The first author then conducted the literature search. The available literature was identified by searching three online databases (NCBI PubMed, Embase and Cochrane Library) for publications prior to May 1st 2016. The full electronic search was optimised using NCBI PubMed after which the same search was performed in the other databases. Free text searches were prioritised in each database after exploding relevant index terms (i.e. MeSH) to ensure all articles of interest to be found and to avoid missing articles not yet categorized in index terms. The full electronic search strategy is provided in Table 1 and the supplementary Figure S1 shows the full example of the final search performed in NCBI Pubmed. Identification of the relevant studies based on the described inclusion and exclusion criteria below were made by the first and last author, first by title, then by abstract and finally by full article.

Study criteria

The inclusion criteria were: 1) design: longitudinal/prospective cohort studies with minimum 1 year follow-up time, 2) exposure: organic dust in different settings (wood work, waste work, farming, cotton work etc.), 3) outcome: longitudinal change in the lung function indices of forced expiratory volume in the 1st second (FEV_1), forced vital capacity (FVC), FEV_1 as % of predicted, FVC as % of predicted, FEV_1 and FVC as z-scores predicted from the Global Lung Function Initiative 2012 (GLI2012) equations, 4) study subjects: humans, 5) language: English language full-length, original publications in peer reviewed journals.

Studies were excluded based on i) design: cross-sectional- or cross-shift studies and follow-up time under 1 year, ii) exposure: inorganic dust exposure or “mixed” dust exposure without opportunity to separate organic and inorganic dust exposure, iii) lack of exposure gradient, such as exposed vs. controls or without different levels of exposure among the exposed subjects, iv) prognostic studies of specific patient groups, v) size: studies with <50 exposed subjects, vi) lack of a quantitative estimate of association between lung function change and organic dust exposure, vii) lack of adjustment for smoking.

Several cohorts had been followed up in different cycles and therefore described at different follow-up times (i.e. Shanghai cotton textile study (14), Saskatchewan grain study (15), and farmers in the Doubs Province (16)). In order to avoid repeated cohorts inflating their effect on the review, we have reported (in Tables 3 and 4) the articles with the study aim closest to our interest, namely the greatest exposure contrast (years of exposure) and

the largest number of participants. Other studies concerning the same cohorts have only been shortly described in the text.

The quality of the studies were evaluated based on study design, dimensions, response rate, exposure measure, exposure-response relation, and confounder control (Table 2). Meta-analysis could not be performed due to large heterogeneity of outcome and exposure measures and different methods of statistical analysis across studies.

RESULTS

Study selection

All manuscripts that met the search criteria were assessed in a common database (www.refworks.com). After removal of duplicates (n=589) there were 1580 potentially relevant publications (Figure 1). After screening these we excluded 1511 studies; 1226 based on their title and 285 based on their abstract. Based on a full text-screening of the remaining 69 articles , 49 articles were excluded due to either a lack of suitability in terms of study aim, study design, size of exposed group, exposure or outcome variables, no quantitative estimate of association, or repeated cohorts (Figure 1). From a snowball search in the 20 remaining publications and 8 review articles (17–24) concerning organic dust and respiratory diseases there were no additional papers that fulfilled the inclusion criteria. Therefore 20 publications were included in this review.

Characteristics of studies

The characteristics of the 20 included studies (14–16,25–41) are summarized in Table 3. All studies were prospective cohort studies with follow-up times between 1 and 20 years (median 6.5 years). Sample sizes ranged from 97 to 11,827 exposed individuals, with a median of 218. Ten studies (14,16,27,30–32,36,38,40,41) had an external control group for comparison, and ten (15,25,26,28,29,33–35,37,39) had an internal control group within the occupation (such as swine farmers compared to dairy farmers) or compared levels of exposure within the exposed group. The mean age at baseline ranged from 18.3 to 50.8 years. The cohorts originated from Canada (15,27,28,30,36,41), China (14,26), Denmark (31,33,38,40), France (16,32,34), The Netherlands (29,37) and USA (25,35,39).

The exposures of interest were 1) cotton dust (14,25,26), 2) grain dust (15,27–30), 3) farm dust (16,31–37), 4) paper dust (38), and 5) wood dust (39–41). In 12 studies (14,25–27,29,31,35,37–41) the exposure was measured with personal or stationary dust collectors with individual exposure estimates for each subject. In 8 studies (15,16,28,30,32–34,36) the exposure was assessed from questionnaires as time spent working in different exposure

settings, or years working in the industry, or as simple dichotomous variables such as exposed versus control or yes or no to different work characteristics.

The outcome was change in lung function (FEV_1, FVC, FEV_1/FVC) in all studies measured by spirometry at minimum 2 occasions with at least 1 year in between. All studies were adjusted for smoking, the strongest predictor and possible confounder for change in lung function. Most studies adjusted for age, sex, height, and weight, and some studies adjusted for baseline lung function (27,28,36–39), and altitude (16,32,34). However, there were different approaches and selection criteria for included confounders across the studies.

The quality score of each study is summarised in Table 2. The maximum possible score was 6. Ten studies had a total score of 5 or 6 and ten studies a score of 3 or 4.

Findings

The results of the 20 included studies are summarised in Table 4. The reported results were heterogenic. Most studies presented results of multivariable linear regression models. Overall 14 studies found an association between exposure and decline in lung function, either when comparing exposed subjects with controls (14,16,30,35,36,41) and/or as exposure-response relations within an exposed population (14–16,25,27–29,32,35,37,40,41).

Of the three studies on cotton workers, one (14) found that cotton workers had a significant greater decline in FEV_1 of -360 mL compared with silk workers over 20 years of follow-up (corresponding to an additional decline of -18 mL/year). In this study they also found an exposure-response relation between endotoxin level and loss in FEV_1 (highest vs. lowest level: -155.3 mL over 20 years). An exposure-response relation was also seen in the cotton-study by Glindmeyer et al (25), where an increase of 100 $\mu g/m^3$ average cotton dust exposure led to a significant decline in both FEV_1 (-16.2 mL/year) and FVC (-18.0 mL/year) among yarn manufacturing workers. The last study (26) found no association between cotton dust exposure and change in lung function.

Among the 5 studies on grain workers an association was found between increasing time in the industry (years) and annual decline in lung function in three studies (FEV_1: -4 mL/year and FVC: -6 mL/year (15), FEV_1: -0.40 % predicted/year and FVC: -0.34 % predicted/year (27), and FEV_1: -0.6 mL/year per week in the industry (28)). One study (29) found no significant difference in annual decline in lung function between subjects with >5 years of exposure compared to no exposure or <5 years of exposure. This study, however, found a significant increased annual decline in FEV_1 among high grain dust exposed (-58.2 mL/year, excess decline of -22.2 mL/year) compared to low grain dust exposed (-35.8 mL/year). This association was not seen for endotoxin exposure. One study (30) found that grain farmers

had an excess annual decline in FVC of -9.2 mL/year compared to controls, although no significant exposure-response relation was observed between the rate of change in FVC per year and years of grain farming in this study.

Eight studies explored the effect of farming exposure on change in lung function. Three studies (16,35,36) showed a significant difference in lung function decline (FEV_1: -38 mL/year (35), FEV_1: -26.1 mL/year and FVC: -33.5 mL/year (36), and FEV_1/FVC: -0.21 %/year (16)) for farmers compared to controls. Two studies (31,32) found no significant difference for lung function change between farmers and controls. One study (33) looked at the difference in change in lung function between pig farmers and dairy farmers and found no significant difference. Another study (34) compared barn-drying farmers with traditional drying farmers and found no difference in change in lung function between the groups. Exposure-response relation was found in two studies (35,37), the first showing that total endotoxin (EU/m^3) was significantly associated with annual decline in FEV_1 (-26 mL/year), and the second showing that an increase in endotoxin exposure with a factor 2 was significantly associated with an extra decline in FEV_1 (-19.4 mL/year). FVC was significantly associated with a factor 2 increase in both endotoxin (-40.7 mL/year) and inhalable dust exposure (-41.2 mL/year). One study (31) found no relation between either dust or endotoxin exposure and change in lung function.

A study of paper workers (38) showed no significant excess loss of lung function among workers exposed to up to 200 EU/m^3 of endotoxin compared to controls.

Of the three studies on wood workers one (41) showed that sawmill workers had a significantly greater annual decline in FEV_1 (-12.1 mL/year) and FVC (-14.6 mL/year) compared with controls. They also found a significant exposure-response relation between wood dust and decline in FVC, where high exposed subjects had an additional decline of -21.3 mL/year, and medium exposed subjects -15.8 mL/year compared with the lowest exposed. Another study (39) showed no association between wood dust exposure and adverse effects on lung function. However, in a sub analyses they found an exposure-response relation between respirable residual particulate matter and decline in FEV_1 and FVC. The last study (40) found a significant exposure-response relation between cumulated wood dust exposure (mg/m^3/year) and decline in FEV_1 and FVC for female workers (FEV_1: -3.1 mL/mg/m^3/year, FVC: -3.0 mL/mg/m^3/year). This was not seen among males.

A few studies explored effect modification by sex and/or smoking. Two studies (31,40) explored effect modification by sex; one study on farmers (31) found that the negative effect on lung function from being a current farmer compared to an ex-farmer was larger for females than for males; the other study on wood workers (40) showed that female wood workers with the highest exposure had a significant excess decline in FEV_1 (-25 mL) compared to the lowest exposure, which was not seen for males. No studies found clear

indications of effect modification by smoking, but one study (36) found that among current smokers only, yearly loss in FEV_1 was significantly greater in swine confinement workers than in control subjects (-100 mL/year vs. -55 mL/year). Two studies (31,41) found no significant interaction between smoking and exposure on the decline in lung function.

When we restricted the analysis to the ten studies (14–16,26,27,31,35,39–41) with the highest quality scores (score 5-6) (Table 2) there were seven studies (14–16,27,35,40,41) that found a significant association between organic dust exposure and change in lung function and the remaining three studies (26,31,39) found no significant associations at all. All ten studies explored exposure-response relations and the same seven studies mentioned above found a significant exposure-response relation between measured exposure levels and decline in lung function. Six of these seven studies compared exposed subjects to controls with 4 reporting a significant difference between exposed and controls (14,16,35,41).

Out of the ten studies (25,28–30,32–34,36–38) with lower quality scores (score 3-4) (Table 2) there were seven (25,28–30,32,36,37) studies that found significant associations overall between organic dust exposure and change in lung function with the remaining three studies (33,34,38) finding no significant associations. Eight studies (25,28–30,32,34,37,38) had explored exposure-response relations, with exposure being estimated without actual dust measurements but based on time in the industry or time spend with different work tasks. Five (25,28,29,32,37) of these eight studies found a significant exposure-response relation. Two other studies (30,36) found a significant difference between exposed and controls.

DISCUSSION

This is, to our knowledge, the first systematic review on long-term change in lung function among populations exposed to organic dust. Overall we found heterogenous results. 14 studies (7 of higher quality and 7 of lower quality) out of 20 found a significant association between exposure to organic dust and change in one or more of the lung function indices (FEV_1, FVC, FEV_1/FVC). Exposure was assessed both as exposed versus controls and as exposure contrast within the exposed cohorts. The remaining six studies found no significant change in lung function in relation to exposure.

The studies with higher quality scores more often showed an exposure-response relation between levels of organic dust exposure and change in lung function than studies with lower quality scores. The higher quality studies showed effects both when comparing exposed with controls and when comparing levels of exposure. The lower quality studies either showed a significant difference between exposed and controls or an exposure-

response relation and the results were not consistent. However, in both the higher and the lower quality score publications there were three studies with no significant association between organic dust exposure and change in lung function.

For all types of exposure (cotton, grain, farming, and wood dust) inconsistent results were reported for the association between exposure and change in lung function. Therefore it is difficult to conclude if any specific exposure type was more evident to be associated with decline in lung function. However, grain dust exposure was the most consistent with all 5 studies finding an association, but not all studies were able to show a significant exposure-response relation. Furthermore, the results do not consistently point towards either endotoxin or dust *per se* as a risk factor for change in lung function. One study showed a significant association for dust but not endotoxin (29), whereas endotoxin in other studies was the significant risk factor for excess lung function decline (14,35).

The size of the effect of exposure to organic dust on change in lung function varied depending on the method of assesment, which makes it challenging to compare effect sizes across studies. The units of exposure-measure used in the studies differed for exposure-response relations between dust/endotoxin and change in lung function. One study reported change in lung function per increase of 100 µg/m^3 average cotton dust (FEV_1: -16.2 mL/year and FVC: -18 mL/year (25)). Another study reported change per factor 2 increase in exposure for both dust (FVC: -41.2 mL/year) and endotoxin (FEV_1: -19.4 mL/year and FVC: -40.7 mL/year (37)). The lung function change per 1 mg/m^3 accumulated increase in wood dust exposure (FEV_1: -3.1 mL/year and FVC: -3.0 mL/year) was reported in one study (40), another study (15) reported change per year in the industry (FEV_1: -4 mL/year and FVC: -6 mL/year) and one study (28) used change per week in the industry (FEV_1: -0.6 mL/week and FVC: -0.7 mL/week). One study compared cumulated high and low dust exposure (FVC: -21.3 mL/year (41)) and one compared cumulated high and low endotoxin exposure (FEV_1: -155.3 mL over 20 years (14)).

The excess FEV_1 decline for dust exposed subjects compared to controls ranged from -12.1 mL/year to -38 mL/year and the excess FVC decline ranged from -9.2 mL/year to -33.5 mL/year in the 6 studies with significant associations for this comparison. Only one study (16) put emphasis on a significant additional decline in FEV_1 as a percent of FVC of -0.21 %/year among dairy farmers compared to controls.

Several studies have explored associations between organic dust exposure and different elements of respiratory health. However, many studies did not show a quantitative estimate of the association between organic dust exposure and change in lung function, and therefore 6 studies (Figure 1) were excluded based on this inclusion criteria.

Strengths and limitations

The strengths of our review are that all 20 of the included studies were follow-up studies (min 1 year), with follow-up rates of >50% in 16 of these, and all had at least 50 exposed particpants. Reliable assessment of lung function decline may, however, require several years of observation to achieve robust estimates of effect, as argued by Burrows in a study on changes in the normal flow-volume curve with growth and aging (42) and by an official statement from the Amarican Thoractic Society on spirometry in the occupational setting (43). Therefore we also did an assessment of results restricted to the 10 studies with the longest follow-up time (>6.5 years) (14–16,27,30–33,38,41), but this restriction revealed similar inconsistent results of the association between exposure and change in lung function.

In this review each cohort is only included once with the paper closest to our interest, namely the greatest exposure contrast (years of exposure) and the largest number of participants. Thus, all cohorts in this review contribute equally to the results. Because of the known detremental effect of smoking on lung function all included studies had to take smoking into account and we believe that the thorough adjustment for smoking in all included papers makes it less likely that the results are biased by smoking. Other confounders considered were sex, height, age, altitude, weight or weight gain, different symptoms, follow-up time, and baseline lung function. The approach of confounder selection was heterogenic between studies. It is debated whether or not one should adjust for baseline lung function when assessing change in lung function over time. Adjusting for the baseline level of lung function is problematic because of correlated errors between lung function level and lung function change (44). The effects of these correlated errors on the regression coefficients may lead to bias due to the “horse-racing effect” (45,46) whereby loss is negatively associated with attained level, i.e. the lower one's function, the more one loses (44). Results adjusted for baseline lung function may therefore be biased, but as several studies chose this approach (27,28,36–39,41) we chose to include them. Studies with and without adjustment for baseline lung function did not differ systematically in terms of finding an association between organic dust exposure and change in lung function or the effect-size.

Limitations include the fact that the studies chose different appraches of analyses, focus on different lung function indices (FEV_1, FVC, FEV_1/FVC), and differed in how the change in lung function was assessed and how the exposure was assessed. Lung function change over time was in most studies presented as absolute change or, in some studies, as change relative to baseline. One study reported change in percent predicted (27). Only one study (31), from our group, used z-scores from the GLI2012 equations (47). Age is an important factor for change in lung function, as lung function decline above the age of 30 is evident (48,49) ,

moreover, the variability of individual measurements around the median is not uniform across all ages and heights (50). It has been argued that conventional multiple regression analysis is not adequate to model the complex relationship between body size, age and lung function (50). The GLI2012 equations account for the effect of aging, height, sex and ethnicity and should be the most precise way to asses lung function across all ages. A z-score approach may also be more meaningful when evaluating FEV_1 over time, as shown in a new study by Vas Fragoso et al. (51), as FEV_1 assessed as z-scores was shown to be more frequently associated with multiple cardiopulmonary predictors than measures of FEV_1 in liters, as % of predicted and as L/m^3.

The methods of exposure assessment also differed between studies. We would judge individual accumulated expsure as actual dust/endotoxin meassurements combined with number of exposed years to be the most precise and valid exposure metric. Exposure assessed from questionnaires giving amount of time spent with different tasks, or simply time employed in the industry is probably less precise.

One study (37) had selected participants for follow-up based on pulmonary symptoms, choosing 200 subjects with symptoms and 200 subjects without. This challenges our selection criteria not to look at prognostic studies of specific patient groups, but because they found no increased decline in lung function among subjects with symptoms compared to subjects without symptoms, and did exposure-response analyses on the full cohort from follow-up, we chose also to include this study.

Articles concerning repeated cohorts

Several cohorts were examined repeatedly during their follow-up time and therefore several articles were published concerning the same cohorts. The Shanghai textile worker study had 6 cycles with 5 year intervals during 1981 to 2011 leading to several results (52–59). They reported both no exposure-response relation but significant differences between cotton workers and silk workers (56), and significant exposure-response relation for both endotoxin level (14,54) as the risk factor for negative change in lung function and, at another follow-up time, dust level as a risk factor (53). The Shanghai textile worker study also studied lung function improvement after exposure cessation and found FEV_1 improvement after cessation of cotton and silk work (57,59) and identified prior occupational exposure levels and sex as important modifiers of FEV_1 recovery (59). The Shanghai study also explored genotypes associated with enzyme activity (60), and found that genotypes associated with "slow" enzyme activity lead to inefficient metabolizing of ROS (reactive oxygen species) generated by endotoxin exposure, and this may eventually induce faster lung function decline. These findings open doors for future research for understanding the biological mechanisms of this issue, but it is out of the scope for this review.

The farmers cohort from 1986 in the Doubs province, France, also reported results at different follow-up cycles. At 6 years of follow-up (61) lung function of farmers had deteriorated slightly more rapidly than that of the control subjects. This was not seen at 12 years of follow-up (32) where the association was non-significant when comparing farmers with controls, though the mean duration of exposure was significantly associated with the decline in FEV_1. Another cohort from 1994 of farmers in the Doubs province showed that dairy farming was associated with an accelerated decline in FEV_1/VC (vital capacity) per year but not VC/year or FEV_1/year after 5 years follow-up (62) compared with controls, and that the modernization of the farm had a beneficial impact on lung function. After 13 years of follow-up (16) a greater decline in FEV_1/FVC ratio remained for dairy farmers.

Grain workers in Saskatchewan, Canada, showed consistent results of annual loss of lung function to increase with number of years in the grain industry both at 6 years (63) and 15 years of follow-up (64), and also was able to measure a beneficial effect on lung function from implementing dust control (15,64).

Biologic plausibility and causality

A negative effect of organic dust on change in lung function is biologicaly plausible. After inhalation and deposition of organic dust and its constituents in the airways the particles may interact with immune cells. A strong inflammatory response may lead to lung function decline in exposed subjects. The individual immunological response to dust and endotoxins is determined by the complex interaction between dose and timing of exposure, other environmental factors, and genetic predisposition (65). The mechanisms that link the immunological response in the lung with possible accelerated loss of lung function are still not fully understood and may also involve factors related to genetics, immune regulation, and mechanisms related to cellular repair and the resolution of inflammation, as hypothesised in the review by Omland et al. (24).

The heterogeneity in exposure, outcome and analyses complicate the assessment of effect size. Though the studies are longitudinal, it cannot be certain that the outcome occurs after the exposure because the lung function decline could have occured in the beginning of the follow-up period, prior to the exposure often assessed at follow-up. One way to cope with this is to include repeated meassurements of lung function and exposure during the follow-up period. Several studies have repeated cycles, but analyses were generaly made at each follow-up time and not modeled for change in exposure and change in lung function during the full follow-up.

The mean age at baseline in the included studies ranged from 18 to 51 years. There were no obvious susceptible age group. One could hypothesize that the effect of the exposure may differ according to age of the subjects, but the results of this review do not support this.

Many longitudinal studies deal with the problem of the healthy workers selcetion bias. A study by Zejda et al (66) showed that the average decline in lung function over the first year with exposure to grain dust was associated with the subsequent exposure duration, i.e. a smaller decline in lung function the first year of exposure predicted a longer subsequent exposure duration. They concluded that the restriction of analyses to the "survivors" may underestimate the relation between the exposure and the respiratory impairment. This could be an evident problem in all the studies included in this review and may even suggest that the associations found are underestimated.

The strength of the associations found in most of the studies in this review lay close to the border of significance ($p<0.05$) and are based on quite large study samples. The size of the effect, however, may sum up to be of importance for the individual, as an excess decline in for example FEV_1 of -12 to -38 mL/year would lead to possible important health issues after many years of exposure. The results in this review are not consistent, but 14 out of 20 studies found results that point toward a negative effect of organic dust exposure on lung function change.

The findings of this review indicate that we must continue to focus on possible adverse health effects associated with occupational exposure to organic dust and to protect the individual working in occupations with high dust exposure. This can be done by securing acceptable levels of organic dust and endotoxin in the worksetting, improving the working environment in terms of better ventilation and cleaning systems, and recommend use of personal protective equipment such as respirators and masks in work tasks with high dust exposure.

Future directions within the field should be to continue with well powered follow-up studies, acquire precise exposure measures, and take healthy worker selection bias into consideration (i.e. follow up young cohorts and examine both active workers and drop-outs). Other exposures such as inorganic dust and vapor, gas, dust and fumes (VGDF) need to be evaluated on their own, as the pathophysiological mechanisms when exposed to these agents may differ from the mechanisms when exposed to organic dust and endotoxin.

CONCLUSION

The studies included in this review were of varying design, applied different measures of exposure and outcome, and were of different population size and area. Although the results were inconsistent they pointed toward a significant association between exposure to

organic dust and change in lung function. Exposure to grain dust showed the most consistent results of an association between grain dust exposure and decline in lung function. We conclude that there is some evidence of a causal association between exposure to organic dust and long-term excess decline in lung function.

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Contributorship statement

Anneli C. S. Bolund, corresponding author, has contributed to the literature search, the identification of relevant articles, full text-screening, drafting the article and final approval of the version to be published.

Vivi Schlünssen has contributed to the identification of relevant articles, drafting the article, revising it critically for important intellectual content, and final approval of the version to be published.

Torben Sigsgaard and Martin Miller have contributed to revising the article critically for important intellectual content, and final approval of the version to be published.

Anneli C. S. Bolund and Vivi Schlünssen are responsible for the overall content as guarantor(s).

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FIGURES AND TABLES for Paper I – The REVIEW

Table 1 Overview of full electronic search strategy

Overall term	Pubmed	Embase	Cochrane Library
Lung function	("lung function" OR "pulmonary function" OR "FEV1" OR "forced expiratory volume" OR "FVC" OR "forced vital capacity" OR "vital capacity" OR "spirometry" OR "spirometer" OR "bronchial provocation" OR "bronchial provocation test" OR "vitalograph")	Same as Pubmed	Same as Pubmed
Exposures	AND ("Endotoxin" OR "endotoxins" OR "dust" OR "dusts" OR "agriculture" OR "farmer" OR "farmers" OR "farms" OR "farming" OR "livestock" OR "organic dust" OR "organic dusts" OR "wood" OR "wood dust" OR "pine" OR "pine wood" OR "western red cedar" OR "beech" OR "beech wood" OR "birch" OR "birch wood" OR "oak" OR "oak wood" OR "waste loaders" OR "waste workers" OR "compost workers" OR "wheat" OR "wheat flour" OR "flour" OR "baker" OR "bakers" OR "grain" OR "grain dust" OR "microbial exposure" OR "microbial agents" OR "woodworker" OR "woodworkers" OR "millworkers" OR "mill workers" OR "sawmill" OR "sawmills")	Same as Pubmed	Same as Pubmed
Longitudinal	AND ("longitudinal" OR "follow-up" OR "follow up" OR "prospective" OR "cohort")	Same as Pubmed	Same as Pubmed
Filters	NOT "*searchword*"[Author] AND "humans"[MeSH Terms] AND English[lang]	NOT "*searchword*"[Author] AND 'human'/de	NOT "*searchword*"[Author]
Results	Only filter ´NOT "*searchword*"[Author]´: **742** records, With all filters: 636 records	No filter: **1112** records, With filter: 1004	**315** records

(Bold numbers illustrate the number of articles that were assessed in a common database)

Table 2 Quality scores of the included studies

Exposure, 1st author (year)	**Study design**	**Dimensions**	**Response rate**	**Exposure measure**	**Exposure-response relation**	**Confounder control**	**Total score**
Cotton workers							
Glindmeyer, Henry W., et al, (1991) (25)	1	1	0	1	1	0	4
Wang, Xiao-Rong, et al, (2003) (26)	1	0	1	1	1	1	5
Wang, Xiao-Rong, et al, (2005) (14)	1	1	1	1	1	1	6
Grain workers							
Huy, Thomas, et al (1991) (27)	1	1	1	1	1	0	5
Olfert, S. M., et al, (2005) (28)	1	1	0	0	1	1	4
Pahwa, Punam, et al (2008) (15)	1	1	1	0	1	1	5
Post, Wendel, et al, (1998) (29)	1	0	0	1	1	1	4
Senthilselvan, Ambikaipakan, et al, (2010) (30)	1	0	1	0	1	1	4
Farmers							
Bolund, ACS, et al (2015) (31)	1	1	1	1	1	1	6
Gainet, M., et al. (2007) (32)	1	0	1	0	1	1	4
Iversen, M., Dahl, R., (2000) (33)	1	0	1	0	0	1	3
Mauny, F. et al, (1997) (34)	1	0	1	0	1	0	3
Schwartz, David A., et al, (1995) (35)	1	0	1	1	1	1	5
Senthilselvan, Ambikaipakan, et al, (1997) (36)	1	0	1	0	0	1	3
Thaon, I, et al, (2011) (16)	1	1	1	0	1	1	5
Vogelzang, Peter F, et al (1998) (37)	1	0	1	1	1	0	4
Paper workers							
Sigsgaard, Torben, Et al., (2004) (38)	1	0	1	1	1	0	4
Wood workers							
Glindmeyer, Henry W., et al, (2008) (39)	1	1	0	1	1	1	5
Jacobsen, Gitte, et al 2008 (40)	1	1	1	1	1	1	6
Noertjojo, H. Kukuh, et al, (1996) (41)	1	1	1	1	1	1	6

Study design (longitudinal=1), Dimensions (>218 exposed subjects=1, <218 exposed subjects=0), Response rate (>50%=1, <50% or unknown=0), Exposure measure (Dust measurements=1, no dust measurements=0), Exposure-response relation (Analysed=1, Not analysed=0), and confounder control (All of smoking, sex, age and height=1, other=0)

Table 3 Characteristics of studies included in the review

Exposure, 1st author (year)	Design, follow-up time	Study population (Country), n, response rate, age-range	Organic dust exposure Measurement, (time of assessment)	Outcome: Lung function change Measurement	Covariates accounted for
Cotton workers					
Glindmeyer, Henry W., et al, (1991) (25)	Prospective cohort study, 5 years follow-up.	Cotton textile workers n=1353 and Synthetic textile workers n=464 with same work through follow-up, in total n=1817 (30%) (USA). Mean age at follow-up: 38.7.	Dust exposure measured as lint free respirable dust (1982-1987).	Spirometry: FEV_1, FVC, FEF_{25-75}.	Age, sex, smoking, employment duration, mill type.
Wang, Xiao-Rong, et al, (2003) (26) (Archives of Environmental Health)	Prospective cohort study, 1 year follow-up.	Newly hired textile workers (Only female non-smokers) n=136 (60%) (Shanghai, China), Mean age at baseline: 18.3 years.	Environmental airborne cotton dust and gram negative bacteria endotoxin in various work areas were measured using vertical elutriators. High- or low-level.	Spirometry. FEV_1, FVC.	Age, height, mill, atopic status, exposure level, respiratory symptoms, serum IgE concentrations. (Only female non-smokers).
Wang, Xiao-Rong, et al, (2005) (14)	Prospective cohort study, 20 years follow-up (5 year intervals).	Cotton workers n=346 (84%), Silk workers n=342 (77%). In total 559 workers (61%) participated in all five surveys (Shanghai, China) Mean age at baseline: 36.1 years.	Area samples of dust and endotoxin. High/Low exposure and accumulative exposure.	Spirometry. FEV_1, FVC.	Age, height, sex, smoking, and exposure.
Grain workers					
Huy, Thomas, et al (1991)(27)	Prospective cohort study, 6-12 years follow-up.	Grain workers (Canada) n= 384 (>85%), controls n=52 (>85%), Mean age at follow-up: 42.5 years.	Personal air sampling.	Spirometry. FEV_1, FVC, FEV_1/FVC, Mid-maximal exp. flow rate (MMF). Change in percent predicted.	Age, height, smoking, average lung function.

Olfert, S. M., et al, (2005) (28)	Prospective cohort study, 5 years follow-up.	Newly employed young male grain workers n=299 (% unknown), Mean age at baseline: 20.3 years. (Saskatchewan, Canada).	No measurements. Number of exposure weeks.	Spirometry. FEV_1, FVC.	Age, height, weight, smoking status, previous pulmonary function test value, number of exposure weeks. (Only male).
Pahwa, Punam, et al (2008) (15)	Prospective cohort study, 15 years follow-up (Cycles of 3 years).	Grain workers n=11827 (with repeated measurements) (79%). Mean age at baseline: 33.3 years (Saskatchewan, Canada).	No measurements. Number of exposure years in three categories.	Spirometry. FEV_1, FVC.	Age, height, weight, smoking status, and cycle effect. (Only male).
Post, Wendel, et al, (1998)(29)	Prospective cohort study, 5 years follow-up.	Male grain processing and animal feed workers n=144 (45%) (The Netherlands), Mean age at baseline: 37.7 years.	Personal inspirable dust samples (1986-1988 and less intensively in 1990-1992).	Spirometry. FEV_1, FVC, PEF, MMEF, MEF_{25-75}.	Age, height, and smoking. (Only male).
Senthilselvan, Ambikaipakan, et al, (2010)(30)	Prospective cohort study, 13 years follow-up.	Male grain farmers n=159 (60.5%) Male controls n=119 (45.6%), (Canada), Mean age at baseline: 42.6 years.	No measurements. Years of grain farming.	Spirometry. FEV_1, FVC, FEV_1/FVC, FEF_{25-75}.	Age, height, weight, and smoking. (Only male).
Farmers					
Bolund, ACS, et al (2015)(31)	Prospective cohort study, 15 years follow-up.	Young farmers: n=962, Controls: n=172 (Denmark), 51,6%, Median age at baseline: 18.6 years (16-25).	Personal exposure samples (2008-2009).	Spirometry FEV_1, FVC, z-scores calculated from GLI2012 taking sex, age, height and ethnicity into consideration.	Smoking, second-hand smoking, being raised on a farm, BHR, BMI, sex (age and height in GLI2012 equations).
Gainet, M., et al. (2007)(32)	Prospective cohort study, 12 years follow-up.	Dairy farmers n=157 (62.8%), controls n=159 (63.6%) (Doubs province, France) Mean age at baseline: 50.8 years.	Questionnaire based exposure indicators of size of farm and handling of fodder.	Spirometry: VC, FEV_1, FEV_1/VC, FEF_{25-75}, PEF.	Age, sex, height, smoking, altitude.

Iversen, M., Dahl, R., (2000)(33)	Prospective cohort study, 7 years follow-up.	Farmers in total n=132 (73 %) (Pig farmers n=94, dairy farmers n=38) (Denmark) Mean age at follow-up: 43.3 years.	No sampling. Exposure evaluated by hours of work in stable.	Spirometry FEV_1, FVC, absolute and relative change.	Age, height, smoking. (Only male).
Mauny, F. et al, (1997)(34)	Prospective cohort study, 5 years follow-up.	Barn-drying farmers n=113 (92%) Traditional drying farmers n=231 (84%) (Doubs province, France) Mean age at baseline: 48.1 years.	Cumulated exposure was evaluated by bale-years.	Spirometry. FEV_1, VC, FEF_{25-75}.	Age, smoking status, altitude and cumulative exposure. (Only male).
Schwartz, David A., et al, (1995)(35)	Prospective cohort study, 2 years follow-up.	Swine farmers n=168 (81%) Controls n=127 (80%) (Iowa, USA) Mean age at baseline: 38.4 years.	Total dust samples were collected over the work-shift in the breathing zone of each subject.	Spirometry. FEV_1, FVC, FEF_{25-75}.	Age, height, sex, smoking, follow-up time.
Senthilselvan, Ambikaipakan, et al, (1997)(36)	Prospective cohort study, 5 years follow-up.	Swine confinement workers n=217 (71.9%), Controls n=171 (65.5%), (Canada), Mean age at baseline: 40.2 years.	No measurements. Farmers vs controls.	Spirometry. FEV_1, FVC, FEV_1/FVC, FEF_{25-75}.	Age, height, smoking, and baseline FEV_1. (Only male).
Thaon, I, et al, (2011)(16)	Prospective cohort study, 13 years follow-up.	Dairy farmers n=219 (82.6%) Non-dairy agricultural workers n=130 (62.5%) Controls n=99 (66.4%), (Doubs province, France), Mean age at baseline: 41.8 years.	Handling hay or straw vs. Handling animal feed (grain).	Spirometry. FEV_1, FVC, FEF_{25-75}, PEF.	Smoking, age, height, sex and altitude.
Vogelzang, Peter F, et al (1998)(37)	Prospective cohort study, 3 years follow-up.	Pig farmers n=171 (86.4%) (The Netherlands), Mean age at baseline: 39.6 years.	Long-term average exposure to dust and endotoxin was determined by personal monitoring in summer and winter 1991-1992.	Spirometry. FEV_1, FVC.	Age, baseline FEV_1 or FVC and smoking. (Only male).
Paper workers					
Sigsgaard, Torben, Et al., (2004)(38)	Prospective cohort study, 11 years follow-up.	Male paper workers n=97 (65%) Controls n=55 (51%), (Denmark), Mean age at baseline: 40.2 years.	Personal samples included total dust, endotoxins and microorganisms.	Spirometry. FEV_1, FVC.	Age, FEV_1 at baseline, smoking and cough. (Only male).

Wood workers					
Glindmeyer, Henry W., et al, (2008)(39)	Prospective cohort study, 5 years follow-up.	Wood processing workers n=1164 with follow-up lung function (37%). No controls. (USA), Mean age at baseline: 40.4 years.	Size-fractionated dust exposures were determined by personal monitoring.	Spirometry: FEV_1, FVC, FEF_{25-75}, FEV_1/FVC.	Age, baseline pulmonary function, sex, percent of body-weight change, smoking amount, wood type, height and height squared.
Jacobsen, Gitte, et al 2008(40)	Prospective cohort study, 6 years follow-up.	Wood workers n=1112 (61%), controls n=235 (57%) (Denmark), Mean age at baseline: 38.6 years.	Personal dust sampling with passive dust samplers at baseline and follow-up.	Spirometry FEV_1, FVC, absolute and relative change.	Smoking, sex, age, height, weight-gain.
Noertjojo, H. Kukuh, et al, (1996)(41)	Prospective cohort study, 11 years follow-up.	Male sawmill workers n=243 (80% of invited), Male controls n=140 (70% of invited) with follow-up. (Vancouver, Canada) Mean age at baseline: 46.1 years.	Cumulative wood dust exposure calculated based on personal and area samples of dust (12 year period). Low, medium, high exposure.	Spirometry. FEV_1, FVC.	Age, race, height, smoking, and initial lung function. (Only male).

Table 4 **The effect of exposure to organic dust on the change in lung function over time, study results.**

W: worker, C: control, ER: Exposure-Response for dust/endotoxin level or exposure-time, -: No association, +: Significant association

Exposure, 1st author (year)	Exposure	Results, Adjusted Exposed vs. Controls	Results, adjusted Male and female	Results, adjusted, Non-smoking and Smoking	Exposure response	Main findings	Results summarised
Cotton workers							
Glindmeyer, Henry W., et al, (1991) (25)	Cotton textile workers and synthetic textile workers. Cotton split in yarn manufacturing (YM) and slashing and weaving (S/W), no controls.	Multivariable linear regression showed steeper annual decline of FEV_1: coeff. ± SEM -13.14 ± 3.13 mL/year ($p<0.01$) in synthetic workers vs. cotton workers (opposite of expected). FVC: coeff. ± SEM -6.45 ± 3.72 mL/year (NS).	N/A	Stratified analyses, Current smokers: YM vs. S/W FEV_1: coeff. ± SEM -23.10 ± 4.92, FVC: -19.16 ± 6.06. Never smokers: YM vs. S/W FEV_1: -3.17 ± 4.75, FVC: 1.55 ± 5.84, unadjusted.	Multivariable linear regressions in YM showed exposure-response relationship: FEV_1: coeff. ± SEM: -16.2 mL/year ± 3.27 ($p<0.01$) and FVC: -18.0 mL/year ± 3.94 ($p<0.01$) per 100microg/m3 average cotton dust exposure.	Despite lower overall dust exposures, cotton yarn manufacturing workers exhibited steeper annual declines in lung function than did workers in slashing and weaving, indicating a difference in dust potency.	+ ER for dust on FEV_1 and FVC
Wang, Xiao-Rong, et al, (2003) (26)	Newly hired cotton workers, no controls.	N/A	N/A (Only female).	N/A - All non-smokers.	Level of exposure to endotoxin was not associated to change in FEV_1 and FVC, but dust was marginally significant in the model for estimating change in FVC (coeff. ± SE: -103.8 mL/year ± 58.8, $p=0.08$).	Exposure to cotton dust was not associated with longitudinal changes in lung function after 1 year follow-up.	- ER
Wang, Xiao-Rong, et al, (2005) (14)	Cotton workers/silk workers.	Cotton workers had greater decline in FEV_1 compared with silk workers over 20 years follow-up: coeff. ± SE: -359.7 mL ± 175.7 ($p<0.01$).	N/A	N/A	Greater loss in FEV_1 over 20 years of follow-up with increasing levels of exposure to endotoxin: highest vs. lowest level: coeff. ± SE: -155.3 mL ± 76.8,$p<0.05$.	Cotton work and increasing levels of endotoxin exposure was associated with greater decline in FEV_1.	+ ER for endotoxin on FEV_1

Grain workers							
Huy, Thomas, et al (1991) (27)	Grain elevator workers/Controls.	Controls showed annual changes in FEV_1 (-21.1 mL/year (SEM ± 5.4)) and FVC (-24.7 mL/year (SEM ± 6.4)) comparable to grain workers in the intermediate (4-9 mg/m3) grain dust exposed group: FEV_1 (-20.7 mL/year (SEM ± 2.3)) and FVC (-20.8 mL/year (SEM ± 2.8)) NS.	N/A	N/A	Grain dust exposure (mg/m3) was associated with a decline in FEV_1 %pred: coeff. ± SEM: -0.89 ± 0.39, $p<0.05$, FVC %pred: -0.73 ± 0.35, $p<0.05$. Also duration of exposure (years in grain industry) was associated with FEV_1 %pred: -0.40 ± 0.08, $p<0.01$, FVC %pred: -0.34 0.07, $p<0.01$.	Both level and duration of dust exposure were significantly associated with reductions in lung function among grain elevator workers, but no significant difference was seen in lung function change between grain elevator workers and controls.	- W vs. C + ER for dust on FEV_1
Olfert, S. M., et al, (2005) (28)	Newly employed young grain workers (NGWS).	N/A	N/A (only male).	Annual decline for FVC: non-smoking: coeff. ± SD: -73.82 mL/year ± 202.27, ever-smoking: -34.87 mL/year ± 252.83. FEV_1: non-smoking: -87.41 mL/year ± 169.71, ever-smoking: -68.60 mL/year ± 181.90.	Exposure weeks were predictive for decline in FEV_1 (L): coeff. (95%CI) -0.0006 (-0.0008 to -0.0003) and FVC (L): -0.0007 (-0.0010 to -0.0003) for the NGWS workers.	Young, non-smoking grain workers had the greatest predicted annual decline in lung function, larger decline with increasing exposure weeks.	+ ER for exp-weeks on FEV_1 and FVC
Pahwa, Punam, et al (2008) (15)	Grain workers. No controls.	N/A	N/A (only male).	N/A	Years in grain industry was associated with annual decline in FEV_1 (L) (-0.004 ± SE 0.0002), $p<0.0001$) and FVC (L) (-0.006 ± SE 0.0003, $p<0.0001$).	Decline in lung function was associated with years in the grain industry. Grain dust control was effective in reducing this decline.	+ ER for exp-years on FEV_1 and FVC

Post, Wendel, et al, (1998) (29)	Grain processing and animal feed workers.	No significant difference in annual decline in lung function between >5 years of exposure compared to no exposure or <5 year (no size of estimate).	N/A	N/A	Annual decline in FEV_1 (mL): high dust exposure: -58.2 mL vs. low dust exposure: -35.8 mL, ($p<0.05$), high endotoxin exposure: -59.0 mL vs. low endotoxin exposure: -36.8 mL, ($p<0.10$, NS).	Higher exposure to dust resulted in higher declines in FEV_1.	+ ER for dust on FEV_1
Senthilselvan, Ambikaipakan, et al, (2010) (30)	Grain farmers/Controls.	Grain farmers had an excess annual decline in FVC of -9.2 mL/year (95% CI: -2.7 to -15.8, p = 0.006) compared to controls.	N/A	N/A	No significant exposure-response relationship was observed between the rate of change in FEV_1 or FVC per year and years of grain farming (no size of estimate).	Excess decline of -9.2 mL/year in FVC in grain farmers compared to controls.	+ W vs. C for FVC - ER
Farmers							
Bolund, ACS, et al (2015) (31)	Farmer/Control. Farmer status - never, ex, current. Dust and endotoxin accumulated during the follow-up period.	Multivariable linear regression: $\Delta zFEV_1$: control ref., ex-farmer: 0.05 (-0.07 to 0.16) NS, current farmer: -0.07 (-0.19 to 0.05) NS.	Female current farmers had a greater negative effect on lung function than male current farmers.	Among farmers, smoking had a greater deleterious effect on $\Delta zFEV_1$: Female: smokers: -0.09 vs. non-smokers: 0.30, $p<0.015$, male: smokers: 0.02 vs. non-smokers: 0.22, $p<0.001$, unadjusted, compared to controls.	No exposure-response found for neither dust ($\Delta zFEV_1$: 4^{th} quart vs. 1^{st} quart: -0.02, p=0.733) nor endotoxin ($\Delta zFEV_1$: 4^{th} quart vs. 1^{st} quart: -0.03, p=0.662) among farmers.	No differences in lung function Δz-scores between farmers and controls.	- W vs. C - ER

Gainet, M., et al. (2007) (32)	Farmers/Controls.	Multivariable linear regression: Farmers vs. controls: annual change FEV_1: coeff. ± SEM: -1.75 mL ± 4.08 (NS), FVC: 2.92 mL ± 5.02 (NS).	N/A	N/A	The mean duration of exposure was significantly associated with the decline in FEV_1 (Coeff. -0.16, $p<0.05$).	No difference seen in lung function change between farmers and controls, but an exposure-response relation was seen between work duration and FEV_1 decline.	- W vs. C + ER for duration on FEV_1
Iversen, M., Dahl, R., (2000) (33)	Pig farmers/Dairy farmers.	Multivariable linear regression: decline in FEV_1 for pig farmers vs. dairy farmers ± SE: -11.0 mL/year ± 6.33 ($p=0.084$, NS).	N/A (Only male).	Multivariable regression: decline in FEV_1 for non-smoking pig farmers vs. dairy farmers: -15.2 mL/year ($p=0.036$).	N/A	Non-smoking pig farmers experience an increased decline in FEV_1 compared to non-smoking dairy farmers, who are comparable to the general population.	- W vs. C
Mauny, F. et al, (1997) (34)	Barn-drying farmers (BD)/Traditional drying farmers (TD).	Decline in lung function was not significantly different between BD farmers and TD farmers for VC (mean±SD): -25.5 mL/year ± 97.6 vs. -8.4 mL/year ± 101.8, ($p=0.27$) or for FEV_1: -36.3 mL/year ± 67.7 vs. -35.5 mL/year ± 80.0 ($p=0.50$).	N/A (Only male).	N/A	Cumulated exposure (bale-years) was not correlated with the changes in lung function, VC: coeff. ± SE: 6.4 mL ± 6.9, NS. FEV_1: 2.09 mL ± 5.5, NS.	The mode of fodder drying does not significantly influence the decline in lung function.	- W vs. C - ER
Schwartz, David A., et al, (1995) (35)	Swine confinement workers and neighbourhood control farmers.	Swine confinement work was associated with decline in FEV_1 (coeff. ± SE: -0.19 L over 5 years ± 0.06, $p<0.01$) compared to control farmers.	N/A	N/A	Decline in FEV_1 (mL) was associated with total endotoxin (EU/m^3) (coeff. ± SE: -0.13 ± 0.04, $p<0.01$).	Positive association between the concentration of endotoxin and accelerated lung function decline.	+ W vs. C for FEV_1 + ER for endotoxin on FEV_1

Senthilselvan, Ambikaipakan, et al, (1997) (36)	Swine farmers/Controls.	Swine confinement workers had excess annual decline of -26.1 mL in FEV_1 (p=0.0005) and -33.5 mL in FVC (p=0.0002) compared to controls.	N/A (Only male).	Among current smokers yearly losses in FEV_1 were greater in swine confinement workers than in control subjects (-100 mL/year vs. -55 mL/year, p<0.05), adjusted for age only.	N/A	Accelerated yearly loss in lung function seen among swine confinement workers compared to controls.	+ W vs. C for FEV_1 and FVC
Thaon, I, et al, (2011) (16)	Dairy farmers/non-dairy agricultural workers/controls.	Dairy farmers had no increased loss in FEV_1 or FVC compared to controls (p>0.10), however greater decline in FEV_1/FVC ratio (coeff. ± SD: -0.21 %/year ± 0.08, p=0.01) was seen for dairy farmers vs. controls.	N/A	N/A	An increased decline in FEV_1 for all agricultural workers was associated with years of exposure with animal feed handling (Coeff. ± SD: -0.71 ± 0.32, p=0.03).	Years of exposure with animal feed handling was associated with decline in FEV_1.	- W vs. C, + ER for yrs with animal feed on FEV_1
Vogelzang, Peter F, et al (1998) (37)	Pig farmers. No controls.	N/A	N/A (Only male).	N/A	An increase in endotoxin exposure with a factor 2 was associated with an extra decline of FEV_1: Coeff. ± SE: -19.4 mL/year ± 10.9, p=0.04. FVC was associated with both endotoxin exposure (-40.7 mL/year ± 14.2, p=0.002) and inhalable dust (-41.2 mL/year ± 21.0, p=0.03).	Long-term average exposure to endotoxin was found to be associated with decline of FEV_1 and FVC, whereas exposure to dust was associated with decline of FVC alone.	+ ER for endotoxin on FEV_1 and FVC

Paper workers							
Sigsgaard, Torben, Et al., (2004) (38)	Paper workers/Controls.	The lung function decline among the controls was comparable to that of the exposed (FEV_1: non-smokers -37 mL/year, smokers: -51 mL/year, unadjusted).	N/A – (Only male).	For both smokers and non-smokers no increase was found in lung function decrements with increasing exposure.	Decline in FEV_1 was not associated with endotoxin exposure (no size of estimate), however an increase in FVC was seen with increasing endotoxin exposure (high exposure: 23.4 mL/year ± 8.9, p=0.009). (Opposite of expected)	No significant excess loss of lung function was seen among workers exposed to paper dust.	- W vs. C - ER
Wood workers							
Glindmeyer, Henry W., et al, (2008) (39)	Wood processing workers, No controls. Wood solids (WS) and Respirable residual particulate matter (RRPM).	N/A	N/A	N/A	Exposure to wood solids was not associated with significant adverse effects on lung function (no size of estimate).	Exposure to wood solids was not associated with change in lung function. RRPM was associated with a negative effect in the milling facility FEV_1: -32 mL/year ($p<0.05$).	- ER
Jacobsen, Gitte, et al 2008 (40)	Woodworkers/ controls.	Only female woodworkers had a larger decline in FEV_1 than controls (coeff. ± SE: -10.6 mL/year ± 4.97, p=0.03). This was not seen among men.	Linear regression: Female wood workers with highest exposure vs. lowest exposure FEV_1 coeff. ± SE: -24.97 ± 9.59 (p=0.01)	Significantly greater decline in FEV_1 was seen for smoking female woodworkers (coeff. ± SEM: -37.0 mL/year, ± 45.3) compared with smoking reference workers (-23.5 mL/year ± 36.9) ($p<0.05$), unadj.	An exposure–response relationship between cumulative wood dust exposure (mg/m^3) and annual decline in FEV_1 and FVC was suggested for female workers FEV_1: -3.1 mL/year (p=0.01) FVC: -3.0 mL/year (p=0.02).	Females, but not males, have an accelerated decline in lung function in a cohort exposed to relatively low concentrations of wood dust.	+ W vs. C, + ER for dust on FEV_1 and FVC for ♀

Noertjojo, H. Kukuh, et al, (1996) (41)	Western red cedar sawmill workers/Controls.	Sawmill workers had a significantly greater annual decline in FEV_1 (-12.1 mL/year, p=0.01) and FVC (-14.6 mL/year, p<0.05) compared with the control subjects.	N/A	No interaction between smoking and exposure on the decline in FEV_1 and FVC was found.	An exposure-response relationship was observed between wood dust exposure and annual decline in FVC: high exposure: coeff. ± SE: -21.3 mL/year ± 10.3, p<0.05. Annual decline in FEV_1 was significant only for medium wood dust: -16.9 ± 6.0, p<0.05.	Sawmill workers had a greater decline in FEV_1 and FVC than office workers.	+ W vs. C for FEV_1 and FVC + ER for dust on FVC

Supplementary material for Paper I – The REVIEW

Supplementary Figure S1

Example of full search strategy from NCBI Pubmed search:

("Lung function" OR "pulmonary function" OR "FEV1" OR "forced expiratory volume" OR "FVC" OR "forced vital capacity" OR "vital capacity" OR "spirometry" OR "spirometer" OR "bronchial provocation" OR "bronchial provocation test" OR "vitalograph")

AND ("Endotoxin" OR "endotoxins" OR "dust" OR "dusts" OR "agriculture" OR "farmer" OR "farmers" OR "farms" OR "farming" OR "livestock" OR "organic dust" OR "organic dusts" OR "wood" OR "wood dust" OR "pine" OR "pine wood" OR "western red cedar" OR "beech" OR "beech wood" OR "birch" OR "birch wood" OR "oak" OR "oak wood" OR "waste loaders" OR "waste workers" OR "compost workers" OR "wheat" OR "wheat flour" OR "flour" OR "baker" OR "bakers" OR "grain" OR "grain dust" OR "microbial exposure" OR "microbial agents" OR "woodworker" OR "woodworkers" OR "millworkers" OR "mill workers" OR "sawmill" OR "sawmills")

AND ("longitudinal" OR "follow-up" OR "follow up" OR "prospective" OR "cohort") = 807 records

To avoid catching author names (i.e. Baker, Wood etc.):

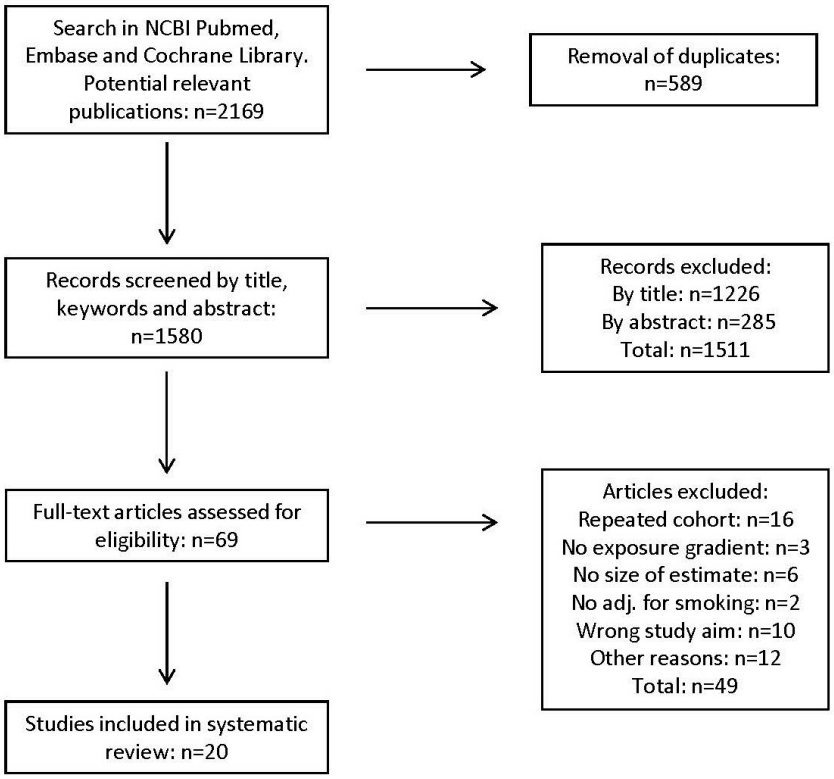
NOT "searchword"[Author] = 807 – 65 = **742 records**

AND "humans"[MeSH Terms] AND English[lang] = 742 – 105 = 637 records.

Because MESH Terms are not assigned immediately for each article, some of the newest articles are lost in this search. The final search included was therefore the above resulting in **742 records** in Pubmed.

Supplementary Figure S2

Flow diagram of study selection for the systematic review



Appendix II

Paper II: The SUS-study

The effect of occupational farming on lung function development in young adults: a 15-year follow-up study. *Occup Environ Med 2015;72:707–713.*

Bolund ACS, Miller MR, Basinas I, Elholm G, Omland Ø, Sigsgaard T, Schlünssen V.

ORIGINAL ARTICLE

The effect of occupational farming on lung function development in young adults: a 15-year follow-up study

Anneli C S Bolund,[1] Martin R Miller,[2] Ioannis Basinas,[1] Grethe Elholm,[1] Øyvind Omland,[3] Torben Sigsgaard,[1] Vivi Schlünssen[1]

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[1]Department of Public Health, Section of Environment Occupation and Health, Danish Ramazzini Centre, University of Aarhus, Aarhus C, Denmark
[2]Institute of Occupational and Environmental Medicine, University of Birmingham, Birmingham, West Midlands, UK
[3]Department of Occupational Medicine, Danish Ramazzini Centre, Aalborg University Hospital, Aalborg, Denmark

Correspondence to
Dr Anneli Clea Skjelmose Bolund, Department of Public Health, Section of Environment Occupation & Health, Danish Ramazzini Centre, University of Aarhus, Bartholins Allé 2, Aarhus C DK-8000, Denmark; abol@ph.au.dk

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ABSTRACT

Objectives Longitudinal studies on the effect of farming on lung function in young participants are few. Our objective was to explore if exposure to farming impaired lung function in young adults.

Methods We studied 1964 farming students and 407 controls in 1992/2004, and carried out follow-up in 2007/2008. Spirometry, skin prick test and bronchial hyper-responsiveness (BHR) were assessed, height and weight measured, and questionnaires covering health and occupation were collected. Cumulative dust and endotoxin exposures were estimated from modelled personal dust measurements. Lung function effect was expressed as change in z-score during follow-up using the Global Lung Initiative 2012 project prediction equations. Longitudinal data were available for 1134 young participants ≤25 years at baseline.

Results We found no differences in lung function Δz-scores between farmers and controls, however, adjusted multivariable linear regression showed a negative effect among current farmers on ΔzFEV1 (forced expiratory volume in 1 s; −0.12, p=0.006) and ΔzFEV1/FVC (forced vital capacity; −0.15, p=0.009) compared to ex-farmers. An interaction was found between sex and farming, showing that current farming suppresses ΔzFEV1 and ΔzFVC more among females. Smoking in farmers had a deleterious effect on ΔzFEV1, which was not seen in controls, though no significant interaction was found. Farm upbringing protected against impairment of lung function, and BHR at baseline had a deleterious effect on ΔzFEV1 only in those not raised on a farm.

Conclusions We conclude that being a current farmer is associated with a negative effect on lung function, when compared to ex-farmers, with females being more susceptible. Being raised on a farm protects against the adverse effect of BHR on change in lung function.

What this paper adds

- Exposure to organic dust in farming has been shown to have a negative effect on lung function among older adults, especially among pig and dairy farmers.
- The effect on the lungs of young adult farmers may be different as their lungs have not yet been fully developed, but only a few longitudinal studies have looked at this.
- We found no differences in lung function Δz-scores between farmers and controls; however, being a current farmer was associated with a negative effect on lung function compared to ex-farmers, with females being more susceptible.
- Smoking was associated with a negative effect on lung function, and being raised on a farm was protective against the adverse effect of BHR on change in lung function.
- Preventive measures to reduce occupational exposures from farming should include those of a young age, and advice against smoking should be given at every opportunity.

INTRODUCTION

Exposure to organic dust in farming has a negative effect on lung function, especially among pig and dairy farmers.[1–5] The effect on the lungs of young adult farmers may be different as their lungs have not yet been fully developed. Lung function keeps increasing continuously until the mid-20s, and then plateaus before starting to decline from about 30 to 35 years of age.[6] To compare a participant's lung function against reference equations from late teens to adulthood has previously been difficult because of the need to use separate equations for individuals up to their late teens, and for adults aged from 18 years onwards. Recently, the Lambda-Mu-Sigma equations[7] from the Global Lung Initiative (GLI-2012)[8] project have become available. With one set of equations, they cover lung function from the age of 3 to 95 years in the general population. This permits lung function comparisons in population terms (as z-scores), making it possible to assess lung function changes in young adults in a consistent way without using discordant equations.

Prospective studies of the specific effect of farming exposure on lung function among young adults have been few and sparse in terms of population size and time of follow-up. Prior *et al*[9] showed a slight overall decrease in FVC over a 4-year follow-up period in a group of 42 young farmers. A larger population-based study showed that after 9 years of follow-up, young individuals exposed to dusts, gases and fumes in different occupations (including farming) did not have a steeper overall decline in lung function than individuals without occupational exposure.[10] We have applied the data from the large Danish SUS cohort[11] (Sund Stald/

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Healthy Farm Project) of young farming students in a 15-year follow-up study to explore the effect of farming exposure on lung function at a young age.

METHODS

Study population

The SUS cohort was established in 1992 with the intention to study the health effects of exposure to farming. The original cross-sectional study consisted of 2478 farming students identified during their second stay at the farming schools in Denmark from February 1992 to February 1994. Of 2004 students (81%) who indicated that they wished to participate, 40 (2%) did not attend for their baseline clinical assessment. The final group consisted of 1734 male and 230 female farming students. Male army draftees examined prior to decision of recruitment were invited to participate as controls, provided they came from rural areas and had no intention of becoming farmers. There were 967 eligible army draftees of whom 592 (61%) agreed to participate. A random sample of 407 of these male recruits was chosen and used as controls in the study.

The cohort consisted of young adults with a median (IQR) age of 18.7 years (18.2–19.5). In 2007 and 2008, we performed a large follow-up of the cohort with the participation of 51.7% of the 2262 cohort members who could be identified.[11]

This paper considers the effect of exposure to occupational farming on lung function during young adulthood. We therefore excluded all participants above 25 years of age at baseline (male farmers n=15, female farmers n=11). Additionally, 10 participants had missing spirometry measurements and were therefore excluded. The follow-up group (n=1134) comprised 866 male farmers, 96 female farmers and 172 male controls (see online supplementary figure S1).

The SUS study and the SUS follow-up study were both approved by the ethics committee of Aarhus County (AA-19912197 and AA-20070074, respectively) and the Danish Data Protection Agency. Informed written consent was obtained from all participants.

Questionnaires

Participants answered questionnaires based on the modified British Medical Research Council questionnaire[12] supplemented with questions from the European Community Respiratory Health Survey[13] at follow-up. The questionnaires dealt with general health issues, respiratory symptoms, asthma, allergy, smoking habits and occupational history. All participants with occupational farming experience filled in a farming-specific occupational questionnaire covering the type and level of farming exposure for all employment since age 15 years.

Measurements

Lung function was assessed at baseline and follow-up by measuring forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC) and FEV1/FVC, according to guidelines of the American Thoracic Society/European Respiratory Society,[14] using a dry wedge spirometer (S-type spirometer number 20.600, Vitalograph, Buckingham, UK) with a maximum volume of 7.7 L. The GLI-2012 equations[8] were applied to calculate the main outcome measures as change in z-scores (Δz-scores) during follow-up for the three lung function indices taking age, height, sex and ethnicity into consideration. The study lacks female controls; however, z-scores have been calculated comparing female farmers with women in a reference population (GLI2012), thereby taking sex into account before comparing z-scores between farmers (female and male) and controls (male).

Height was measured at baseline and follow-up, and weight at follow-up, from which body mass index (BMI) was calculated. Bronchial hyper-responsiveness (BHR), expressed as PD20 for FEV1, was assessed with histamine at baseline and methacholine at follow-up using a modified[15] Yan method.[16] Studies have shown that these agents are comparable.[17] At baseline and follow-up, skin prick testing was performed as previously described.[15] Positive atopic status was defined as at least one positive reaction of ≥3 mm in mean diameter to any of 10 common aero-allergens,[11] provided a negative control.

Exposure assignment

The cumulative exposures to dust and endotoxin during follow-up were estimated for every participant based on personal exposure measurements (n=507 among 327 farmers) performed in 2008–2009.[18] Mean time-weighted average (TWA, 8 h) dust and endotoxin concentrations for pig, cattle and field farming, were estimated. For every participant, the total number of work years in swine, cattle and field farming, standardised to a 40 h work week, were calculated from the occupational questionnaires. Cumulative exposures were calculated as the sum of the products of the TWA dust and endotoxin concentrations and the total work years during follow-up for each type of work.

Smoking was expressed as pack-years (equal to 20 cigarettes/day years) during the follow-up period. Participants were divided into never-smokers, ex-smokers and current smokers at follow-up. Smokers who quit smoking less than 2 years before follow-up were defined as current smokers. Current smokers were split into low (0–10 pack-years) and high (10–59 pack-years) accumulated smoking exposure during the follow-up period.

Data analyses

Statistical analysis was carried out using STATA V.12.1. For normally distributed data, mean±SD was reported, and for non-normally distributed data, median (minimum–maximum or IQR) was reported.

Baseline z-scores (zFEV1, zFVC and zFEV1/FVC) and height were normally distributed, and comparisons between participants and non-participants were made using a Students t test. The continuous variables for absolute lung function values and changes in z-scores during follow-up were normally distributed, and here comparisons between farmers and controls were made using analysis of variance. Mann-Whitney within-group comparison was used to compare changes in z-scores between smokers and non-smokers. For the categorical variables 'Smoker', 'Raised on a farm', 'Asthma', and 'BHR', χ^2 tests were used to compare farmers and controls. Spearman correlation coefficients were estimated to examine the relationship of exposure between dust and endotoxin.

Possible confounders considered for multivariable linear regression analysis were sex, side job with exposure to high dust, other dust exposures, lung disease, BMI, smoking, farm upbringing, urban upbringing, bronchitis, BHR, atopy, self-reported asthma and second-hand smoking (smokers in childhood home). The final models included a priori selected confounders with plausible effect on lung function change. First-order interactions between exposures and confounders were explored. The level of significance was set at $p<0.05$.

RESULTS

Comparing baseline characteristics of participants and non-participants aged <25 years at baseline showed few differences

(see online supplementary table S1). The participation rate at follow-up was 51.7%. Those failing to participate in the follow-up were more likely to have been smokers at baseline. Participating male farmers were more likely to have been raised on a farm, had less asthma at baseline, and had significantly better zFEV1 and zFEV1/FVC at baseline compared to non-participating male farmers. The same tendencies were seen for controls.

The demographics and exposure characteristics of the follow-up cohort can be seen in table 1.

The cumulative exposures to dust and endotoxin among farmers (men and women) had a median (min-max) of 8.2 (0–71) mg/m^3 years and 2400 (0–32 000) EU/m^3 years, respectively. The exposures were divided into quartiles for further analysis. The distribution of male and female farmers in the different quartiles of endotoxin was even, but for dust quartiles, the distribution was a bit uneven, with more females in the lower quartiles of dust exposure ($p<0.05$, χ^2 test). Dust and endotoxin exposures were strongly correlated (correlation between quartiles: Spearman $r=0.93$, $p<0.001$). A few controls (8/172) reported a small amount of farming exposure, but were, for further analyses, still treated as controls. Compared with male controls, the proportion of high current smoking was greater, and the proportion of ex-smoking was lower, among male farmers ($p<0.05$, χ^2 test).

Comparing ex-farmers and current farmers (see online supplementary table S2) showed differences concerning height (current farmers were, on average, 2 cm taller at both baseline and follow-up), asthma, which was less prevalent among current farmers at baseline, z-score for FEV1/FVC, which started significantly higher at baseline for current farmers despite zFEV1 and zFVC not being significantly different at baseline between the groups. Also, smoking showed differences, with current farmers being more likely to be never-smokers and less likely to be

Table 1 Demographics and exposure characteristics of the cohort

Characteristics	Farmers		Controls	
Sex	Male	Female	Male	Missing, %
n (%)	866 (90.0)	96 (10.0)	172 (100)	
Baseline characteristics				
Age years (median (minimum–maximum))	18.5 (16.8–24.8)	19.0 (17.3–24.2)	19.0 (18.5–23.1)	0.0
Height, cm (mean±SD)	180.4±6.98	167.4±7.08	180.9±6.86	0.0
Atopy	13.7	11.5	25.0	0.5
Asthma	3.2	6.3	5.2	0.2
BHR	9.1	7.3	6.4	2.2
Follow-up characteristics				
Follow-up time years (median (minimum–maximum))	15.1 (13.8–16.8)	15.2 (13.8–16.7)	14.8 (14.3–15.7)	0.0
Height, cm (mean±SD)	181.4±6.92	168.1±7.04	181.8±6.74	0.0
BMI, kg/m^2 (median (minimum–maximum))	27.2 (18.4–48.9)	26.9 (19.8–50.7)	26.6 (18.3–37.1)	0.0
Smoking history				1.8
Never-smoker	63.7	65.2	63.7	
Ex-smoker (quit >2 years ago)	6.6	12.0	11.3	
Current smoker low (0–10 pack-years)	11.3	15.2	13.1	
Current smoker high (10–59 pack-years)	18.4	7.6	11.9	
Raised on a farm	58.7	30.2	16.9	0.1
Farm work years (median (minimum–maximum))	13.0 (0–25.0)	7.0 (0–20.0)	0 (0–15.0)	1.9
Farming status				1.8
Never	0.0	0.0	95.3	
Ex	52.6	60.0	4.1	
Current	47.4	40.0	0.6	
Farm type				1.9
Only worked with cattle	21.1	14.9	–	
Worked with mixed animals	54.6	59.6	–	
Only worked with pigs	24.3	25.5	–	
Dust, mg/m^3 years				2.6
1st quartile: 0 to <3.8	24.5	30.1	–	
2nd quartile: 3.8 to <8.2	24.0	33.3	–	
3rd quartile: 8.2 to <16.1	26.0	16.1	–	
4th quartile: 16.1 to 71.0	25.5	20.5	–	
Endotoxin EU/m^3 years				2.6
1st quartile: 0 to <900	25.4	22.6	–	
2nd quartile: 900 to <2400	24.6	28.0	–	
3rd quartile: 2400 to <6000	25.1	23.6	–	
4th quartile: 6000 to 32 000	24.9	25.8	–	
Total per cent missing				6.7

Expressed as per cent unless otherwise specified.
BHR, bronchial hyper responsiveness; BMI, body mass index.

Table 2 Change during follow-up in z-scores for lung function stratified by follow-up smoking status

Participants (n)	Smoker (n)	ΔzFEV1	ΔzFVC	ΔzFEV1/FVC
Controls (172)	Yes (44)	0.16±0.65	0.37±0.55	−0.32±0.82
	No (128)	0.23±0.61	0.44±0.61	−0.31±0.82
Female farmers (94)	Yes (23)	−0.09±0.54*	0.10±0.80	−0.27±1.02
	No (71)	0.30±0.81	0.25±0.69	0.01±0.93
Male farmers (865)	Yes (262)	0.02±0.66**	0.25±0.68	−0.35±0.85**
	No (603)	0.22±0.65	0.33±0.69	−0.16±0.81

All values shown as mean±SD.
*p≤0.015, **p≤0.001 (Mann-Whitney within group comparison between smokers and non-smokers).
FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

current smokers at follow-up compared to ex-farmers. Significantly more current farmers were raised on a farm and had more years of active farm work during follow-up compared to ex-farmers. Similarly, current farmers were more likely to be in the higher quartiles of exposure to dust and endotoxin than ex-farmers.

The absolute lung function values at baseline and follow-up expressed as mean±SD are shown in online supplementary table S3 together with baseline z-scores and the change in z-scores during follow-up. The absolute values for FVC increased during follow-up and decreased slightly for FEV1. The baseline z-scores for FEV1 and FVC in male farmers were slightly but non-significantly lower than in controls. For change in z-scores during follow-up, there was no significant difference between farmers and controls. When stratified by smoking status at follow-up, there was a significant difference in ΔzFEV1 between smokers and non-smokers, but only among the farmers (table 2).

For both female and male farmers, the positive change in ΔzFEV1 during follow-up was significantly smaller among smokers compared to non-smokers. The ΔzFEV1/FVC during follow-up was more negative in male farmers who smoked compared to non-smokers. There was a similar non-significant trend in the female farmers. Smoking did not affect the controls to the same degree as it did the farmers.

Multivariable linear regression analyses to predict change in lung function over the follow-up period for farmers compared to controls can be seen in table 3.

Predictors included in the models were a priori selected based on plausible confounding effects on the change in lung function z-scores. Being a current or an ex-farmer compared to a control was not associated with a significant difference in change in z-score. High current smoking and follow-up BMI had a negative effect on both ΔzFEV1 and ΔzFVC, and being raised on a farm mitigated the effect. In order to quantify the absolute impact on lung function, we found that a change of 1 z-score in FEV1 equates to 515.5 mL for a man aged 25 years with height 175 cm,[8] and for FVC it equates to 622.8 mL. From table 3, the effect of being a high current smoker equates to the FEV1 being 124 mL (95% CI 72 to 180 mL) lower than a non-smoking control at the end of follow-up, and the FVC being 62 mL (95% CI 2 to 131 mL) lower. Sensitivity analyses adjusting for smoker/non-smoker at follow-up did not change the estimates of effect. Sensitivity analyses excluding the female farmers also showed similar results (data not shown).

Being raised on a farm was associated with a larger positive ΔzFEV1 and ΔzFVC compared with those not raised on a farm, whereas, baseline BHR significantly reduced ΔzFVC. Looking at BHR-stratified analyses we found that among those without BHR at baseline (n=965), being raised on a farm no longer had a significant effect on ΔzFEV1 and ΔzFVC, but in those with BHR at baseline (n=93), being raised on a farm was associated with a larger positive ΔzFEV1 (0.39, p=0.020) and ΔzFVC (0.32, p=0.035). This interaction between baseline BHR and being raised on a farm was explored by rerunning the regression with an interaction term. This showed that if you were not raised on a farm, having baseline BHR had a negative effect on the ΔzFEV1 and ΔzFVC compared to those without baseline BHR; however, if you were raised on a farm, ΔzFEV1 and ΔzFVC were not significantly affected irrespective of BHR status. The coefficient for the interaction term was 0.24 (p=0.074) in the model for ΔzFEV1 and 0.27 (p=0.044) in the model for ΔzFVC. Figure 1A illustrates the separate combinations of baseline BHR (yes/no) and being raised on a farm (yes/no) for FEV1 and FVC for all the study subjects together. Figure 1B and C illustrate this for FEV1 and FVC, respectively, with controls and farmers separated. Atopy and BHR were correlated (Spearman r=0.19, p<0.001), and exactly the same interaction was seen between baseline atopy and being raised on a farm. These findings were stronger when controlling for

Table 3 Estimates of the expected change during follow-up for lung function z-scores per unit change in predictor variables, when comparing farmers with controls (n=1058)

	ΔzFEV1 (R^2=0.13)		ΔzFVC (R^2=0.23)		ΔzFEV1/FVC (R^2=0.06)	
Regression model	**E (95% CI)**	**p Value**	**E (95% CI)**	**p Value**	**E (95% CI)**	**p Value**
Control	0	–	0	–	0	–
Farmer status						
Ex	0.05 (−0.07 to 0.16)	0.414	−0.05 (−0.16 to 0.06)	0.393	0.14 (−0.01 to 0.29)	0.074
Current	−0.07 (−0.19 to 0.05)	0.272	−0.09 (−0.21 to 0.03)	0.139	−0.01 (−0.17 to 0.15)	0.932
Smoking						
Ex-smoker	0.04 (−0.10 to 0.18)	0.573	−0.06 (−0.20 to 0.08)	0.402	0.15 (−0.04 to 0.34)	0.123
Current low	−0.11 (−0.23 to 0.01)	0.081	−0.01 (−0.12 to 0.11)	0.906	−0.15 (−0.31 to 0.01)	0.065
Current high	**−0.24 (−0.35 to −0.14)**	**0.000**	**−0.10 (−0.21 to −0.003)**	**0.044**	**−0.20 (−0.33 to −0.06)**	**0.005**
Second-hand smoking	−0.08 (−0.16 to 0.01)	0.069	**−0.09 (−0.17 to −0.01)**	**0.025**	0.04 (−0.07 to 0.15)	0.465
Raised on a farm	**0.10 (0.01 to 0.18)**	**0.025**	**0.08 (−0.000 to 0.16)**	**0.050**	0.06 (−0.05 to 0.17)	0.274
Baseline BHR	−0.09 (−0.22 to 0.04)	0.190	**−0.13 (−0.26 to −0.005)**	**0.042**	0.01 (−0.16 to 0.19)	0.876
Follow-up BMI	**−0.05 (−0.06 to −0.04)**	**0.000**	**−0.07 (−0.08 to −0.06)**	**0.000**	**0.03 (0.02 to 0.05)**	**0.000**

Bold shows significance p<0.05.
BHR, bronchial hyper responsiveness; BMI, body mass index; E, estimate; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

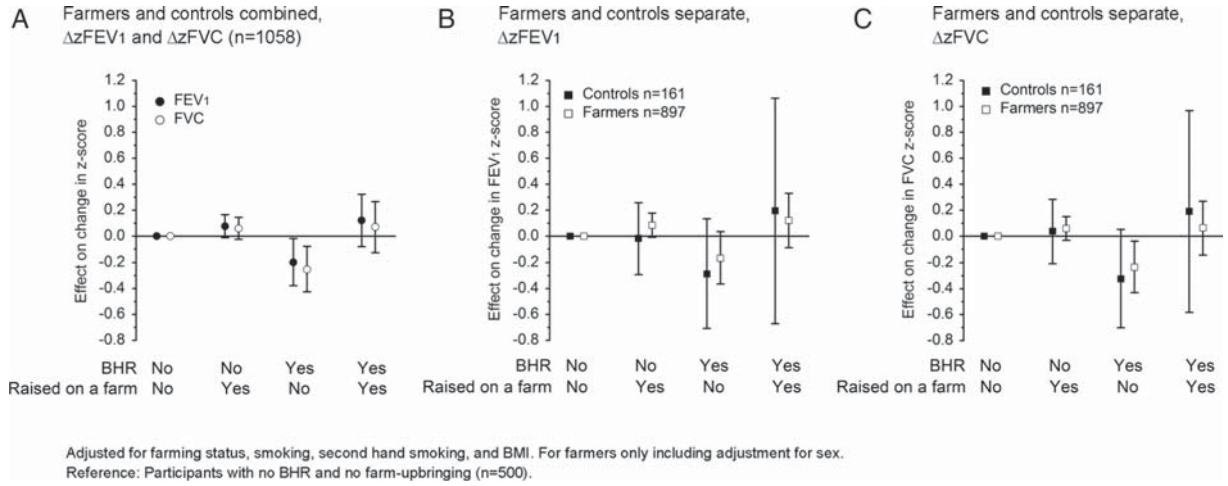

Figure 1 Mean estimates of the effect, with 95% CI, on change in z-score for FEV1 and FVC during follow-up according to the status of baseline BHR and being raised on a farm.

baseline values of zFEV1 and zFVC, arguing that the protective effect of being raised on a farm was not due to pre-existing impairment of lung function.

Multivariable linear regression analyses (equivalent to table 3) stratified by baseline atopy did not change the estimates of effect of farmer status but showed a stronger negative effect of BHR among atopics (n=158) than non-atopics (n=895), especially on ΔzFVC (–0.23, p=0.05 vs –0.07, p=0.41, respectively).

Table 4 presents linear regression analyses of the effect of exposures to cumulative farming during follow-up on lung function for farmers only, using the same confounders as in previous analyses (table 3) and including sex. When farming exposure was assessed by quartiles of dust and endotoxin (analysis A and B), no significant differences were seen among the different quartiles in farmers and change in lung function, and the quartiles of exposure in farmers were not associated with change in lung function compared to the controls (data not shown).

Analyses by type of farming did not show systematic differences between only cattle, only pig, or mixed farmers (table 4C), and the type of farming was not associated with change in lung function compared to the controls (data not shown).

Multivariable linear regression analysis comparing only current farmers with ex-farmers (controls excluded) showed a significant worse effect among current farmers on ΔzFEV1 (–0.12, p=0.006) and ΔzFEV1/FVC –0.15, p=0.009) (table 4 D). The total cumulative exposure to dust was significantly lower among the ex-farmers (mean±SD 6.3 mg/m^3 years ±6.7) compared to the current farmers (17.5 mg/m^3 years

Table 4 Estimates of the expected change during follow-up for lung function z-scores per unit change in exposure variables in separate analytical models including (A) dust exposure, (B) endotoxin exposure, (C) farm type and (D) farm status (A, B, C for current and ex-farmers together, D comparing current and ex-farmers)

	ΔzFEV1			ΔzFVC			ΔzRatio		
Regression models	**R^2**	**E (95% CI)**	**p Value**	**R^2**	**E (95% CI)**	**p Value**	**R^2**	**E (95% CI)**	**p Value**
(A) Dust: (n=892)	0.14			0.24			0.06		
1st quartile		0	–		0	–		0	–
2nd quartile		–0.06 (–0.17 to 0.06)	0.359		–0.01 (–0.13 to 0.10)	0.816		–0.06 (–0.21 to 0.10)	0.468
3rd quartile		–0.03 (–0.15 to 0.09)	0.590		–0.04 (–0.15 to 0.08)	0.529		–0.01 (–0.17 to 0.14)	0.880
4th quartile		–0.02 (–0.14 to 0.10)	0.733		0.03 (–0.09 to 0.15)	0.619		–0.09 (–0.25 to 0.06)	0.245
(B) Endotoxin: (n=892)	0.13			0.24			0.06		
1st quartile		0	–		0	–		0	–
2nd quartile		–0.10 (–0.22 to 0.02)	0.104		–0.09 (–0.21 to 0.02)	0.117		–0.01 (–0.17 to 0.14)	0.865
3rd quartile		–0.07 (–0.19 to 0.05)	0.233		–0.07 (–0.19 to 0.04)	0.205		–0.003 (–0.16 to 0.15)	0.964
4th quartile		–0.03 (–0.15 to 0.09)	0.662		–0.02 (–0.13 to 0.10)	0.754		–0.03 (–0.18 to 0.13)	0.739
(C) Farm type: (=896)									
Only cattle	0.13	0	–	0.23	0	–	0.05	0	–
Mixed pigs/cattle		–0.03 (–0.13 to 0.08)	0.619		–0.06 (–0.16 to 0.04)	0.264		0.07 (–0.11 to 0.17)	0.659
Only pigs		–0.02 (–0.14 to 0.11)	0.786		–0.03 (–0.15 to 0.09	0.667		0.00 (–0.16 to 0.17)	0.955
(D) Farm status: (n=897)	0.14			0.23			0.06		
Ex-farmers		0	–		0	–		0	–
Current farmers		**–0.12 (–0.21 to –0.03)**	**0.006**		–0.04 (–0.13 to 0.04)	0.334		**–0.15 (–0.26 to –0.04)**	**0.009**

Mutually adjusted for smoking, second hand smoking, sex, being raised on a farm, baseline BHR and follow-up BMI.
Bold shows significance p<0.05.
BHR, bronchial hyper responsiveness; BMI, body mass index; E, estimate; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

±11.4, p<0.001 rank sum test), and the same was true for the cumulative endotoxin exposure (2300 EU/m^3 years±2900 vs 6500 EU/m^3 years±5400, p<0.001 rank sum test). However, when assessing only current farmers, no exposure–response relationship between change in z-scores and their quartiles of exposure to dust and endotoxins was seen, and the same was true for ex-farmers and their quartiles of exposures (data not shown).

A significant term of interaction between sex and current farming was seen in the model for ΔzFVC (−0.30, p=0.034), and a borderline significant interaction term was seen in the model for ΔzFEV1 (−0.25, p=0.08). This indicates that sex modifies the effect of current farming on change in lung function compared to ex-farming, with females being more susceptible (references in the model with the interaction term were male ex-farmers).

DISCUSSION

In this study, the longitudinal change in lung function among young farmers did not differ significantly from the lung function change among controls when compared with population reference levels. Being a current farmer, compared to being an ex-farmer, had a larger negative impact on lung function. This might suggest a negative effect of high levels of exposures to dust and endotoxins. However, looking at the quartiles of exposure, no exposure–response relation was seen, though it may represent a more acute effect on lung function among current farmers.

The lung function change was negatively associated with baseline BHR, smoking and not being raised on a farm. Smoking had a negative effect on ΔzFEV1 and ΔzFEV1/FVC, but only among farmers. This finding could suggest the effect of smoking to be modified by farming exposures, though we found no significant interaction between farming exposure and smoking (data not shown). The lack of an effect of smoking in the controls could be that there were too few participants to detect a true effect. However, smoking prevalence in the 94 female farmers was 24.5% and lower than in the controls (25.6%), and yet an effect on ΔzFEV1 was seen in the female farming smokers compared to non-smokers (see online supplementary table S2).

We found a significant interaction between baseline BHR and place of upbringing. This suggests that farm upbringing attenuates the negative effect of BHR on lung function. The protective effect of being raised on a farm was not due to pre-existing impairment of lung function among those raised on a farm, and neither among farmers nor controls were there significant differences in prevalence of BHR between those raised on a farm and those not. Terho *et al*[19] and Schulze *et al*[20] similarly found no difference in BHR prevalence among those raised on a farm and those not. To the best of our knowledge, no other study has seen an interaction between BHR and place of upbringing, and the effect of this on the development of lung function.

We also found a significant interaction between sex and farming status, showing a negative effect on ΔzFVC and ΔzFEV1 primarily among female current farmers. This could be a reflection of a higher susceptibility in females towards the chronic occupational exposure. We have shown, in earlier studies, that female workers in the wood industry also seem to be more susceptible to occupational exposure.[21]

We cannot predict when the peak of lung function was reached during follow-up for each individual. Therefore, we do not know if the impaired lung function seen among current farmers compared to ex-farmers depicts actual impaired lung development during follow-up, or a deleterious effect from farming exposure on fully developed lungs. But as the absolute values show an increase in FVC during follow-up, we know that some of the participants have been exposed during lung development. To be able to say more about the actual impact on lung development, multiple repeated measurements of lung function during the follow-up period would have been needed.

Assessing the lung function change of young people using the GLI-2012 equations covers the change in one continuous set of prediction equations. We found that z-scores for lung function increased during follow-up in farmers and in controls, confirming that longitudinal lung function change in the young is different from that estimated from cross-sectional lung function prediction equations,[22 23] possibly due to cohort effects. One problem that may still be present is that the error between longitudinal and cross-sectional equations might be sex dependent. A limitation of our study is the lack of female controls, and this restraint might have affected our results. However, the estimated effects did not change when all women were excluded from the analyses (data not shown).

Adjusting for the baseline level of lung function in regression analyses estimating the effect on lung function change is a controversial and highly debated practice because of correlated errors between lung function level and lung function change.[24] The effects of these correlated errors on the regression coefficients may lead to bias due to the 'horse-racing effect',[25 26] whereby loss is negatively associated with attained level, that is, the lower one's function, the more one loses.[24] To avoid overadjustment, we did not adjust for baseline z-scores and this choice was supported by a sensitivity analysis including baseline z-scores for lung function in the regression models, which did not change the estimates of effect (data not shown).

In our study, occupational history was based on self-reported questionnaires introducing possible recall bias concerning the reported details of each employment. In our exposure assessment, we included actual personal dust measurements, allowing us to calculate individual quantitative cumulated exposure estimates during follow-up. The estimates of exposure from dust and endotoxins were, however, strongly correlated. Other non-assessed exposures in the farming environment, such as ammonia, moulds and disinfectants, may be similarly correlated to our measured exposures.[3 27] Therefore, it is not possible to state the exact elements of the farming exposure that have an effect on the lung function changes over time. We may have underestimated the true total exposures because of possible improvements in the farming environment that were accomplished during course of the follow-up in terms of better ventilation and cleaning systems; but a recent review suggests that no clear downward trend in exposure of livestock farmers has been observed during the last three decades.[28]

The strength of this study is the reliable information on health, smoking habits, working history and specific exposure in a large follow-up cohort of young adults. Thorough lung function testing was performed with good internal consistency and reproducibility.[15] The lung function measurements were assessed the same way at baseline and follow-up by the same small group of assessors. The baseline farming cohort comprised 79% of all farming students in Denmark during 1992–1994.[29] A participation rate at follow-up of only 51.7% may relate to the follow-up examination no longer taking place at the individuals' place of work (as it did at baseline), and that the study individuals were all young with changing personal circumstances. Differences in demographics between participants and non-participants at follow-up were small, and we do not expect these to influence

the results. We consider our results to be representative for Danish farmers, as well as farmers in temperate zones with similar farming methods.

We found no differences in lung function Δz-scores between farmers and controls; however, we can conclude that continuing farming exposure in young adults is associated with a negative effect on lung function development when compared with those who quit farming, with this effect being more prominent in females. Being bronchial hyper-responsive has a negative effect on longitudinal lung function, which is attenuated in those raised on a farm. Already, at this young age, smoking has a clear deleterious effect on lung function development, so preventive measures to reduce occupational exposures from farming should include those of a young age, and advice against smoking should be given at every opportunity.

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Contributors ACSB contributed to conception and design, acquisition of the data, analysis and interpretation of the data; drafting the article; and final approval of the version to be published. MRM contributed to analysis and interpretation of the data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. IB contributed to conception and design, acquisition of the data; revising the article critically for important intellectual content; and final approval of the version to be published. GE contributed to analysis and interpretation of the data; revising the article critically for important intellectual content; and final approval of the version to be published. ØO contributed to conception and design, acquisition of the data; revising the article critically for important intellectual content; and final approval of the version to be published. TS contributed to conception and design, acquisition of the data, analysis and interpretation of the data; revising the article critically for important intellectual content; and final approval of the version to be published. VS contributed to conception and design, acquisition of the data, analysis and interpretation of the data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. ACSB and VS are responsible for the overall content as the guarantors.

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Supplementary material for Paper II – The SUS-study

Supplementary Table S1 Baseline characteristics of the follow-up participants and non-participants.

Characteristics	**Participants**			**Non-participants**		
Sex	**Female farmer**	**Male farmer**	**Male control**	**Female farmer**	**Male farmer**	**Male control**
n (% of total)	96 (4.2)	866 (37.8)	172 (7.5)	111 (4.8)	815 (35.5)	234 (10.2)
Age yrs median (min-max)	19.0 (17.3 - 24.1)	18.5 (16.8 - 24.8)	19.0 (18.5 - 23.1)	18.8 (16.9 - 24.9)	18.5 (16.8 - 24.8)	18.9 (17.8 - 23.2)
Height cm mean±SD	167.4 ± 7.08	180.4 ± 6.98	180.9 ± 6.86	168.3 ± 6.93	180.0 ± 6.74	180.4 ± 7.02
Smoker n(%)	22 (22.9)	235 (27.1)**	49 (28.5)	38 (34.2)	291 (35.7)	86 (36.8)
Raised on a farm n(%)	29 (30.2)	507 (58.6)**	29 (16.9)	25 (23.2)	285 (38.1)	21 (9.0)
Asthma n(%)	8 (8.3)	40 (4.6)*	11 (6.4)	8 (7.2)	58 (7.1)	15 (6.4)
BHR n(%)	7 (7.3)	79 (9.1)	11 (6.4)	12 (10.8)	80 (9.8)	21 (9.0)
zFEV1 mean±SD	0.00 ± 0.82	-0.11 ± 0.89**	-0.04 ± 0.92	0.02 ± 0.96	-0.28 ± 0.93	-0.21 ± 0.91
zFVC mean±SD	0.03 ± 0.88	-0.17 ± 0.87	-0.14 ± 0.88	-0.02 ± 0.96	-0.22 ± 0.90	-0.26 ± 0.88
zFEV1/FVC mean±SD	-0.06 ± 1.00	0.07 ± 1.00**	0.10 ± 0.97	0.06 ± 1.04	-0.12 ± 1.00	0.03 ± 0.95

*p≤0.030, **p≤0.001 between participants and non-participants among male farmers.

Supplementary Table S2 Demographics and exposure characteristics of ex- and current farmers.

Characteristics	Farmers (n=962)		% Missing
Status	**Ex**	**Current**	
n (%)	503 (52.3)	439 (45.6)	2.1
Baseline characteristics			
Age yrs (median (min-max))	18.6 (16.8 - 24.8)	18.6 (16.9 - 24.6)	0.0
Height cm (mean±SD)	178.2 ± 8.5	180.2 ± 7.3 **	0.0
Atopy	14.8	12.4	0.6
Asthma	5.0	2.0 *	0.2
BHR	10.2	8.1	1.8
Lung function z-scores (mean±SD)			0.0
- zFEV1	-0.12 ± 0.88	-0.07 ± 0.90	
- zFVC	-0.12 ± 0.87	-0.19 ± 0.87	
- zFEV1/FVC	-0.03 ± 0.98	0.16 ± 1.01 *	
Follow-up characteristics			
Follow-up time yrs (median (min-max))	15.1 (13.8 - 16.8)	15.2 (13.8 - 16.7)	0.0
Height cm (mean±SD)	179.2 ± 8.5	181.1 ± 7.3 **	0.0
BMI kg/m^2 (median (min-max))	27.7 (18.6 - 50.7)	26.6 (18.4 – 43.7)	0.0
Smoking history			1.7
- Never smoker	57.7	70.8 *	
- Ex smoker (quit > 2yrs ago)	7.5	6.9	
- Current smoker low (0-10 pkyrs)	13.6	9.4 *	
- Current smoker high (10-59 pkyrs)	21.2	12.9 *	
Raised on a farm	43.6	70.4 *	0.1
Farm work yrs (median (min-max))	7.0 (0 - 20.0)	16.0 (2.3 - 25.0) **	0.0
Farm type			2.2
- Only worked with cattle	23.9	16.4 *	
- Worked with mixed animals	53.9	54.9	
- Only worked with pigs	20.8	28.0 *	
Dust mg·m^{-3}·yrs			2.6
- 1st quartile: 0 to < 3.8	43.5	3.7 *	
- 2nd quartile: 3.8 to < 8.2	31.1	18.0 *	
- 3rd quartile: 8.2 to < 16.1	17.6	33.4 *	
- 4th quartile: 16.1 to 71.0	7.8	44.9 *	
Endotoxin EU·m^{-3}·yrs			2.6
- 1st quartile: 0 to < 900	41.3	6.4 *	
- 2nd quartile: 900 to < 2,400	29.5	19.6 *	
- 3rd quartile: 2,400 to < 6,000	20.4	30.2 *	
- 4th quartile: 6,000 to 32,000	8.8	43.8 *	
Total % missing			7.4

Expressed as % unless otherwise specified
* p<0.05, χ^2 test
** p<0.05, rank-sum test

Appendix III

Paper III: The WOOD-study

New-onset COPD and decline in lung function among wood dust exposed workers – a 6 year follow-up study.

(Submitted manuscript in 2nd round review).

Bolund ACS, Miller MR, Jacobsen G, Sigsgaard T, and Schlünssen V.

Appendix III

Paper III: The WOOD-study

New-onset COPD and decline in lung function among wood dust exposed workers – a 6 year follow-up study

(Submitted manuscript in 2nd round review).

Author of correspondence:

MD Anneli Clea Skjelmose Bolund,

Department of Public Health, Section of Environment Occupation & Health, Danish Ramazzini Centre,

University of Aarhus, Bartholins Allé 2, DK-8000 Aarhus C, Denmark

abol@ph.au.dk Tel.:+45 87 16 80 19 Mobile: +45 21 25 54 47

Anneli C S Bolund[a], Martin R Miller[b], Gitte Jacobsen[c], Torben Sigsgaard[a] and Vivi Schlünssen[ad].

[a] Department of Public Health, Section of Environment Occupation and Health, Danish Ramazzini Centre, University of Aarhus, 8000 Aarhus C, Denmark.

[b] Institute of Occupational and Environmental Medicine, University of Birmingham, UK.

[c] Department of Occupational Medicine, Regional Hospital West Jutland - University Research Clinic, Herning, Denmark.

[d] National Research Centre for the Working Environment, Copenhagen, Denmark.

Keywords: Lung function, COPD, occupational exposure, wood dust, organic dust, epidemiology.

Word counts: Abstract = 240; Text =4273; References: 40

What this paper adds

- Wood dust exposure may be associated to COPD and change in lung function; however, there is a lack of longitudinal studies exploring this association.
- We found a significant exposure-response relation between inhalable wood dust and new-onset COPD as well as excess decline in lung function among female woodworkers only.
- Among male woodworkers only smoking and asthma were significant predictors for new-onset COPD and excess decline in lung function, whereas a positive association was found for level of wood dust exposure, probably due to healthy worker selection bias.
- In conclusion female woodworkers seem more susceptible to wood dust exposure than male woodworkers and the importance of reduction in both smoking and wood dust exposure should continuously be an effort to prevent adverse pulmonary health effects.

ABSTRACT

Objectives: Organic wood dust exposure may be associated to COPD and change in lung function. However, there is a lack of longitudinal studies exploring this association. We have investigated the association between exposure to inhalable wood dust and new-onset COPD and change in lung function during 6 years of follow-up.

Methods: A large 6 year follow-up study of 1,112 woodworkers (participation rate 63%) and 235 controls (participation rate 57%) was conducted between 1998-2004. Forced expiratory volume in the 1st second (FEV_1), forced vital capacity (FVC), and height and weight were measured at baseline and follow-up, and questionnaire data on respiratory symptoms, wood dust exposure and smoking habits were collected. Exposure was assessed as cumulative inhalable wood dust using a study-specific job exposure matrix and exposure time.

Results: New-onset COPD for woodworkers compared to the controls showed an exposure-response for female smokers with an OR (95% CI) of 8.47 (0.9-82.4) in the highest exposed group and a significant test for trend, p=0.049. No such association was seen among males for whom only smoking was strongly associated to new-onset COPD. For change in lung function a significant exposure-response was seen for women with increasing decline in lung function with increasing levels of wood dust exposure. An opposite association was seen for men, probably due to healthy workers selection bias.

Conclusion: In conclusion we found that female woodworkers have a dose-dependent increased OR of new-onset COPD and an excess decline in lung function.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a global health problem with increasing prevalence, leading to a substantial economic and social burden worldwide (1). COPD is closely associated to morbidity and mortality. In 2002 it was the fourth leading cause of death worldwide and expected to be the third leading cause of death by 2030 (2,3). Level and change in lung function *per se* predict morbidity and mortality (4–6) in the general population. Smoking is the leading cause for lung function decline and development of COPD, but also occupational exposures such as organic dust particles is regarded to be causally related to the disease.

Many workers are exposed to wood dust. In 2000-2003 this was approximated to 3.6 million workers in the European Union, of which 700,000 were employed in the furniture industry (7). Dry wood dust, which contains different compounds including low levels of endotoxins, terpenes, and moulds (8), is a well-known risk factor for different pulmonary symptoms and diseases (9–13), and impairment in lung function (11,13,14) evaluated primarily in cross-sectional studies. The association between occupational exposure to wood dust and prevalence of COPD has been studied before but displaying conflicting results (14,15). To our knowledge, no other study has looked at wood dust and new-onset COPD in a longitudinal study. Change in lung function, however, has in several longitudinal studies been associated to the level of organic dust exposure in different occupations (16–18), and also for wood dust exposed subjects (19). In previous analyses of the present cohort (described later) it was found that cumulated wood dust exposure during the follow-up period was significantly associated with the absolute decline in FEV_1 and FVC for female workers only (20).

The main characteristic of COPD is persistent airflow limitation in combination with respiratory symptoms, but a clear diagnostic definition of COPD has been debated during the last decades. Using a fixed ratio of the forced expiratory volume in the first second (FEV_1) to forced vital capacity (FVC) (FEV_1/FVC) < 0.7 leads to an over diagnosis of COPD in the elderly and an under diagnosis in the younger subjects (1,21). The Global Lung Function Initiative 2012 (GLI2012) equations account for the effect of aging, height, sex, and ethnicity implementet in one set of equations that cover lung function from the age of 3 to 95 years in the general non-smoking population (22). Using GLI2012 and the lower limit of normal (LLN , which is the lower 5^{th} percentile of a healthy non-smoking population) accompanied by respiratory symptoms is regarded a valid way of estimating COPD (21).

The aim of this study was to investigate the association between inhalable wood dust exposure and both new-onset COPD and change in lung function during 6 years of follow-up using the GLI2012 equations. We hypothesized that exposure to wood dust was associated to an increased risk of developing COPD and excess decline in lung function.

METHODS

Study population

The cohort of wood workers in the Danish furniture industry was established in 1997-1998 with the objective to study the impact of wood dust exposure on pulmonary health effects described in detail by Schlünssen et al (23). The study population is presented in a flow chart in Figure 1 and more detail is available in the article by Jacobsen et al. (20). In brief, 54 furniture factories out of 86 identified in Viborg County with >4 employees participated in the baseline study. The population consisted of 2,380 workers employed in the wood processing, product assembly and storage units. As a control population, 619 employees from three factories producing refrigerators and hearing aids were invited. Valid lung function tests, personal characteristics (sex, age, height and ethnicity) and questionnaire data were available for 1,776 woodworkers and 410 controls at baseline. In 2003-2005 a follow-up was conducted. Visits to 38 of the original 54 furniture factories as well as 14 new furniture factories and the three control factories were performed. Subsequently baseline subjects not identified at these factories were traced and contacted. This resulted in an overall participation at follow-up of 1,112 woodworkers (63%) and 235 controls (57%) with valid lung function measurements at both baseline and follow-up. Informed written consent was obtained from all participants and the study was approved by the ethics committee of Viborg County and the Danish Data Protection Agency.

Lung function and COPD

Lung function was assessed at baseline and follow-up by measuring FEV_1 and FVC 3 times according to guidelines of the European Respiratory Society (24). The same dry wedge spirometer (Vitalograph Ltd, Buckingham, UK) with a maximum volume of 7.7 L was used for all measurements conducted by the same two individuals from the research group. 98.6% of the lung function measurements met the criteria for reproducibility of <200 mL difference between the largest and the second largest lung function value and the highest obtained spirometry values were included in the analyses. Height and weight were likewise measured at baseline and follow-up.

The GLI-2012 equations (8) were applied taking age, height, sex and ethnicity into consideration to calculate the z-scores for FEV_1, FVC, and FEV_1/FVC at both baseline and follow-up. The average of the baseline and follow-up height for each participant was used in calculations of z-scores at both baseline and follow-up in order to minimise any possible errors related to measuring height.

The definition used for newly developed cases of COPD (new-onset COPD) during the follow-up period was defined as FEV_1/FVC falling below the LLN (lower 5^{th} percentile) at

follow-up, having previously been above the LLN, accompanied by symptoms of chronic cough or chronic sputum or breathlessness at follow-up.

Change in z-scores during the follow-up period for the three lung function indices FEV_1 ($\Delta zFEV_1$), FVC ($\Delta zFVC$), and FEV_1/FVC ($\Delta zFEV_1/FVC$) were calculated by subtracting the baseline z-score from the follow-up z-score.

Exposure assignment of inhalable dust

Personal dust sampling was performed at both baseline and follow-up using passive dust monitors described previously (27). In brief, sampling was performed on transparent sticky foils and light extinction was measured before and after sampling. The difference in light extinction expressed the dust-covered foil area and was converted into equivalent inhalable dust levels by linear regression models based on calibration measurements (27). As described by Schlünssen et al. (28) an internal job exposure matrix (JEM) was constructed at baseline (12 groups, 2,217 measurements, 1,581 individuals) and at follow-up (7 groups, 1,355 measurements, 1,044 individuals) based on factory size and work task. The groups were identified in a random effect analysis, where grouping by task and factory size achieved the greatest contrast between groups.

Participants answered questionnaires on respiratory health, smoking habits, and occupational history including work tasks, and these questionnaires were based on the modified British Medical Research Council questionnaire (25) supplemented with questions from the European Community Respiratory Health Survey (26) regarding asthma at both baseline and follow-up.

Wood dust exposure was evaluated at baseline and during the follow-up period. Wood dust exposure at baseline was assessed from the internal baseline JEM together with the number of years working in the wood industry from the age of 15 until baseline. Individual cumulative inhalable wood dust exposure during the follow-up period was calculated as the sum of the exposure for the first half of the period (the product of the level of exposure at baseline and years in the industry during 1997-2000), and the exposure for the second half of the follow-up period (the product of the level of exposure at follow-up and years in the industry during 2001-2004).

For the analyses, woodworkers were divided into groups based on the distribution of the wood dust exposure levels to create the greatest possible contrast of exposure between groups and to ensure enough power for analyses. The cut off points for the baseline wood dust exposure (Supplementary Figure S1), which was not normally distributed, therefore led to two groups (low/high) for analyses on new-onset COPD and three groups (low/medium/high) for change in lung function. Exposure during the follow-up period was

normally distributed and divided into groups based on quartiles of the cumulative dust exposure during the follow-up period; two groups (low/high) for analyses on new-onset COPD, and 4 groups for analyses on change in lung function. Control workers were in all analyses evaluated as non-exposed.

Smoking

For analyses of new-onset COPD a dichotomous smoking variable was used (current smoker yes/no at baseline), and similarly for smoking during the follow-up period (current smoker yes/no during the follow-up period). For analyses on change in lung function smoking was assessed as packyears (equal to 20 cigarettes·day^{-1}·yrs) prior to baseline and as packyears during the follow-up period. Subsequently, smoking was categorised as never smoker, ex-smoker, low smoker, and high smoker for both smoking exposure prior to baseline and during the follow-up period. Ex-smoking prior to baseline was defined as quitting ≥2 years prior to baseline assessment and ex-smoking during the follow-up period as quitting <2 years prior to baseline and ≥2 years prior to follow-up assessment. Low and high smoking exposure was defined based on the median accumulated smoking exposure both prior to baseline (17 pack years) and during the follow-up period (6 pack years).

Data analyses

Statistical analyses were carried out using *STATA V.14* (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). For continuous variables *mean ± SD* was reported for normally distributed data, and median (min.-max. range) for non-normally distributed data. For nominal variables numbers (%) was reported.

Two-sample t-test was used to compare group means for normally distributed data with equal variance and ANOVA with Bonferroni correction when multiple comparisons were made. For groups with unequal variance Mann-Whitney (Wilcoxon) rank sum test was used for comparison between groups. Pearson χ^2 test was used to compare categorical variables.

The association between exposure to wood dust and new-onset COPD was assessed using logistic regression. The association between exposure to wood dust and the longitudinal change in z-score for lung function was assessed using linear regression. Both analyses were conducted in two ways; as 1) follow-up analyses with current exposure level at baseline, and 2) analyses with cumulative exposure during the follow-up period. The final models included the *a priori* selected confounders smoking, self-reported doctor diagnosed asthma at baseline and, for analyses on exposure during the follow-up period, also weight change during the follow-up period. The outcomes of new-onset COPD and change in lung function were through the GLI2012 equations standardised for sex, age, height and ethnicity. The

effect of cumulative exposure prior to baseline (the product of exposure level at baseline and years in the industry from the age of 15) was explored through sensitivity analyses. Data were presented stratified by sex, as effect modification by sex was evident in the analyses. First order interactions between exposure and sex, and between exposure and smoking were explored. The level of significance was set at $p<0.05$.

RESULTS

Baseline characteristics of follow-up participants and non-participants showed important differences (see online supplementary Table S1) with the non-participants having significantly lower levels of zFEV1, zFVC, and zFEV1/FVC at baseline compared to the participants ($p<0.05$). More of the non-participating male woodworkers (9.9%) had FEV1/FVC<LLN at baseline compared to participating male woodworkers (5.5%) ($p<0.05$). This was also seen among male controls. Non-participants were in general 3-6 years younger and non-participating woodworkers had worked in the industry fewer years, but had comparable baseline levels of inhalable wood dust exposure. Non-participants were also more likely to be current and heavier smokers compared to participants.

The demographics and exposure characteristics of the follow-up cohort can be seen in Table 1. Only few differences were seen between woodworkers and controls, and between females and males. The median exposure level at baseline for inhalable wood dust was 1.1 mg/m^3 for male woodworkers and 0.7 mg/m^3 for female woodworkers, and for exposure during the follow-up period the numbers were 3.8 mg/m^3*year for males and 3.3 mg/m^3*year for females. More females were placed accordingly in the lower exposure categories at both baseline ($p<0.001$) and in the follow-up period ($p<0.001$). Male woodworkers had worked more years in the industry prior to baseline compared to female woodworkers, (10 years vs. 4 years, $p<0.0001$). Similar baseline smoking history was seen for woodworkers and controls, but female woodworkers tended to smoke less at baseline than male woodworkers ($p<0.05$), and the same was true for female controls compared to male controls ($p<0.05$). Smoking history during the follow-up period did not differ between groups, neither did asthma prevalence.

Lung function values at baseline and follow-up as well as new-onset COPD are shown in supplementary Table S2. The absolute values of FEV1 and FVC decreased slightly during the follow-up period. The baseline standardized z-scores for FEV1 and FVC were significantly lower for male woodworkers than for female woodworkers, and for male controls than for female controls for FVC ($p<0.05$). No significant differences for change in z-scores during the follow-up period were seen between woodworkers and controls for either sex. For new-onset COPD a significant difference was seen among females only ($p<0.05$). There was a significant difference between smokers and non-smokers for ΔzFEV1 and ΔzFVC, primarily

among woodworkers, Table 2. New-onset COPD was significantly associated with smoking ($p<0.05$). Among females only there was a significantly higher number of subjects with new-onset COPD among smoking woodworkers compared to smoking controls ($p<0.05$).

New-onset COPD for woodworkers compared to the controls can be seen in Table 3. The adjusted analyses for females were based on smokers only (n=139), as none of the non-smoking females developed new-onset COPD. An exposure-response was seen for women in both crude and adjusted analyses with an adjusted OR (95% CI) of 8.47 (0.9-82.4) in the highest exposed group and a significant test for trend, p=0.049. The confidence intervals were wide, as the estimates were based on only 10 female cases. No association between wood dust exposure and new-onset COPD was seen among males, whereas smoking was strongly associated to new-onset COPD with an adjusted OR (95% CI) of 6.2 (2.5-15.6). Time spent in the industry was taken into account in sub analyses, by analyses of the effect of cumulative exposure prior to baseline (exposure level at baseline * years in the industry prior to baseline), well aware of the risk of healthy worker selection bias, and the assumption of constant exposure levels during a career in the wood industry. Among female smokers the adjusted OR (95% CI) was 7.66 (0.9-63.4, p=0.059) for low cumulative exposure ($<50^{th}$ percentile) compared to controls, whereas the adjusted OR (95% CI) of high cumulative exposure ($>50^{th}$ percentile) was 2.94 (0.2-49.8, p=0.453) compared with controls. No association was seen for males.

Table 4 shows multivariable linear regression analyses of change in $zFEV_1$ ($\Delta zFEV_1$) during the follow-up period comparing woodworkers and controls by sex. Similar results for $\Delta zFVC$ and $\Delta zFEV_1/FVC$ can be seen in supplementary Table S3. Female woodworkers had a significantly larger decline in $\Delta zFEV_1$ than female controls with a decline of -0.32 $\Delta zFEV_1$-score (95% CI: -0.56 to -0.08, p=0.009) for 3^{rd} quartile exposure group during the follow-up period compared to controls, (test for trend, p=0.005). Heavy smoking was strongly associated to decline in $\Delta zFEV_1$ (Estimate (E) (95% CI): -0.44 (-0.66 to -0.23), p=0.000). A change of 1 z-score in FEV_1 for an average woman aged 40 years and with height 167 cm equates to 389.8 mL. This corresponds to a -125 mL (95% CI: -203 to -35 mL) excess FEV_1-decline for a female exposed to 3^{rd} quartile wood dust compared to a female control during the 6 years of follow-up. Similarly the effect of smoking was equivalent to an excess decline in FEV_1 of -172 mL (-257 to -90 mL).

For males heavy smoking was also significantly associated to decline in $\Delta zFEV_1$ in both analyses, but wood dust exposure level at baseline was associated with an increase in $\Delta zFEV_1$ (E (95% CI): 0.14 (0.006 to 0.28), p=0.041) for the highest exposure group compared to male controls with a significant test for trend, p=0.026.

Analyses of the effect of cumulative exposure prior to baseline (exposure level at baseline*years in the industry prior to baseline) on decline in lung function showed similar results for both sexes without reaching statistical significance (data not shown).

A significant interaction was confirmed in the adjusted models between wood dust exposure and sex on the effect of ΔzFEV1 (exposure to wood dust*sex (female): E (95% CI) -0.29 (-0.53 to -0.06), p=0.014, compared to male controls). A similar interaction between exposure and sex was seen on the effect of ΔzFVC. The interaction between wood dust exposure and smoking was not significant.

Analyses of ΔzFVC showed the same tendencies for both female and male, whereas no significant associations were seen for wood dust exposure and ΔzFEV1/FVC (Supplementary Table S3). Asthma was only significantly associated with a decline in zFEV1 and zFVC for males. A positive weight change was associated with a decline in zFEV1 and zFVC during the follow-up period.

All analyses were repeated with exposure level as continuous variables, showing no significant association. To explore the possible issues of cohort effects by comparing longitudinal data with cross-sectional data (29) we repeated the analysis using change in lung function in relation to height^3 ($\Delta FEV_1/m^3$) as the outcome of interest and saw similar results, but with increased levels of significance.

DISCUSSION

To our knowledge this is the first longitudinal study to explore the risk of new-onset COPD in association with wood dust exposure. An evident difference between female and male woodworkers was seen. Only the female woodworkers had an increased dose-dependent OR of new-onset COPD compared to controls. Also change in zFEV1 and zFVC during the follow-up period was negatively associated with wood dust exposure among females in a dose-dependent manner, whereas the change in zFEV1 and zFVC among males was positively associated with wood dust exposure. This is considered to reflect a strong healthy worker selection bias among the male woodworkers. The difference between sexes was supported by a significant interaction term between wood dust exposure and sex, suggesting effect modification by sex on the effect of wood dust exposure. Furthermore, the distribution of male and female woodworkers in the different categories of exposure was not equal (spearman $\rho=0.19$, $p<0.0001$), with more female woodworkers in the lower exposure categories at both baseline and follow-up. Females were also more negatively affected by smoking than men with larger effects of heavy smoking on change in lung function in linear regression analyses (Table 4) supporting the theory of increased susceptibility to adverse effects of exposure among females. We have, in a previous study of

farming exposure, shown a similar difference in susceptibility between male and female farmers (37).

The baseline z-scores for female woodworkers started higher compared to male woodworkers (see Table S2), potentially leaving more lung function to be lost during the follow-up period for female woodworkers. However, considering the horse racing effect (30,31), i.e. the lower one's lung function, the more one loses (32) this is probably not the explanation for our results. Whether the difference in effect seen in females and males depicts an actual difference in susceptibility of wood dust exposure on lung function or a healthy workers selection bias among male woodworkers not present among female woodworkers is uncertain. Suggestive of the later theory was that the higher baseline levels of zFEV$_1$, zFVC and zFEV$_1$/FVC for participants compared to non-participants was more pronounced for males than for females (supplementary Table S1). Healthy worker selection bias is therefore highly probable in the cohort, possibly leading to an underestimation of the effect of exposure to wood dust on new-onset COPD and change in lung function especially among the males.

Another sign of a healthy worker selection bias was seen in the analysis of new-onset COPD and cumulative exposure prior to baseline. The highest effect was seen among the lowest exposed, pointing towards a selection of healthy workers that stay in jobs with high levels of exposure for more years.

Smoking had a negative effect on change in lung function, most clearly for woodworkers, suggestive of effect modification by wood dust exposure on the effect of smoking. However, we found no significant interaction between wood dust exposure and smoking. It may be due to power issues that we were unable to detect a synergistic negative effect of smoking and wood dust exposure on change in lung function. In regression models heavy smoking was a significant predictor of decline in zFEV$_1$ and zFVC for both male and female as well as decline in zFEV$_1$/FVC and new-onset COPD for males.

It must be emphasized that the participants in this cohort were quite young at follow-up (average age 45 years) and therefore only few developed COPD within the follow-up time. As COPD is a disease that increases in prevalence with increasing age it would be of great scientific interest to continue following the cohort.

ΔzFEV$_1$/FVC showed no significant association to wood dust exposure, even though female woodworkers had an increased OR for new-onset COPD. However, only 10 females had new-onset COPD, for which the baseline value of zFEV$_1$/FVC was significantly lower ($p<0.0001$) and ΔzFEV$_1$/FVC showed a significantly larger decline ($p<0.0001$) compared to females without new-onset COPD during the follow-up period (n=291). The effect of this small group of females only slightly affected ΔzFEV$_1$/FVC of the full cohort.

The fact that asthma was only significant for change in zFEV1 and zFVC among males and not among females may hypothetically be due to two issues. The first, that the self-reporting of asthma in males reflects a more reliable asthma diagnosis than among females, which in return is associated to a negative effect on lung function change; and second, that female asthma patients may have a higher compliance with regards to asthma-medication, hereby counterbalancing the adverse effect of asthma on lung function change.

A possible weakness in our study was the use of pre-bronchodilator lung function measures. 20-30% classified as having COPD pre-bronchodilator will not meet the criteria post-bronchodilator (36), which indicates reversible obstruction. In fact a weak correlation between asthma and baseline exposure was found (spearman ρ=0.17, p=0.02) with an increase of asthmatic subjects with increasing wood dust exposure. However, only one female participant (out of 14) with baseline doctor diagnosed asthma developed new-onset COPD during the follow-up period, and it is unlikely that the possible overestimation of new-onset COPD from using pre-bronchodilator measurements has biased the results.

For calculating the lung function z-scores using the GLI2012-equation the average height from baseline and follow-up of an individual was chosen in order to level out possible errors related to measuring height. The younger subjects increased only slightly in height during the follow-up period (<22 years (n=70), change in height=+0.1 cm) whereas the older decreased slightly in height (>22 years (n=1,277), change in height=-0.23 cm, p<0.01).

Assessing exposure in two time windows, at baseline and exposure during the follow-up period, allowed us to evaluate the relevance of different exposure metrics for COPD and change in lung function. It is pivotal to know whether exposure occurred before, after or during disease onset, to be able to infer causality and avoid bias due to healthy worker selection. In general, the analyses assessing the effect of current exposure at baseline were comparable to the analyses assessing the effect of accumulated exposure during the follow-up period. The two exposure metrics were strongly correlated (Spearman ρ=0.66, p<0.0001). A similar correlation was seen for smoking prior to baseline and smoking during the follow-up period. Due to these correlations the two types of analyses tend to show similar associations between exposure and outcome.

Other non-assessed exposures in the wood industry such as endotoxins, terpenes and moulds, may be correlated to our measured exposure. However, the levels of these substances are low in the dry wood industry (10). Endotoxin levels were evaluated in the present study with 29 measurements in three factories and showed low levels with a median (range) of 0.9 EU/m^3 (0.3-6.3) (37).

A genuine strength of our study was the use of quantitative exposure measurements. Although the occupational exposure history was based on self-reported questionnaires, it

allowed us to calculate individual quantitative exposure estimates using measured exposure both at baseline and as accumulated exposure during the follow-up period.

A few other longitudinal studies have shown a significant association between wood dust exposure and decline in lung function. In a study by Noertjojo et al. they found that sawmill workers exposed to western red cedar had a significantly greater annual decline in FEV_1 (-12.1 mL/year) and FVC (-14.6 mL/year) compared with controls (19). A significant exposure-response relation between wood dust and decline in FVC was also seen. In another study by Glindmeyer et al. they found no association between total wood dust exposure and adverse effects on lung function (39). However, in a sub analysis using respirable residual particulate matter they found a significant exposure-response relation with decline in FEV_1 and FVC.

To our knowledge no other studies on wood dust have shown a clear difference in the effect on lung function between male and female. Other studies have, however, seen this discrepancy between sexes on effect on lung function, especially in relation to smoking (40).

We consider our results to have internal validity with possible causal inference. We have available reliable information on health, smoking habits, working history and quantitative exposure estimates. Lung function testing was performed the same way at both baseline and follow up, using the same spirometer and the same group of researchers, resulting in good internal consistency and reproducibility. External validity is also considered strong, our results being representative for other Danish woodworkers, and woodworkers from similar geographical areas with similar industrial methods.

In conclusion our results indicate that female woodworkers are more susceptible to wood dust exposure in terms of new-onset COPD and accelerated decline in FEV_1 and FVC than male woodworkers. Efforts must continue to reduce dust exposure in the wood industry to prevent these adverse pulmonary health effects. However, smoking still seems to be the most significant risk factor for COPD and accelerated lung function decline in our subjects, which also emphasizes the importance of smoking reduction and abstention.

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Contributorship statement

Anneli C. S. Bolund, corresponding author, has contributed to 1) analysis and interpretation of data; 2) drafting the article; and 3) final approval of the version to be published.

Martin R. Miller has contributed to 1) analysis and interpretation of data; 2) drafting the article and revising it critically for important intellectual content; and 3) final approval of the version to be published.

Gitte Jacobsen has contributed to 1) conception and design, acquisition of data; 2) revising the article critically for important intellectual content; and 3) final approval of the version to be published.

Torben Sigsgaard has contributed to 1) analysis and interpretation of data; 2) revising the article critically for important intellectual content; and 3) final approval of the version to be published.

Vivi Schlünssen has contributed to 1) conception and design, acquisition of data, analysis and interpretation of data; 2) drafting the article and revising it critically for important intellectual content; and 3) final approval of the version to be published.

Anneli C. S. Bolund and Vivi Schlünssen are responsible for the overall content as guarantors.

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Figures and Tables for Paper III

Table 1 Characteristics of the WOOD-cohort

Group status	**Wood workers**		**Controls**		**Missing n (%)**
Sex	**Female**	**Male**	**Female**	**Male**	
Number (%)	185 (16.6)	927 (83.4)	131 (55.7)	104 (44.3)	0
Baseline characteristics					
Age, years (median (min-max))	37.9 (20.2-59.3)	38.1 (16.6-64.9)	36.3 (20.6-59.0)	39.4 (18.4-61.2)	0
Height, cm (mean ± SD)	166.4 ± 6.0	178.8 ± 7.1	167.2 ± 5.4	178.6 ± 7.5	0
Asthma[$], number (%)	15 (8.1)	50 (5.4)	10 (7.6)	6 (5.8)	55 (4.1)
Worked in industry[€], years (median (min-max))	4.0 (0-29)	10.0 (0-45)	-	-	13 (1.0)
Smoking history^ number (%):					28 (2.8)
Never smoker	75 (40.5)	389 (42.0)	47 (35.9)	37 (35.6)	
Ex-smoker (quit>2 years prior to baseline)	18 (9.7)	165 (17.8)	19 (14.5)	18 (17.3)	
Smoker low (≤17 pack years)	56 (30.3)	157 (16.9)	43 (32.8)	17 (16.4)	
Smoker high (>17 pack years)	30 (16.2)	192 (20.7)	18 (13.7)	28 (26.9)	
Level of inhalable dust: number (%)					24 (1.8)
Low exp.: >0 to ≤0.822 mg/m3	132 (71.4)	409 (44.1)	-	-	
Medium exp.: >0.822 to ≤1.144 mg/m3	40 (21.6)	363 (39.2)	-	-	
High exp.: >1.144 up to 1.61 mg/m3	12 (6.5)	132 (14.2)	-	-	
Follow-up characteristics					
Age, years (median (min-max))	44.8 (26.7-66.1)	44.4 (23.0-71.8)	42.5 (27.1-65.4)	45.6 (25.0-67.1)	0
Height, cm (mean ± SD)	166.1 ± 6.0	178.6 ± 7.1	167.1 ± 5.4	178.6 ± 7.5	0
Follow up time, years (mean ± SD)	6.3 ± 0.6	6.3 ± 0.6	6.3 ± 0.3	6.3 ± 0.4	0
Smoking history† number (%):					81 (6.0)
Never smoker	73 (39.5)	377 (40.7)	46 (35.1)	36 (34.6)	
Ex-smoker (quit before or during the follow-up period)	36 (19.5)	222 (24.0)	28 (21.4)	22 (21.2)	
Smoker low (≤6.0 pack years)	39 (21.1)	142 (15.3)	23 (17.6)	16 (15.4)	
Smoker high (>6.0 pack years)	23 (12.4)	134 (14.5)	26 (19.9)	23 (22.1)	
Cumulated inhalable dust[β]: number (%)					72 (5.3)
1st quartile: >0 to ≤2.97 mg/m^3*years	61 (33.0)	197 (21.3)	-	-	
2nd quartile: >2.97 to ≤3.75 mg/m^3*years	52 (28.1)	209 (22.6)	-	-	
3rd quartile: >3.75 to ≤4.71 mg/m^3*years	38 (20.5)	234 (25.2)	-	-	
4th quartile: >4.71 to 7.55 mg/m^3*years	19 (10.3)	230 (24.8)	-	-	

[$] Self-reported doctor diagnosed asthma at baseline

^ Smoking prior to baseline: Pack-years (20 cigarettes/day/yr)

† Smoking during the follow-up period: Pack-years (20 cigarettes/day/yr)

[€] Years of work in the industry prior to baseline since age 15.

[β] Cumulated inhalable dust during the follow-up period

Table 2 Changes in z-scores for lung function during the follow-up period and new-onset COPD stratified by sex and smoking status during the follow-up period

Participants (n)	Smoker (n)	ΔzFEV1	ΔzFVC	ΔzFEV1/FVC	New-onset COPD[$] number/total (%)
Female Woodworkers (185)[η]	Yes (96)	-0.22 ± 0.76**	-0.17 ± 0.70*	-0.11 ± 0.84	9/94 (9.6)^[#]
	No (87)	0.06 ± 0.60	0.05 ± 0.42	0.04 ± 0.86	0
Female controls (131)[η]	Yes (72)	-0.03 ± 0.53	-0.07 ± 0.48*	0.02 ± 0.66	1/68 (1.5)
	No (58)	0.12 ± 0.53	0.09 ± 0.41	0.02 ± 0.64	0
Male Woodworkers (927)[η]	Yes (429)	-0.06 ± 0.53***	-0.04 ± 0.48	-0.05 ± 0.65**	22/398 (5.5)^^
	No (494)	0.06 ± 0.50	0.02 ± 0.44	0.06 ± 0.61	4/488 (0.8)
Male controls (104)[η]	Yes (55)	-0.15 ± 0.60	-0.07 ± 0.53	-0.14 ± 0.57	4/53 (7.6)^
	No (49)	0.05 ± 0.60	-0.02 ± 0.47	0.14 ± 0.79	0

All values showed as mean ± SD unless otherwise specified; [η] Cases may vary due to missing values.

[$] New onset COPD: follow-up FEV1/FVC<LLN + symptoms if at baseline not FEV1/FVC<LLN

* p<0.05, ** p<0.005, *** p<0.001 (Mann-Whitney within group comparison between smokers and non-smokers).

^p<0.05, ^^p<0.001 (Pearson χ^2 within group comparison between smokers and non-smokers).

[#] p<0.05 Female smoking woodworkers compared to female smoking controls (Pearson χ2).

Table 3 Logistic regression of new-onset COPD during the follow-up period, comparing woodworkers with controls stratified by sex

	New-onset COPD[$]							
	Female				Male			
Regression models	**OR, Crude**	***R²§***	**OR^ (95% CI)**	**p value**	**OR, Crude**	***R²§***	**OR^ (95% CI)**	**p value**
Exposure at baseline	n=307	*0.07*	n=139 (smokers only)!		n=971	*0.10*	n=916	
Controls: 0 mg/m3	1 (ref.)		1 (ref.)	-	1 (ref.)		1 (ref.)	-
Low exp.: >0 & ≤0.972 mg/m3	5.08*		5.49 (0.6-48.7)+	0.126	0.90		0.94 (0.3-3.0)	0.917
High exp.: >0.972 up to 1.61 mg/m3	**10.72***		8.47 (0.9-82.4)+	0.066	0.58		0.66 (0.2-2.2)	0.490
Baseline smoking[#]			-	-			**6.24 (2.5-15.6)**	**0.000**
Asthma[¤]			0.90 (0.09-9.1)	0.926			**3.38 (1.1-10.5)**	**0.036**
Exposure during the follow-up period	n=293	*0.10*	n=141 (smokers only)!		n=937	*0.09*	n=900	
Controls: 0 mg/m3*year	1 (ref.)		1 (ref.)	-	1 (ref.)		1 (ref.)	-
Low exp.: >0 & ≤3.75 mg/m3*year	4.71[µ]		5.57 (0.6-52.2)[¥]	0.132	0.92		0.82 (0.3-2.7)	0.745
High exp.: >3.75 up to 7.55 mg/m3*year	**12.60[µ]**		**12.00 (1.3-111.0)[¥]**	**0.029**	0.62		0.72 (0.2-2.4)	0.593
Follow-up smoking[£]			-	-			**7.05 (2.4-20.76)**	**0.000**
Asthma[¤]			0.99 (0.1-10.3)	0.991			2.74 (0.8-9.8)	0.123
Weight change~			0.97 (0.9-1.1)	0.534			0.98 (0.9-1.04)	0.535

Bold shows significance p<0.05. Cases vary due to missing values.

FEV_1, forced expiratory volume in 1st second; OR, Odds Ratio; FVC, forced vital capacity

§ Pseudo R2; ^ Adjusted Odds Ratio; € Years in industry since age 15

$ New-onset COPD: follow-up $FEV_1/FVC<LLN$ + symptoms if at baseline not $FEV_1/FVC<LLN$

Smoking status at baseline (current smoker yes/no); ¤ Doctor diagnosed asthma at baseline (yes/no)

! Female analysis only among smokers as smoking predicts failure perfectly (for female 10/10 new-onset COPD among smokers)

£ Smoking status during the follow-up period (current smoker during the follow-up period yes/no)

~ Weight change during the follow-up period

* Test for trend, OR (95% CI): 2.9 (1.2-7.1), p=0.021

+ Test for trend, OR (95% CI): 2.5 (1.01-6.3), p=0.049

µ Test for trend, OR (95% CI): 3.3 (1.3-8.1), p=0.011

¥ Test for trend, OR (95% CI): 3.1 (1.2-7.8), p=0.017

Table 4 **Multivariable linear regression of change in lung function z-score for FEV1 during the follow-up period, comparing different levels of exposure in woodworkers with the controls**

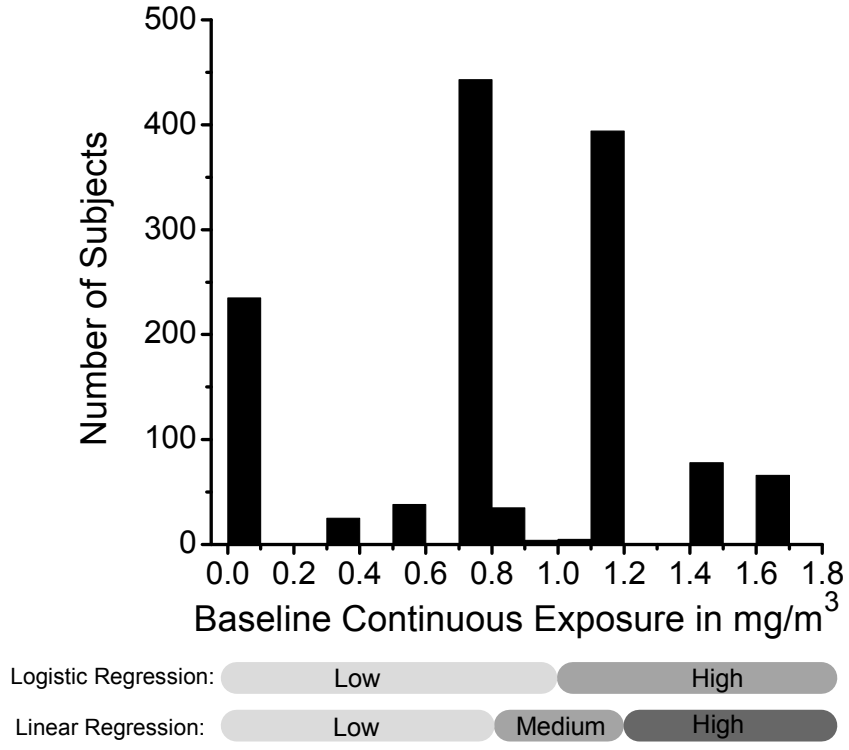
	ΔzFEV1							
	Female				Male			
Regression models	E, Crude	R^2	**E (95% CI)**	**p value**	E, Crude	R^2	**E (95% CI)**	**p value**
Exposure at baseline (n=number)	n=315	*0.03*	n=295		n=1008	*0.04*	n=945	
Control: 0 mg/m3	0 (ref.)		0 (ref.)	-	0 (ref.)		0 (ref.)	-
Low exp.: >0 to ≤0.822 mg/m3	-0.08		-0.07 (-0.23 to 0.09)	0.399	0.03		0.05 (-0.07 to 0.16)*	0.418
Medium exp.: >0.822 to ≤1.144 mg/m3	-0.21		-0.21 (-0.44 to 0.03)	0.081	0.07		0.08 (-0.04 to 0.20)*	0.174
High exp.: >1.144 up to 1.61 mg/m3	-0.05		-0.01 (-0.41 to 0.39)	0.971	0.13		**0.14 (0.006 to 0.28)***	**0.041**
Ex-smoker			0.09 (-0.15 to 0.33)	0.468			0.03 (-0.06 to 0.13)	0.463
Smoker low (≤17 pack years)			-0.15 (-0.32 to 0.03)	0.096			-0.06 (-0.16 to 0.03)	0.193
Smoker high (>17 pack years)			**-0.31 (-0.52 to -0.09)**	**0.006**			**-0.19 (-0.28 to -0.11)**	**0.000**
Asthma¤			0.03 (-0.25 to 0.30)	0.834			**-0.16 (-0.31 to -0.02)**	**0.028**
Exposure during the follow-up period (n=number)	n=301	*0.15*	n=265		n=974	*0.09*	n=911	
Control: 0 mg/m3*year	0 (ref.)		0 (ref.)	-	0 (ref.)		0 (ref.)	-
1st quart exp.: >0 to ≤2.97 mg/m3*year	-0.02		0.02 (-0.18 to 0.23)+	0.818	0.10		0.09 (-0.03 to 0.21)	0.158
2nd quart exp.: >2.97 to ≤3.75 mg/m3*year	-0.05		-0.05 (-0.26 to 0.17)+	0.674	0.02		0.03 (-0.10 to 0.15)	0.672
3rd quart exp.: >3.75 to ≤4.71 mg/m3*year	**-0.25**		**-0.32 (-0.56 to -0.08)+**	**0.009**	0.04		0.05 (-0.07 to 0.17)	0.461
4th quart exp.: >4.71 up to 7.55 mg/m3*year	**-0.31**		**-0.31 (-0.62 to -0.001)+**	**0.049**	0.09		0.08 (-0.04 to 0.20)	0.174
Ex-smoker			**0.25 (0.04 to 0.46)**	**0.018**			0.005 (-0.08 to 0.09)	0.904
Smoker low (≤6 pack years)			-0.13 (-0.33 to 0.07)	0.188			**-0.11 (-0.20 to -0.02)**	**0.026**
Smoker high (>6 pack years)			**-0.44 (-0.66 to -0.23)**	**0.000**			**-0.20 (-0.30 to -0.11)**	**0.000**
Asthma¤			0.07 (-0.22 to 0.36)	0.643			-0.08 (-0.22 to 0.06)	0.280
Weight change~			**-0.01 (-0.02 to -0.002)**	**0.024**			**-0.02 (-0.03 to -0.016)**	**0.000**

Bold shows significance p<0.05. Cases may vary due to missing values.
FEV1, forced expiratory volume in 1st second; E, Estimate
€ Years in industry since age 15.
¤ Doctor diagnosed asthma at baseline (yes/no).
~ Weight change during the follow-up period
* Test for trend, E (95% CI): 0.04 (0.005 to 0.08), p=0.026
+ Test for trend, E (95% CI): -0.08 (-0.14 to -0.03), p=0.005

Supplementary material for Paper III – The WOOD-study

Supplementary Figure S2 Distribution of baseline wood dust exposure

Inhalable wood dust exposure at baseline was assessed from the internal baseline JEM and resultet in 12 unique values. To create the greatest possible contrast of exposure between groups and due to power issues the cut off points led to two groups (low/high) for analyses on new-onset COPD (logistic regression) and three groups (low/medium/high) for change in lung function (linear regression).



Supplementary Table S1 Baseline characteristics of the WOOD-cohort comparing participants and non-participants

	Participants					Non-participants				
	Wood workers		**Controls**		**Missing n (%)**	**Wood workers**		**Controls**		**Missing n (%)**
	Female	**Male**	**Female**	**Male**		**Female**	**Male**	**Female**	**Male**	
n (%)	185 (16.6)	927 (83.4)	131 (55.7)	104 (44.3)	0	126 (19.0)	538 (81.0)	59 (33.7)	116 (66.3)	0
Baseline characteristics										
Age years (median (min-max))	37.9 (20.2-59.3)	38.1 (16.6-64.9)	36.3 (20.6-59.0)	39.4 (18.4-61.2)	0	33.0 (16.8-63.4)*	34.5 (16.6-63.0)*	31.5 (19.4-54.7)*	33.1 (19.3-61.6)*	0
Height cm (mean ± SD)	166.4 ± 6.0	178.8 ± 7.1	167.2 ± 5.4	178.6 ± 7.5	0	165.6 ± 6.4	178.9 ± 7.3	168.0 ± 6.0	178.5 ± 7.6	0
Asthma[$] (n (%))	15 (8.1)	50 (5.4)	10 (7.6)	6 (5.8)	55 (4.1)	6 (4.8)	26 (4.8)	4 (6.8)	8 (6.9)	38 (4.5)
Worked in industry years[€] (median (min-max))	4.0 (0-29)	10.0 (0-45)	-	-	13 (1.0)	1.0 (0-21)*	7.0 (0-43)*	-	-	10 (1.5)
Smoking history^ (n (%)):					28 (2.8)					29 (3.5)
Never smoker	75 (40.5)	389 (42.0)	47 (35.9)	37 (35.6)		48 (38.1)	215 (40.0)	14 (23.7)	37 (31.9)	
Ex-smoker (quit>2 years prior to baseline)	18 (9.7)	165 (17.8)	19 (14.5)	18 (17.3)		11 (8.7)	73 (13.6)	7 (11.9)	12 (10.3)	
Smoker low (<=17 pack years)	56 (30.3)	157 (16.9)	43 (32.8)	17 (16.4)		46 (36.5)	101 (18.8)	21 (35.6)	26 (22.4)	
Smoker high (>17 pack years)	30 (16.2)	192 (20.7)	18 (13.7)	28 (26.9)		20 (15.9)	130 (24.1)	15 (25.4)	34 (29.3)	
Level of inhalable dust[β] mg/m^3: (GM (GSD))	0.90 (1.85)	0.92 (2.1)	-	-	140 (12.6)	0.87 (2.1)	0.98 (2.14)	-	-	83 (12.5)
Baseline Lung function (mean ± SD)					0					0
FEV_1 L	3.13 ± 0.51	4.13 ± 0.71	3.20 ± 0.41	4.13 ± 0.71		3.11 ± 0.49	4.06 ± 0.76	3.27 ± 0.54	4.16 ± 0.71	
FVC L	3.85 ± 0.54	5.16 ± 0.81	3.93 ± 0.47	5.12 ± 0.78		3.77 ± 0.50	5.12 ± 0.84	3.97 ± 0.62	5.26 ± 0.82	
FEV_1/FVC	0.81 ± 0.06	0.80 ± 0.07	0.82 ± 0.06	0.81 ± 0.06		0.83 ± 0.07*	0.79 ± 0.07*	0.82 ± 0.55	0.79 ± 0.07	
zFEV_1	-0.13 ± 1.03	-0.30 ± 0.93	-0.09 ± 1.00	-0.21 ± 1.01		-0.25 ± 0.89	-0.54 ± 1.01*	-0.25 ± 0.91	-0.43 ± 0.98	
zFVC	-0.03 ± 0.88	-0.23 ± 0.85	-0.01 ± 0.85	-0.23 ± 0.92		-0.16 ± 0.77	-0.36 ± 0.91*	-0.13 ± 0.86	-0.20 ± 0.85	
zFEV_1/FVC	-0.24 ± 0.82	-0.14 ± 0.97	-0.18 ± 0.92	-0.02 ± 0.87		-0.17 ± 0.95	-0.34 ± 0.99*	-0.26 ± 0.77	-0.39 ± 1.03*	
FEV_1/FVC<LLN (n (%))	6 (3.2)	51 (5.5)	7 (5.3)	3 (2.9)		9 (7.1)	53 (9.9)[#]	1 (1.7)	17 (14.7)[#]	

[$] selfreported doctor diagnosed asthma at baseline
[€] years of work in the industry prior to baseline since age 15.
^ smoking prior to baseline: Pack-years (20 cigarettes/day/yr)
[β] Inhalable dust level based on individual dust meassurements
* p<0.05 between nonparticipants and participants (Mann-Whitney comparison for same sex and group status)
[#] p<0.05 between nonparticipants and participants (Mann-Whitney comparison for same sex and group status)

Supplementary Table S2 Lung function expressed as absolute values, baseline z-scores, change in z-scores during the follow-up period and new-onset COPD

Participants (n)	Index	FEV_1	FVC	FEV_1/FVC
Female Woodworkers (185)	Absolute value baseline (L)	3.13 ± 0.51	3.85 ± 0.54	0.81 ± 0.06
	Absolute value follow-up (L)	2.95 ± 0.55*	3.70 ± 0.58	0.79 ± 0.07
	Baseline z-score	-0.13 ± 1.03^	-0.03 ± 0.88^	-0.24 ± 0.82
	Δz-score during the follow-up period	-0.08 ± 0.70	-0.06 ± 0.59	-0.03 ± 0.85
	New-onset COPD[$] (n/N (%))			9/181 (5.0)[#]
Female controls (131)	Absolute value baseline (L)	3.20 ± 0.41	3.93 ± 0.47	0.82 ± 0.06
	Absolute value follow-up (L)	3.07 ± 0.47	3.82 ± 0.50	0.80 ± 0.06
	Baseline z-score	-0.09 ± 1.00	-0.01 ± 0.85^	-0.18 ± 0.92
	Δz-score during the follow-up period	0.03 ± 0.54	0.001 ± 0.46	0.02 ± 0.65
	New-onset COPD[$] (n/N (%))			1/126 (0.8)
Male Woodworkers (927)	Absolute value baseline (L)	4.13 ± 0.71	5.16 ± 0.81	0.80 ± 0.07
	Absolute value follow-up (L)	3.95 ± 0.78	5.01 ± 0.88	0.79 ± 0.07
	Baseline z-score	-0.30 ± 0.93	-0.23 ± 0.85	-0.14 ± 0.97
	Δz-score during the follow-up period	0.002 ± 0.52	-0.01 ± 0.46	0.01 ± 0.63
	New-onset COPD[$] (n/N (%))			26/890 (2.9)
Male controls (104)	Absolute value baseline (L)	4.13 ± 0.71	5.12 ± 0.78	0.81 ± 0.06
	Absolute value follow-up (L)	3.92 ± 0.78	4.93 ± 0.88	0.79 ± 0.07
	Baseline z-score	-0.21 ± 1.01	-0.23 ± 0.92	-0.02 ± 0.87
	Δz-score during the follow-up period	-0.06 ± 0.60	-0.05 ± 0.50	-0.01 ± 0.70
	New-onset COPD[$] (n/N (%))			4/102 (3.9)

All values showed as mean ± SD unless otherwise specified.

[$] New onset COPD: follow-up FEV_1/FVC<LLN + symptoms if at baseline not FEV_1/FVC<LLN + symptoms.

* p<0.05 Female woodworkers compared to female controls (ANOVA with Bonferroni correction).

^ p<0.05 Female compared to male of same exposure group (ANOVA with Bonferroni correction).

[#] p<0.05 Female woodworkers compared to female controls (Pearson $\chi 2$).

Supplementary Table S3 **Multivariable linear regression of change in z-scores for FVC and FEV1/FVC during the follow-up period, comparing woodworkers with controls**

	ΔzFVC							
	Female				Male			
Regression models	E, Crude	R^2	**E (95% CI)**	**p value**	E, Crude	R^2	**E (95% CI)**	**p value**
Exposure at baseline (n)	n=315	*0.05*	n=295		n=1008	*0.02*	n=945	
Control: 0 mg/m3	0 (ref.)		0 (ref.)	-	0 (ref.)		0 (ref.)	-
Low exp.: >0 to ≤0.822 mg/m3	-0.02		-0.01 (-0.15 to 0.12)	0.838	0.01[$]		0.02 (-0.08 to 0.12)	0.696
Med exp.: >0.822 to ≤1.144 mg/m3	**-0.22**		**-0.22 (-0.42 to -0.02)**	**0.030**	0.07[$]		0.07 (-0.03 to 0.18)	0.180
High exp.: >1.144 up to 1.61 mg/m3	-0.10		-0.08 (-0.42 to 0.25)	0.632	0.08[$]		0.08 (-0.04 to 0.20)	0.199
Ex-smoker			-0.09 (-0.29 to 0.12)	0.392			0.04 (-0.04 to 0.13)	0.281
Smoker low (≤17 pack years)			**-0.17 (-0.31 to -0.02)**	**0.026**			-0.02 (-0.10 to 0.07)	0.663
Smoker high (>17 pack years)			**-0.25 (-0.43 to -0.07)**	**0.007**			**-0.12 (-0.20 to -0.04)**	**0.003**
Asthma[¤]			0.07 (-0.16 to 0.30)	0.537			-0.12 (-0.25 to 0.005)	0.059
Exposure during the follow-up period (n)	n=301	*0.14*	n=265		n=974	*0.09*	n=911	
Control: 0 mg/m3*year	0 (ref.)		0 (ref.)	-	0 (ref.)		0 (ref.)	-
1st quart exp.: >0 to ≤2.97 mg/m3*year	0.04		0.06 (-0.12 to 0.23)^	0.522	0.07		0.07 (-0.04 to 0.18)	0.201
2nd quart exp.: >2.97 to ≤3.75 mg/m3*year	-0.05		-0.03 (-0.21 to 0.15)^	0.726	-0.01		-0.01 (-0.11 to 0.10)	0.912
3rd quart exp.: >3.75 to ≤4.71 mg/m3*year	**-0.23**		**-0.27 (-0.47 to -0.08)^**	**0.007**	0.05		0.06 (-0.04 to 0.16)	0.266
4th quart exp.: >4.71 up to 7.55 mg/m3*year	-0.14		-0.13 (-0.39 to 0.13)^	0.319	0.07		0.07 (-0.03 to 0.17)	0.189
Ex-smoker			0.07 (-0.10 to 0.25)	0.389			-0.01 (-0.08 to 0.06)	0.825
Smoker low (≤6 pack years)			-0.16 (-0.32 to 0.01)	0.067			-0.07 (-0.15 to 0.01)	0.088
Smoker high (>6 pack years)			**-0.39 (-0.57 to -0.21)**	**0.000**			**-0.11 (-0.20 to -0.03)**	**0.006**
Asthma[¤]			0.06 (-0.18 to 0.30)	0.633			-0.05 (-0.17 to 0.07)	0.432
Weight change~			**-0.015 (-0.02 to -0.006)**	**0.001**			**-0.019 (-0.024 to -0.015)**	**0.000**

Bold shows significance p<0.05

E, Estimate; FVC, forced vital capacity

[€] Years in industry since age 15.

[¤] Doctor diagnosed asthma at baseline (yes/no).

~ Weight change during the follow-up period

^ Test for trend, E (95% CI): -0.06 (-0.10 to -0.008), p=0.023

[$] Test for trend, E (95% CI): 0.04 (0.002 to 0.07), p=0.040

Supplementary Table S3 Continued

	ΔzFEV1/FVC							
	Female				Male			
Regression models	E, Crude	R^2	**E (95% CI)**	**p value**	E, Crude	R^2	**E (95% CI)**	**p value**
Exposure at baseline (n)	n=315	*0.03*	n=295		n=1008	*0.01*	n=945	
Control: 0 mg/m3	0 (ref.)		0 (ref.)	-	0 (ref.)		0 (ref.)	-
Low exp.: >0 to ≤0.822 mg/m3	-0.09		-0.08 (-0.27 to 0.12)	0.422	0.01		0.02 (-0.12 to 0.16)	0.786
Med exp.: >0.822 to ≤1.144 mg/m3	0.02		0.04 (-0.25 to 0.32)	0.807	0.01		0.00 (-0.14 to 0.14)	0.996
High exp.: >1.144 up to 1.61 mg/m3	0.20		0.27 (-0.21 to 0.75)	0.272	0.05		0.08 (-0.09 to 0.25)	0.332
Ex-smoker			0.28 (-0.01 to 0.57)	0.062			-0.01 (-0.12 to 0.10)	0.878
Smoker low (≤17 pack years)			0.02 (-0.19 to 0.23)	0.832			-0.06 (-0.18 to 0.05)	0.280
Smoker high (>17 pack years)			-0.16 (-0.43 to 0.10)	0.218			**-0.16 (-0.27 to -0.05)**	**0.004**
Asthma[¤]			-0.05 (-0.38 to 0.28)	0.776			-0.11 (-0.28 to 0.07)	0.225
Exposure during the follow-up period (n)	n=301	*0.03*	n=265		n=974	*0.01*	n=911	
Control: 0 mg/m3*year	0 (ref.)		0 (ref.)	-	0 (ref.)		0 (ref.)	-
1st quart exp.: >0 to ≤2.97 mg/m3*year	-0.07		-0.03 (-0.30 to 0.23)	0.813	0.000		-0.02 (-0.18 to 0.14)	0.794
2nd quart exp.: >2.97 to ≤3.75 mg/m3*year	-0.007		-0.03 (-0.30 to 0.25)	0.853	0.04		0.03 (-0.13 to 0.19)	0.696
3rd quart exp.: >3.75 to ≤4.71 mg/m3*year	-0.03		-0.09 (-0.39 to 0.22)	0.583	-0.01		-0.03 (-0.19 to 0.12)	0.674
4th quart exp.: >4.71 up to 7.55 mg/m3*year	-0.15		-0.18 (-0.57 to 0.22)	0.386	0.04		0.03 (-0.12 to 0.19)	0.692
Ex-smoker			0.26 (-0.001 to 0.53)	0.051			0.01 (-0.09 to 0.12)	0.826
Smoker low (≤6 pack years)			-0.03 (-0.28 to 0.23)	0.846			-0.05 (-0.17 to 0.07)	0.420
Smoker high (>6 pack years)			-0.07 (-0.35 to 0.20)	0.608			**-0.12 (-0.25 to 0.-0.002)**	**0.046**
Asthma[¤]			0.03 (-0.34 to 0.40)	0.872			-0.10 (-0.28 to 0.09)	0.300
Weight change~			0.002 (-0.01 to 0.02)	0.761			-0.003 (-0.01 to 0.003)	0.335

Bold shows significance p<0.05

E, Estimate; FEV_1, forced expiratory volume in 1st second; FVC, forced vital capacity

[€] Years in industry since age 15

[¤] Doctor diagnosed asthma at baseline (yes/no)

~ Weight change during the follow-up period

Appendix IV

Paper IV: The TWIN-study

Lung function discordance in monozygotic twins and associated differences in blood DNA methylation.
(Submitted manuscript).

Bolund ACS, Starnawska A, Miller MR, Schlünssen V, Backer V, Børglum A, Christensen K, Tan Q, Christiansen L, Sigsgaard T.

Appendix IV

Paper IV: The TWIN-study

Lung function discordance in monozygotic twins and associated differences in blood DNA methylation

(Submitted manuscript).

Anneli C S Bolund[a], Anna Starnawska[bcd], Martin R Miller[e], Vivi Schlünssen[af], Vibeke Backer[g], Anders D. Børglum[bcd], Kaare Christensen[hijk], Qihua Tan[hj], Lene Christiansen[hi], Torben Sigsgaard[a].

[a] Department of Public Health, Section for Environment Occupation and Health, Danish Ramazzini Centre, Aarhus University, Denmark.
[b] Department of Biomedicine, Aarhus University, Denmark
[c] The Lundbeck Foundation Initiative for Integrative Psychiatric Research, iPSYCH, Denmark
[d] Center for Integrative Sequencing, iSEQ, Aarhus University, Denmark
[e] Institute of Occupational and Environmental Medicine, University of Birmingham, UK.
[f] National Research Centre for the Working Environment, Copenhagen, Denmark.
[g] Dept. of respiratory medicine, Bispebjerg University Hospital, Copenhagen, Denmark
[h] The Danish Twin Registry, Institute of Public Health, University of Southern Denmark, Odense, Denmark
[i] The Danish Aging Research Center, Epidemiology, Biostatistics and Biodemography, Institute of Public Health, University of Southern Denmark, Odense, Denmark
[j] Department of Clinical Genetics, Odense University Hospital, Odense, Denmark
[k] Department of Clinical Biochemistry and Pharmacology, University Hospital, Odense, Denmark

Running Title
Lung function and blood DNA methylation in MZ twins

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Abstract

The role of DNA methylation for lung function, allowing for interplay between environmental and genetic factors, needs exploring, as it may help identify genes and pathways of importance for lung function. The aim of this study was to explore the association between blood DNA methylation and lung function in monozygotic (MZ) twins.

A sample of 169 middle-aged MZ twin-pairs was included in an epigenome-wide association study. They were collected from the Danish Twin Register and examined at baseline (1998-1999) and follow-up (2008-2011). Using the twin-design, we correlated intra-pair differences in cross-sectional and longitudinal lung function with intra-pair blood DNA methylation differences at follow-up by linear regression analyses adjusted for sex, age, BMI, smoking, and blood-cell-composition.

We identified several differentially methylated CpG sites associated with Forced Expiratory Volume the 1st second (FEV1) and Forced Vital Capacity (FVC). Three probes identified for level of FVC were located in *GLIPR1L2* (lowest p-value=7.14x10^{-8}), a gene involved in innate immunity, and tumour-suppressor/pro-oncogenic mechanisms. Change in FEV1 during the follow-up period was associated with blood DNA methylation level in *TRIM27* (p-value=1.55x10^{-6}), a negative regulator of CD4-T-cells also involved in cancer development. Several enriched pathways were identified, especially for FEV1, with one being "TGFBR" (Benjamini-Hochberg$_{adj}$ p-value=0.045), the receptor for TGFβ, a growth-factor involved in normal lung tissue repair through pro-fibrotic effects.

Our findings suggest that blood DNA methylation signatures are associated with lung function, identifying immunological- and cancer-related genes, as well as TGF-β-receptor related genes to be possibly involved in the level and change in lung function.

Introduction

Accelerated decline in lung function and associated diseases can have immense consequences for the individual and profound economic and social consequences for society (1,2). Lung function is an important predictor of morbidity and mortality (3), as well as cognitive and physical wellbeing (4) in the general population. Even though lung function slowly and continuously declines with age in adulthood (5), an accelerated decline in lung function is associated with chronic pulmonary diseases, such as asthma and Chronic Obstructive Pulmonary Disease (COPD). Both diseases impose a high burden on society; COPD is estimated to be the 3rd leading cause of death worldwide (6), and the prevalence of asthma is increasing, now affecting more than 10% of the population in some developed countries (1).

Age-dependent lung function decline is a consequence of anatomical structural changes, such as decreased lung recoil and decreased respiratory muscle strength, in combination with physiological and immunological changes (7). Furthermore, factors such as smoking (8) and various occupational exposures (9) are known to lead to an accelerated decline in lung function.

Studies on monozygotic (MZ) and dizygotic twins have previously shown that there is a substantial genetic component influencing level of lung function, with a heritability estimate for forced expiratory volume in the first second (FEV_1) ranging from 61% to 69% and for forced vital capacity (FVC) from 55% to 63% (10,11). Part of this heritability is linked to body composition, such as height and chest size (12). Furthermore, several Genome-Wide Association Studies (GWAS) have identified numerous genetic variants associated with lung function level and change (13–15), as well as important integrative pathways for lung function and airflow obstruction (16,17). However, also novel molecular mechanisms, such as epigenetics, may help understand the variance in liability and systems biology responsible for change in lung function across lifetime.

DNA methylation is the most widely studied epigenetic modification, which primarily occurs at CpG sites (18). Genome-wide DNA methylation patterns change along lifetime, and specific CpG sites become either hyper- or hypo-methylated with age depending on their genomic location (19). Underlying hereditary factors, as well as environmental and stochastic factors are likely to influence these age-related DNA methylation changes (20).

Only few studies have explored the association between DNA methylation and the cross-sectional level and longitudinal change in lung function. Significant associations have been found between methylation of the inflammatory genes *CRAT, F3, TLR2, IFNγ* and *IL6,* as well as *SERPINA1, ATP6V1E2*, *FXYD1*, *FUT7*, *STAT5A, TRPM2,* and *LRP3* and lung function (21,22). Also the methylation of the repetitive elements *Alu* and LINE-1 was found to be associated with lung function (23). There are also various environmental factors that may influence

lung function and associated DNA methylation patterns. The possible mediating effect of smoking for any association between DNA methylation and lung function is crucial, as smoking is considered to be one of the strongest environmental modifiers of DNA methylation (24) and the greatest predictor of lung function decline (8). Several studies (25,26) have found effects of smoking to be associated with both hypo- and hyper-methylation signatures depending on the CpG site, although global hypo-methylation seems to dominate in smokers (26,27).

In this study we performed an epigenome-wide association study (EWAS) between lung function and genome-wide DNA methylation using a study sample of 169 middle-aged MZ twin pairs, thus enabling us to control for underlying genetic and shared environmental factors. We explored blood DNA methylation signatures in association with both cross-sectional lung function level and long-term change in lung function during an 11-year follow-up period.

Materials and Methods

The studied population is a sub-population of twins from the Middle Aged Danish Twins study (MADT) (28) collected as a part of the Danish Twin Register (DTR) (29). MADT was initiated with a baseline survey in 1998-1999 as a Danish nation-wide study of 4.314 twins randomly selected from birth cohorts spanning 1931-1952 (28). A follow-up study was conducted in 2008-2011 of all eligible twin pairs (9.9% deceased) originally enrolled (30). The present study included 169 MZ twin pairs (83 female and 86 male pairs) that participated at both baseline and follow-up and with full data available.

Lung function was assessed for all participants at baseline at the participant's home (10), and at the follow-up approximately 11 years later (min-max: 9.6-13.4 years) at five study centres (30). The three lung function measures FEV_1, FVC and the ratio FEV_1/FVC (differentiating between obstructive and restrictive pulmonary disease) were assessed by spirometry using the micro DL device at baseline and "EasyOne" device at follow-up. The quality of each attempt was evaluated and the highest obtained spirometry values out of three acceptable attempts for each individual were accepted and included for further analyses according to spirometry guidelines (31). Height and weight was self-reported at baseline and measured at follow-up. Body mass index (BMI) was calculated at both time points as weight (kg) divided by height squared (m^2). At follow-up whole blood samples were collected from all participants. Informed written consent was obtained from all participants.

In order to standardize each participant's individual lung function, the important predictors sex, age, height and ethnicity as well as the lung function measures of the individual were

applied to the GLI2012-equations (32) providing z-scores for FEV_1, FVC and FEV_1/FVC for both baseline and follow-up measures. The standardized z-scores hence describe how many standard deviations (SD's) an individual's lung function is away from the lung function of a healthy, non-smoking reference population of same sex, age, height, and ethnicity. We used follow-up height for both baseline and follow-up z-score calculations, as height was only objectively measured at the follow-up visit. Moreover, for this middle-aged group of twins, it was not expected that changes in height during the follow-up period would occur. Calculation of change in z-score during the follow-up period for all three lung function measures was done by subtracting the baseline z-score from the follow-up z-score.

Intra-pair (IP) differences in z-score for level of lung function ($\Delta zFEV1_{IP}$, $\Delta zFVC_{IP}$, $\Delta zFEV1/FVC_{IP}$) were calculated for follow-up values as the absolute difference in e.g. $zFEV_1$ between the "superior" twin (the twin with higher $zFEV_1$) and the "inferior" twin (the twin with lower $zFEV_1$):

$$\Delta zFEV1_{IP} = zFEV1_{superior} - zFEV1_{inferior}$$

Similarly $\Delta zFVC_{IP}$ and $\Delta zFEV1/FVC_{IP}$ was calculated as the difference between the "superior" and the "inferior" twin. The same was calculated for intra-pair differences of the changes in lung function z-scores during the follow-up period ($\Delta zFEV1\text{-change}_{IP}$, $\Delta zFVC\text{-change}_{IP}$, $\Delta zFEV1/FVC\text{-change}_{IP}$).

All analyses were adjusted for smoking status and smoking history of the investigated twins. Smoking was expressed as pack years (equal to 20 cigarettes/day*years) in total and during the follow-up period. Participants were also divided into current smokers, non-current smokers, and never smokers at follow-up. Smokers who quit smoking less than 2 years prior to assessment were defined as current smokers at the time of assessment in order to account for smoking effects that persist for some time after smoking cessation.

DNA methylation analysis

The semi-automated salt precipitation protocol with Autopure System (Qiagen) was used to extract genomic DNA from leukocytes in the buffy-coat. Genomic DNA (500 ng/sample) was bisulfite converted with EZ Methylation Gold Kit (Zymo Research) and analysed using the Infinium HumanMethylation450 BeadChips (Illumina) array according to the manufacturer's protocol. Quality control for obtained DNA methylation data was performed with two different pipelines, a combination of MethylAid (33) and minfi tools (34). Probes with low bead count (<3 beads), high detection p-value (> 0.01), zero signal, and missing in > 5% of samples were removed from further analysis. Additionally, cross-reactive probes identified previously by Chen et al (35) were removed from the dataset. 453,014 good quality probes

remained for further EWAS analyses. Normalisation of DNA methylation data, to control for technical variation, was done with the use of functional normalization (FunNorm) (36) and obtained β-values (the proportion of DNA methylation) were further logit transformed giving an M-value=$\log_2(\beta/1-\beta)$ for each probe. The data have been deposited to the NCBI Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under series accession number XXXXXXXX (soon available).

Blood cell composition

Blood cell counts were measured from the same blood samples that were used for DNA methylation profiling. Blood cell counts were available for 332 individuals, for which blood leukocyte subtypes (monocytes, lymphocytes, basophils, neutrophils, and eosinophils) were counted using a Coulter LH 750 Haematology Analyser. Blood cell counts were not available for 6 individuals, and thus they were imputed based on the methylome dataset as described previously by van Iterson, pipeline provided on GitHub (37). Blood cell counts were used to adjust for individual differences in blood cell composition from which genomic DNA was extracted.

Statistical analyses

Distributions of data were evaluated using histograms and quantile-quantile plots. For normally distributed data, mean ± SD was reported and comparisons were made using Student's t-test. For non-normally distributed data, median (min-max) was reported and Mann-Whitney (Wilcoxon) rank-sum test was used to compare groups with unequal variance.

Epigenome Wide Association Study (EWAS) analyses were performed for intra-pair (IP) differences of both *cross-sectional* and *longitudinal* lung function z-scores.

The intra-pair differences in DNA methylation level (M-value) for each probe was calculated as the "superior" minus the "inferior" twin in accord with the explanatory variable (e.g. $\Delta zFEV1_{ip}$). The same was done for all other included variables for each twin pair. In EWAS analyses, using linear regression models, associations between intra-pair DNA methylation difference and both the cross-sectional and the longitudinal intra-pair lung function difference were investigated. The z-score of lung function can be seen as a relative measure, as it implies the state of the individuals' lung function, compared to the state expected from the reference population (GLI 2012 (32)). With that consideration and also due to the distribution of z-scores, which is around 0, we took the intra-pair difference in z-score as a measurement of quantitative discordance, instead of calculating the proportion of

discordance between twins as done in previous studies of birth-weight discordant MZ twins (38,39).

All regression models were adjusted for sex, age, BMI, ever-smoking history (total pack-years), smoking status at follow-up, as well as blood cell composition difference within each twin pair. The longitudinal models were additionally adjusted for BMI-change during the follow-up period (instead of cross-sectional BMI) and smoking pack-years during the follow-up period (instead of total pack-years).

Log-transformation was applied to independent variables with extremely skewed distributions (IP-differences of zFEV1, zFVC, zFEV1-change and zFVC-change). All analyses were performed in R (http://www.R-project.org/) and STATA14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

Results with a p-value < 10^{-6} were reported as significant in this study (as suggested to be the genome-wide significant threshold for EWAS (40)). The level of significance for the corresponding false-discovery rate (FDR)-adjusted p-value was < 0.05. Results from EWAS with a p-value < 10^{-5} were presented in tables.

In order to explore if associated genes were overrepresented in specific pathways, pathway enrichment analyses were performed. Kyoto Encyclopaedia of Genes and Genomes (KEGG), Gene Ontology pathway (GO), and Pathway Commons (PC) enrichment with the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) (41) were used against the genes included in the 450K DNA methylation array. Probes from EWAS with an unadjusted p-value < 10^{-5} were used for pathway enrichment analyses. P-values from pathway enrichment analyses were corrected for multiple testing with the Benjamini-Hochberg (BH) correction method (42).

Results

A total of 169 twin pairs (83 female and 86 male pairs) with a mean age at follow-up of 66 years (min-max: 56-79 years) were available with complete details on lung function measurements at baseline and follow-up, sex, age, height, smoking history and DNA methylation data. Table 1 shows the demographics of the participants.

In Table 2 the lung function values are presented as absolute lung function values, z-score values, and change in z-score during the follow-up period for the population. Furthermore, calculated intra-pair differences in z-scores at follow-up are given, as well as the intra-pair differences in change in z-score during the follow-up period. As expected, males and females differed in absolute values for FEV1 and FVC, whereas standardized measures of lung function (z-scores) showed no significant difference between the sexes. Absolute lung function measures declined during the follow-up period (Table 2). The observed decrease was slightly higher in males in comparison to females, however it was not higher relative to

the baseline value, for which both males and females decreased with 13% in FEV1 and 5% in FVC during the 11 years of follow-up (data not shown). The variance of intra-pair difference in lung function tended to be higher in male twin pairs in comparison to female twin pairs – possibly due to greater discordance in smoking history between male twins than between female twins (mean difference of 14 pack years vs. 8 pack years ($p<0.05$), respectively, for smoking discordant twin pairs).

Table 3 and 4 present results from EWAS analyses for intra-pair difference in level of lung function at follow-up and for intra-pair difference in change in lung function during the follow-up period, respectively. All probes with p-value < 10^{-5} were annotated with the most proximal gene, genomic position and CpG island context (according to Human Genome Issue hg19) and are presented together with regression estimates and p-values.

Manhattan plots depicting results from all six EWAS analyses are presented in Figure 1 panels A-F. In the supplementary Figure S1 panels A-F corresponding QQ-plots are presented.

Regressing intra-pair DNA methylation differences on intra-pair differences in lung function at follow-up, nine CpG sites with p-value < 10^{-5} were found for zFEV1 (log-ΔzFEV1$_{IP}$) (Table 3) with no sites reaching p-value < 10^{-6}. For intra-pair difference in zFVC (log-ΔzFVC$_{IP}$) ten sites with p-value < 10^{-5} were identified (Table 3), with the most significant probe identified as cg02071292 annotated to GLI (glioma) pathogenesis-related 1 like 2 (*GLIPR1L2*) with p-value=7.14 x 10^{-8} (FDR$_{adj}$ p-value = 0.03). Two other probes with p-value < 10^{-5} in this analysis were also annotated to *GLIPR1L2* (cg07311024 p-value = 1.96 x 10^{-6}, and cg15942481 p-value = 8.77 x 10^{-6}). Intra-pair difference in level of zFEV1/FVC (log-ΔzFEV1/FVC$_{IP}$) identified 11 CpG sites with the only probe reaching p-value < 10^{-6} mapping to no currently known gene (cg00995220, located on chromosome 6) (Table 3).

Regressing intra-pair DNA methylation differences on change in zFEV1 (log-ΔzFEV1-change$_{IP}$) resulted in 2 findings of p-value < 10^{-5} (Table 4), with the most associated probe cg19484381 mapping to Tripartite motif containing 27 (*TRIM27*) with p-value = 1.55 x 10^{-6}. Intra-pair difference for change in zFVC (log-ΔzFVC-change$_{IP}$) also identified two associated probes, with the most highly associated probe cg12796186 mapping to Phosphogluconate dehydrogenase (*PGD*) with p-value = 3.28 x 10^{-6}. Change in zFEV1/FVC (ΔzFEV1/FVC-change$_{IP}$) identified differential methylation at 3 probes (Table 4).

In general, the significant findings in both cross-sectional and longitudinal models showed higher DNA methylation (relative hyper-methylation) for zFEV1 and zFVC for the “inferior” twin, whereas lower DNA methylation (relative hypo-methylation) for zFEV1/FVC was seen for the “inferior” twin in identified probes (Table 3 and 4).

Pathway enrichment analyses for Gene Ontology (GO), KEGG and Pathway Commons (PC) were performed on all EWAS results with p-value < 10^{-5} obtained from both cross-sectional and longitudinal lung function analyses and the results are presented in Table 5 and 6, respectively.

Pathway enrichment analyses for Gene Ontology (GO), based on top results from intra-pair difference in level of zFEV1, identified several enriched pathways (Table 5). The most significant were "Negative regulation of Bone morphogenetic protein (*BMP*) signalling pathway" (BH_{adj} p-value = 0.011), "Ubiquitin protein ligase binding" (BH_{adj} p-value = 0.009), and "Promyelocytic leukaemia protein (PML) body" (BH_{adj} p-value = 0.005). Pathway Commons identified "Transforming growth factor beta (*TGF*-β) receptor (TGFBR)" to be an enriched pathway for zFEV1 (BH_{adj} p-value = 0.045) (Table 5). Several other pathways driven by the same genes reached statistical significance (12 pathways with BH_{adj} p-value < 0.05) (shown in supplementary table S1). Enrichment signal in all pathways for zFEV1 was driven by *SKI* ('Sloan-Kettering Institute') proto-oncogene in combination with either BMP binding endothelial regulator (*BMPER*) or death domain associated protein (*DAXX*). GO and KEGG pathway analyses for intra-pair difference in level of zFEV1/FVC identified enriched pathways to be "Protein homodimerization activity" and "Metabolic pathways" involving other genes than those highlighted for zFEV1 and zFVC (Table 5). Additional pathways found in all enrichment analyses are shown in supplementary table S1.

For intra-pair difference in change in lung function, pathway enrichment analyses identified significant pathways for ΔzFEV1/FVC-$change_{IP}$ using Gene Ontology. "Transferase activity" was identified as the most enriched pathway, driven by the genes xylosyltransferase 1 (*GXYLT1*) and DNA polymerase mu (*POLM*).

Discussion

In this study we investigated possible associations between lung function and genome-wide DNA methylation patterns in a population of Danish middle-aged MZ twin pairs. The lung function of the MADT cohort used in this study was assessed at two different occasions, what allowed us to investigate lung function both as cross-sectional level and as longitudinal change.

The EWAS analyses identified DNA methylation at several CpG sites associated with intra-pair difference in level of lung function for the different metrics. The most significant associated finding for the level of zFVC was DNA methylation of 3 CpG sites positioned in the gene body of *GLIPR1L2* in close proximity to each other (within 150 bp). This gene, situated on chromosome 12, is a member of the cysteine-rich secretory proteins, antigen 5, and

patho-genesis-related 1 proteins (CAP) superfamily. These genes are expressed in the immune tissues and are involved in a variety of physiological processes, including innate immunity, inhibition of ion channels and proteases, and interaction with immunoglobulin proteins, as well as tumour-suppressor and pro-oncogenic genes in different tissues (43,44). The *GLIPR1L2* is a p53 target gene encoding functional p53 response elements that induce tumour-suppression (44). p53 is the most widely and best described tumour-suppressor gene known in humans and p53 mutation is the most frequently described intermediate step in the path between smoking and lung cancer (45). However, as we, in this study, did not find any overlap with previous findings of differentially methylated loci associated with smoking (46), we feel confident that the adjustment for smoking status in these EWAS analyses was successful.

For intra-pair difference in change in lung function during the follow-up period the EWAS analyses identified only few CpG sites to be associated with the studied trait. These were, among others, annotated to genes encoding cellular enzymes i.e. *PGD*, *GXYLT1, POLM* and DEAD box polypeptide 43 (*DDX43*) also known as *HAGE* (DEAD-box helicase antigen), of which the latter has been shown to be an immunogenic cancer-specific antigen present in the protein level of different tumours including lung cancers (47). Also the methylation of *TRIM27*, a negative regulator of CD4 T-cells (48) also involved in development of cancer (49), was identified to be associated with change in lung function.

The observed discrepancy in directions of level of DNA methylation within twin pairs (hypo-/hyper-methylation) for the different lung function measures (Table 3 and 4) may illustrate differences in level of activation of inflammatory genes and pathways, especially for zFEV1/FVC, for which inflammation is known to be important. However, why the direction of methylation level is opposite for zFEV1 and zFVC (hyper-methylation in the “inferior” twin) than for zFEV1/FVC (hypo-methylation in the “inferior” twin) is unknown, though it has been shown that FEV1 and FVC levels are more dependent on growth (12).

Pathway enrichment analyses showed several significant pathways of interest for the level of lung function. ”Negative regulation of BMP signalling pathway” involving the genes *BMPER* and *SKI*, and “TGFBR”-pathway involving *DAXX* and *SKI* were enriched pathways for intra-pair difference in level of zFEV1. These pathways are involved in malignant tumour growth and metastasis (50,51), and angiogenesis (52) and TGF-β plays an important role for normal lung morphogenesis and hence lung function (53). TGF-β is involved in normal lung tissue repair in adults through its pro-fibrotic effects, however over-expression of TGF-β is associated with different lung diseases, including lung fibrosis (53,54). Another possible mechanism for regulating the activity of TGF-β is the expression of the TGF-β receptors (53), making it plausible that the TGFBR-pathway is of high importance for lung function.

These findings suggest that DNA methylation of oncogenic- and tumour suppressor-related genes, as well as TGF-β-receptor related genes could be involved in the level and change in lung function. However, a limitation to the pathway enrichment analyses was the lack of adjustment for probe density. As longer genes are represented by a higher number of probes in the array, the chance of identifying these as associated with the studied phenotype, and hence to be part of enriched pathways is more likely.

Other studies similarly reported significant association between DNA methylation and lung function, however not within the same genes and not involving the same pathways as found in this study, though most of the genes studied previously are included in the 450 BeadChips used in this study. Lepeule et al (21) explored the association between the DNA methylation of nine specific inflammatory genes and the lung function level in a cohort of elderly Caucasian men. Decreased DNA methylation in the inflammatory genes *CRAT*, *F3* and *TLR2* was shown to be associated with lower level of lung function (FEV_1 and FVC). Oppositely, decreased DNA methylation in *IFNγ* and *IL6* was associated with better lung function, insinuating that these genes may be involved in activation of negative feedback in the inflammatory pathways (21). Qiu et al (22) identified 349 CpG sites significantly associated to lung function, of which 330 were hypo-methylated in the presence of lower lung function. Hypo-methylation of CpG sites in *SERPINA1* was negatively associated to both FEV_1 and FEV_1/FVC and this was replicated in another cohort. This gene encodes the acute-phase reactant α1-antitrypsin, a potent circulating anti-elastase produced in the liver but transported to the lung. Variation in the α1-antitrypsin gene *SERPINA1* is known to be a monogenic cause of COPD (55) as deficiency of α1-antitrypsin leads to failing maintenance of the structural integrity of the lung. Other top associations for CpG sites found by Qiu et al were in *ATP6V1E2*, *FXYD1*, *FUT7*, and *STAT5A* for FEV1 and in *ATP6V1E2*, *FXYD1*, *TRPM2*, and *LRP3* for the FEV_1/FVC ratio (22) with no overlap to our study. The specific associations between DNA methylation of the repetitive elements *Alu* and LINE-1 and lung function was studied in the Veterans Administration Normative Aging Study of older Caucasian men (23). Hypo-methylation of *Alu* and LINE-1 was associated to lower cross-sectional FEV_1 level. Faster rate of decline in FEV_1 and FVC was associated with relative hypo-methylation in LINE-1 ($p<0.005$), but not in *Alu*. The mechanism behind this association was suggested to be that hypo-methylation may increase the activity of the repetitive elements as retro-transposable sequences, leading to greater genomic instability and more mutations, which in turn may lead to adverse effects on lung function and lung function decline (23). However, as the above-mentioned previous studies were performed on a general population and not on twin samples their results may be driven by an underlying genetic component. Our study, comparing intra-pair methylation differences in MZ twins, accounts for the genetic factor for lung function and for DNA methylation-levels.

It should be noted that blood samples from the participants in this study were collected only at follow-up. Accordingly, it was only possible to correlate longitudinal lung function change with DNA methylation status at follow-up and not with DNA methylation changes over time. Thus, although significant associations have been found between DNA methylation and lung function, direction of causality cannot be inferred. However, biologically plausible hypotheses may suggest the direction of causality. The possible mechanism behind a causal relation may be that environmental exposures induce oxidative damage and changes in DNA methylation, which may in turn impact lung function due to altered gene-expression, as also suggested by Lepeule et al (23).

Another possible weakness of this study is that DNA methylation was measured in leukocytes, as blood is an easily accessible biological sample. Whether or not the identified methylation differences reflect differential processes also occurring in the lung tissue is not clear. However, leukocytes infiltrate the lung tissue and neutrophilic inflammation can be an early component of lung function decline (21). Furthermore, inflammatory cytokines have been shown to be elevated in circulating blood of COPD patients (56) suggesting a “spill-over” of cytokines from the lung tissue to the systemic circulation. This may also happen as part of the recruitment of leukocytes to the lungs in association with different inhalable exposures (57,58). DNA methylation levels of genes expressed in leukocytes may therefore be a relevant marker of e.g. inflammatory processes in the lungs.

The observed cross-sectional lung function phenotype (z-score level) for this cohort was lower than expected for the population, while the z-score increased during the follow-up period in comparison to the GLI 2012 reference population. There might be several reasons for these cross-sectional and longitudinal differences. First, different spirometer devices were used and several technicians involved at the two time points. Secondly, the increase in z-score during the follow-up period may be due to discrepancies when comparing longitudinal lung function change with that estimated from cross-sectional lung function prediction equations, possibly due to cohort effects as reported before (59). However, we expect any possible misclassifications to be of non-differential type. The rate of absolute lung function decline for this age group was similar to what has been reported in previous studies (60–62).

As for generalizability to other populations it must be emphasized that this unique cohort consisted of Caucasian middle-aged MZ-twins with the unique opportunity to control for all underlying genetic background and shared environment. How DNA methylation in younger cohorts and of other ethnicities would be associated with lung function still remains to be explored. Validation is further needed in order to ensure strength and relevance of our results.

Conclusion

In conclusion, this study shows that DNA methylation patterns in blood are associated to the level and change in lung function in MZ twin pairs, identifying several CpG sites and biological pathways of possible importance for lung function. Specifically, oncogenic- and tumour suppressor-related genes (*GLIPR1L2*, *BMPER*, *SKI*, and *DAXX*), as well as TGF-β-receptor related genes could be involved in level and change in lung function. All these findings point to biological pathways of potential importance for pulmonary physiology.

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FIGURES AND TABLES for Paper IV – The TWIN-study

Table 1 Demographics of the cohort of MZ twin pairs included in this study

	Male (n=172)	Female (n=166)
Participating twin pairs	86	83
Age, years	66 (57-79)	66 (56-78)
Follow-up time, years	11.0 (9.6-13.2)	11.0 (9.8-13.4)
Height, cm	174.0 (158.8-194)*	161.9 (148.5-176.2)
Weight, kg	81.9 (56.2-118.8)*	67.7 (35-102.9)
BMI, kg/m^2	27.0 (19.3-38.0)*	25.5 (13.8-38.1)
Smoking, pack-years (mean ± SD)	20.8 ± 26.9*	7.3 ± 12.8

Numbers are stated as median (min-max) unless otherwise specified

* $p<0.05$ between male and female

Table 2 **Lung function values for the cohort with full data available shown as absolute values (Litres (L)), z-scores and intra-pair differences.**

Lung function (mean ± SD)	**Male (n=172)**	**Female (n=166)**
FEV_1 (L) follow-up	3.0 ± 0.7*	2.2 ± 0.5
FVC (L) follow-up	4.1 ± 0.9*	2.9 ± 0.6
FEV_1/FVC follow-up	0.73 ± 0.09	0.75 ± 0.08
FEV_1-change (L)	-0.45 ± 0.4*	-0.34 ± 0.2
FVC-change (L)	-0.21 ± 0.6	-0.18 ± 0.4
FEV_1/FVC-change	-0.07 ± 0.08	-0.07 ± 0.08
zFEV_1 follow-up	-0.46 ± 1.33	-0.42 ± 1.18
zFVC follow-up	-0.23 ± 1.23	-0.15 ± 1.04
zFEV_1/FVC follow-up	-0.47 ± 1.19	-0.53 ± 0.97
zFEV_1-change	0.03 ± 0.79	0.14 ± 0.73
zFVC-change	0.45 ± 0.92	0.57 ± 0.82
zFEV_1/FVC-change	-0.87 ± 1.18	-0.86 ± 1.12
Intra-pair (IP) differences (median (min-max))	(86 pairs)	(83 pairs)
ΔzFEV_{1IP} follow-up	0.7 (0.01-5.3)	0.6 (0.002-2.8)
ΔzFVC_{IP} follow-up	0.6 (0.03-4.8)	0.6 (0.02-2.9)
ΔzFEV_1/FVC_{IP} follow-up	0.7 (0.02-3.9)	0.5 (0.01-2.5)
ΔzFEV_1-$change_{IP}$	0.7 (0.005-5.2)	0.5 (0.02-2.9)
ΔzFVC-$change_{IP}$	0.7 (0.005-4.7)	0.6 (0.04-3.0)
ΔzFEV_1/FVC_{IP}-$change_{IP}$	1.1 (0.005-5.7)	1.0 (0.02-4.3)

* $p<0.05$ between male and female

Table 3 **EWAS-analyses for intra-pair difference in level of lung function at follow-up (p-value < 10^{-5})**

Lung function measure	Probe	Estimate	P-value	Chromosome	Bp (hg19)	Proximal gene	CGI feature	Methylation* of "inferior" twin
Log-ΔzFEV1$_{IP}$	cg04261072	-0.054	4.33E-06	13	79977499	RBM26	Body - shelf	Hyper
	cg10196163	-0.062	7.62E-06	14	105560628	NA	IGR - shore	Hyper
	cg12552820	-0.033	2.69E-06	1	2231925	SKI	Body - shore	Hyper
	cg13971574	-0.037	9.65E-06	2	39102874	MORN2	TSS1500 - island	Hyper
	cg18582260	-0.079	3.99E-06	13	25085301	PARP4	5'UTR - shore	Hyper
	cg20552903	-0.038	5.81E-06	6	33289678	DAXX	Body - shore	Hyper
	cg23759053	-0.136	5.99E-06	7	34173999	BMPER	Body - open sea	Hyper
	cg23840275	-0.045	2.62E-06	13	20969493	NA	IGR - shore	Hyper
	cg27180671	-0.050	7.51E-06	17	65527566	PITPNC1	Body - open sea	Hyper
Log-ΔzFVC$_{IP}$	cg00008488	-0.0935	8.23E-06	5	175199915	NA	IGR - shore	Hyper
	cg00306721	-0.0797	9.54E-06	11	62477480	BSCL2	TSS1500 - island	Hyper
	cg01028379	0.0617	2.84E-06	7	4798471	FOXK1	Body - shore	Hypo
	cg02071292	-0.2366	**7.14E-08**^{FDR}	12	75785097	GLIPR1L2	1stExon - island	Hyper
	cg07311024	-0.1553	1.96E-06	12	75785089	GLIPR1L2	1stExon - island	Hyper
	cg15909232	-0.1167	5.08E-06	7	156235420	NA	IGR - open sea	Hyper
	cg15942481	-0.1366	8.77E-06	12	75785230	GLIPR1L2	Body – island	Hyper
	cg17154159	0.0832	6.02E-06	6	160401562	IGF2R	Body - open sea	Hypo
	cg22089890	-0.0391	4.20E-06	17	48708077	NA	IGR – shelf	Hyper
	cg25249300	-0.0660	**1.36E-07**	2	54483341	TSPYL6	1stExon – island	Hyper
ΔzFEV1/FVC$_{IP}$	cg00347643	0.1747	7.88E-06	7	75957202	YWHAG	3'UTR – shore	Hypo
	cg00995220	0.1333	**2.13E-07**	6	56259582	NA	IGR - open sea	Hypo
	cg04697953	0.0849	4.43E-06	2	179299404	PRKRA	Body - open sea	Hypo
	cg06244016	0.1917	2.17E-06	6	151186511	MTHFD1L	TSS200 – shore	Hypo
	cg07219303	0.1575	3.92E-06	4	100140905	ADH6	TSS1500 - open sea	Hypo
	cg11980944	0.0863	8.55E-06	1	205399731	NA	IGR - open sea	Hypo
	cg13107302	0.0995	5.69E-06	17	75237970	NA	IGR - open sea	Hypo
	cg13912599	0.1229	2.66E-06	1	150959380	ANXA9	Body - open sea	Hypo
	cg18221862	-0.1038	4.81E-06	2	193059230	TMEFF2	1stExon – island	Hyper
	cg18537205	-0.1205	9.86E-06	10	114575091	VTI1A	Body - open sea	Hyper
	cg19529957	0.1367	5.50E-06	7	4198590	SDK1	Body - open sea	Hypo

Estimate: Intra-pair difference in M-value (logit transformed beta). Bold indicates significance p < 1 x 10^{-6}.FDR: False Discovery Rate < 0.05.

Probes were annotated with the most proximal gene, genomic position Bp (hg19) and CpG island context (CGI-feature).

TSS200: region spanning from Transcription start site (TSS) to 200 bp upstream of TSS;
TSS1500: region spanning from - 200 to - 1500 bp upstream of TSS; 5'UTR: 5' untranslated region; IGR: intergenic region; NA: Not applicable.
*Relative DNA methylation of "inferior" twin compared to "superior" twin; Hyper: Hyper-methylation; Hypo: Hypo-methylation.

Table 4 **EWAS-analyses for intra-pair difference in change in lung function during the follow-up period (p-value < 10^{-5})**

Lung function measure	Probe	Estimate	P-value	Chromosome	Bp (hg19)	Proximal gene	CGI feature	Methylation* of "inferior" twin
Log-ΔzFEV1-change$_{IP}$	cg19484381	-0.055	1.55E-06	6	28890673	TRIM27	Body - shore	Hyper
	cg27261494	-0.081	5.77E-06	6	74104097	DDX43	TSS200 - shore	Hyper
Log-ΔzFVC-change$_{IP}$	cg12796186	-0.066	3.28E-06	1	10458599	PGD	TSS1500 - island	Hyper
	cg14514174	-0.044	6.39E-06	9	99181512	ZNF367	TSS1500 - island	Hyper
ΔzFEV1/FVC-change$_{IP}$	cg00552805	0.0551	9.84E-06	7	44119858	POLM	Body - shore	Hypo
	cg06375580	0.0449	9.25E-06	12	42538820	GXYLT1	TSS200 - island	Hypo
	cg12733656	0.0396	8.82E-06	7	6388695	C7orf70	TSS200 - island	Hypo

Estimate: Intra-pair difference in M-value (logit transformed beta). Bold indicates significance $p < 1 \times 10^{-6}$.
Probes were annotated with the most proximal gene, genomic position Bp (hg19) and CpG island context (CGI-feature).
TSS200: region spanning from Transcription start site (TSS) to 200 bp upstream of TSS;
TSS1500: region spanning from - 200 to - 1500 bp upstream of TSS; 5'UTR: 5' untranslated region; IGR: intergenic region; NA: Not applicable.
*Relative DNA methylation of "inferior" twin compared to "superior" twin; Hyper: Hyper-methylation; Hypo: Hypo-methylation.

Figure 1 **Manhattan plots from EWAS analyses for intra-pair analyses on cross-sectional level (panel A-C) and longitudinal change (panel D-F) in lung function.**

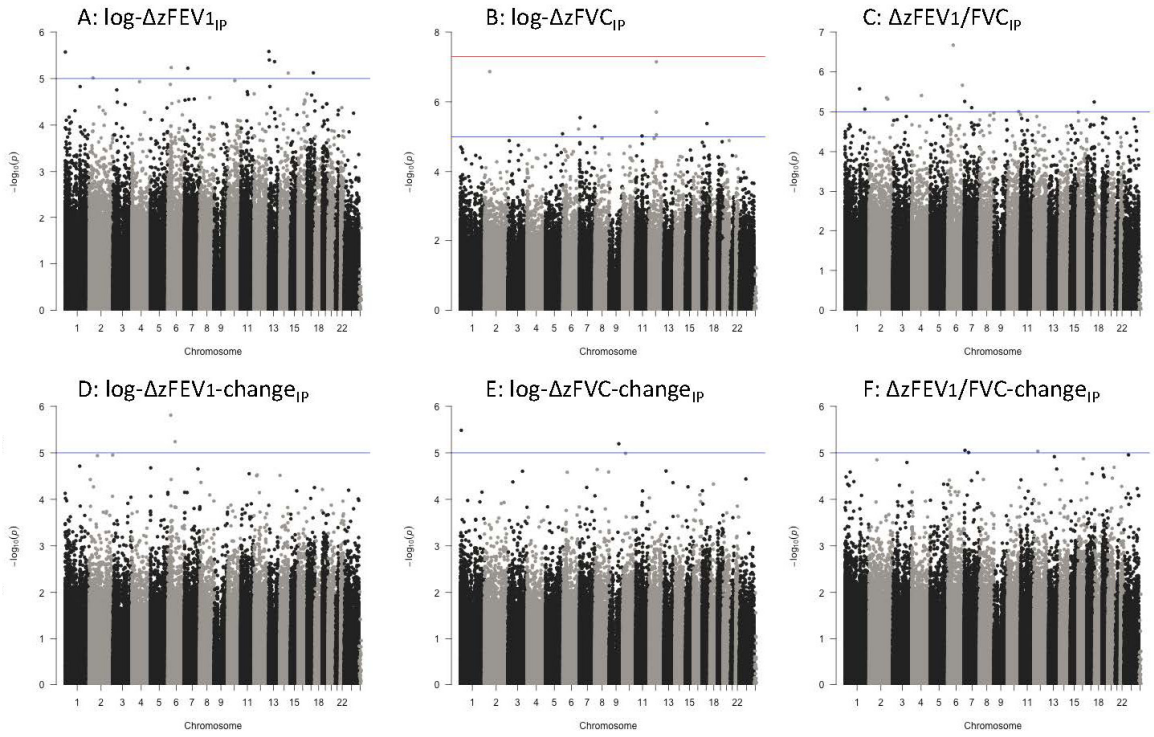


Table 5 **Pathway enrichment analyses for Gene Ontology (GO), KEGG and Pathway Commons (PC) (Separate pathways with BH-corrected p-value < 0.1) based on significant findings from EWAS for level of lung function**

Lung function measure	Data base	Pathway name	#Genes	Genes	Statistics
Log-ΔzFEV1$_{IP}$	GO	Negative regulation of BMP signaling pathway	2	*BMPER SKI*	C=32; O=2; E=0.01; R=142.86; rawP=7.87e-05; **adjP=0.0114**
		Ubiquitin protein ligase binding	2	*DAXX SKI*	C=147; O=2; E=0.05; R=38.69; rawP=0.0010; **adjP=0.0090**
		PML body	2	*DAXX SKI*	C=72; O=2; E=0.02; R=87.37; rawP=0.0002; **adjP=0.0052**
	PC	TGFBR	2	*DAXX SKI*	C=125; O=2; E=0.05; R=43.92; rawP=0.0009; **adjP=0.0450**
Log-ΔzFVC$_{IP}$	No significant pathway enrichment results				
ΔzFEV1/FVC$_{IP}$	GO	Protein homodimerization activity	3	*MTHFD1L ANXA9 PRKRA*	C=554; O=3; E=0.27; R=11.00; rawP=0.0018; **adjP=0.0324**
	KEGG	Metabolic pathways	2	*ADH6 MTHFD1L*	C=1093; O=2; E=0.46; R=4.39; rawP=0.0720; adjP=0.0720

C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category;
E: the expected number in the category; R: ratio of enrichment; rawP: p value from hypergeometric test;
adjP: p-value adjusted by the multiple test adjustment (BH), (**Bold** for adjP<0.05)

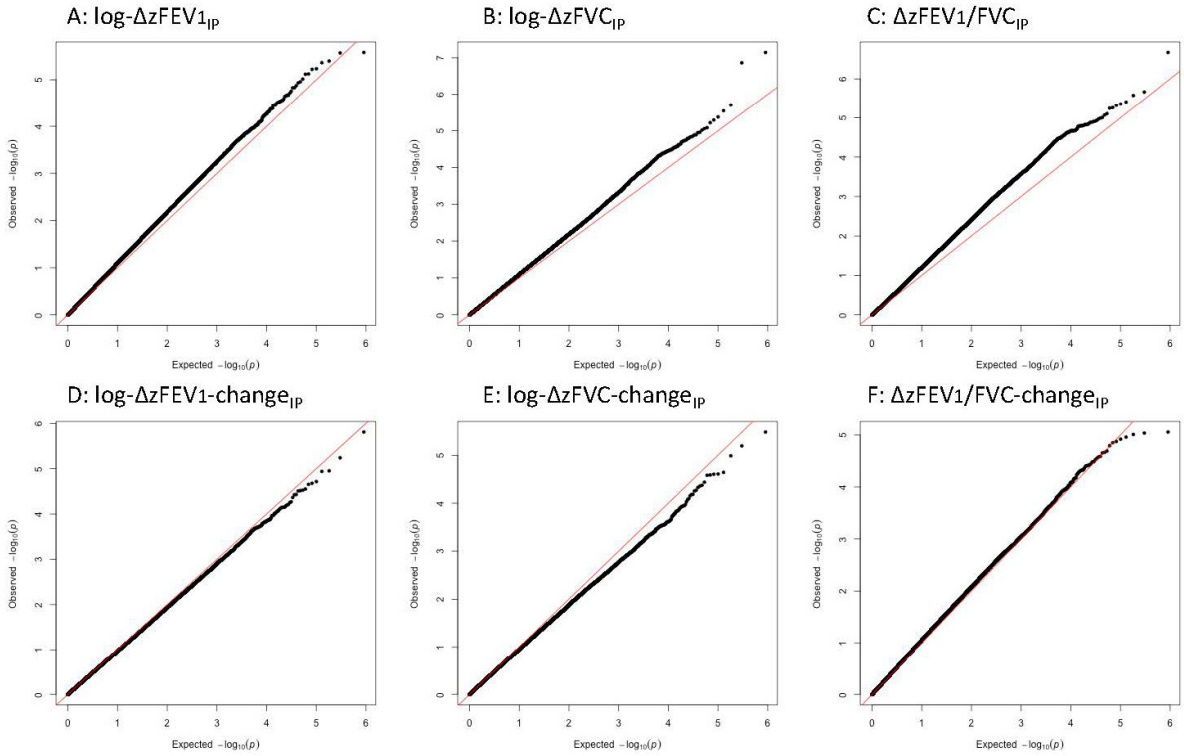
Table 6 **Pathway enrichment analyses for Gene Ontology (GO), KEGG and Pathway Commons (PC) (Separate pathways with BH-corrected p-value < 0.1) based on significant findings from EWAS for change in lung function**

Lung function measure	Data base	Pathway name	#Genes	Genes	Statistics
Log-ΔzFEV1-change$_{IP}$	No significant pathway enrichment results				
Log-ΔzFVC-change$_{IP}$	No significant pathway enrichment results				
ΔzFEV1/FVC-change$_{IP}$	GO	Transferase activity	2	*GXYLT1 POLM*	C=1641; O=2; E=0.23; R=8.67; rawP=0.0133; **adjP=0.0399**

C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category;
E: the expected number in the category; R: ratio of enrichment; rawP: p value from hypergeometric test;
adjP: p-value adjusted by the multiple test adjustment (BH), (**Bold** for adjP<0.05)

Supplementary material for Paper IV – The TWIN-study

Supplementary Figure S1 **QQ-plots from EWAS analyses for intra-pair analyses on cross-sectional level (panel A-C) and longitudinal change (panel D-F) in lung function.**



Supplementary Table S1 **Additional pathways from enrichment analyses for Gene Ontology (GO), KEGG and Pathway Commons (PC) (BH-corrected p-value < 0.1) based on significant findings from EWAS for level of lung function**

Lung function measure	Data base	Pathway name	#Genes	Genes	Statistics
Log-ΔzFEV1_{IP}	GO	Regulation of BMP signaling pathway	2	*BMPER SKI*	C=65; O=2; E=0.03; R=70.33; rawP=0.0003; adjP=0.0217
		Negative regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	2	*BMPER SKI*	C=91; O=2; E=0.04; R=50.24; rawP=0.0006; adjP=0.0290
		BMP signaling pathway	2	*BMPER SKI*	C=106; O=2; E=0.05; R=43.13; rawP=0.0009; adjP=0.0326
		Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	2	*BMPER SKI*	C=163; O=2; E=0.07; R=28.05; rawP=0.0020; adjP=0.0580
		Small conjugating protein ligase binding	2	*DAXX SKI*	C=147; O=2; E=0.05; R=38.69; rawP=0.0010; adjP=0.0090
		Enzyme binding	3	*DAXX SKI PARP4*	C=1052; O=3; E=0.37; R=8.11; rawP=0.0036; adjP=0.0216
		Transcription factor binding	2	*DAXX SKI*	C=410; O=2; E=0.14; R=13.87; rawP=0.0078; adjP=0.0351
		Nuclear body	2	*DAXX SKI*	C=258; O=2; E=0.08; R=24.38; rawP=0.0026; adjP=0.0338
	PC	ALK1 pathway	2	*DAXX SKI*	C=320; O=2; E=0.12; R=17.15; rawP=0.0055; adjP=0.0458
		Regulation of cytoplasmic and nuclear SMAD2/3 signaling	2	*DAXX SKI*	C=301; O=2; E=0.11; R=18.24; rawP=0.0049; adjP=0.0458
		Regulation of nuclear SMAD2/3 signaling	2	*DAXX SKI*	C=301; O=2; E=0.11; R=18.24; rawP=0.0049; adjP=0.0458
		TGF-beta receptor signaling	2	*DAXX SKI*	C=301; O=2; E=0.11; R=18.24; rawP=0.0049; adjP=0.0458

Table S1 continued

Log-ΔzFEV1$_{IP}$	PC	ALK1 signaling events	2	*DAXX SKI*	C=317; O=2; E=0.12; R=17.32; rawP=0.0054; adjP=0.0458
		Integrin-linked kinase signaling	2	*DAXX SKI*	C=637; O=2; E=0.23; R=8.62; rawP=0.0206; adjP=0.0798
		Class I PI3K signaling events mediated by Akt	2	*DAXX SKI*	C=1254; O=2; E=0.46; R=4.38; rawP=0.0718; adjP=0.0798
		Signaling events mediated by VEGFR1 and VEGFR2	2	*DAXX SKI*	C=1262; O=2; E=0.46; R=4.35; rawP=0.0726; adjP=0.0798
		Syndecan-1-mediated signaling events	2	*DAXX SKI*	C=1266; O=2; E=0.46; R=4.34; rawP=0.0730; adjP=0.0798
ΔzFEV1/FVC$_{IP}$	GO	Identical protein binding	3	*MTHFD1L ANXA9 PRKRA*	C=836; O=3; E=0.41; R=7.29; rawP=0.0059; adjP=0.0486
		Protein dimerization activity	3	*MTHFD1L ANXA9 PRKRA*	C=935; O=3; E=0.46; R=6.52; rawP=0.0081; adjP=0.0486

C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category;
E: the expected number in the category; R: ratio of enrichment; rawP: p value from hypergeometric test;
adjP: p-value adjusted by the multiple test adjustment (BH)

Appendix V

Calculation of cigarette equivalents

Cigarette equivalents were calculated based on information from www.sundhed.dk about suggested grams of tobacco in different products:

- 1 cigarette = 1 g of tobacco
- 1 cheroot = 2 g of tobacco
- 1 cigar = 4 g of tobacco
- 1 pipe = 3 g of tobacco

No distinction was made between ”filter cigarettes” and ”without filter cigarettes” or if the participant reports to inhale or not inhale, because the negative effects of smoking are in all situations considered to be of importance to pulmonary health.

Estimating smoking exposure by calculating pack years:

1 pack year: 20 cigarettes/day for one year.

Number of pack years: (number of cigarettes (or equivalent)/day * number of years)/20

For each individual the accumulated amount of smoking was calculated as the sum of all types of tobacco smoked ever, and as amount during the follow-up period.

Appendix VI

Calculation of cumulative exposure in the SUS-study

Cumulative exposures (CE) were calculated as the sum of the products of the mean time-weighted averages (TWA, 8 h) over an 8 hour work day for inhalable dust and endotoxin concentrations and the total work years during the follow-up period (sum of each employment period) for each type of work, collected from questionnaires, standardized as a 40 hour work-week.

As an example consider a person that worked in three different farms for an overall period of ten years (full time with stable work):

a) in a pig farm for 40 hours/week for a period of 3 years

b) in a pig farm for 30 hours/week for a period of 5 years and

c) in a cattle farm for 35 hours/week for a period of 2 years.

First the individual job specific standardized working periods were calculated, i.e.:

a) (40 hours/week * 3 years)/40 hours/week = 3 years with pigs in job a

b) (30 hours/week * 5 years)/40 hours/week = 3.75 years with pigs in job b and

c) (35 hours/week * 2 years)/40 hours/week = 1.75 years with cattle in job c

Then the type-specific overall working periods were calculated as the sum of years spend on working in each type of farming i.e.:

Overall years in pig work = 3 years + 3.75 years = 6.75 “pig” years

Overall years in cattle work = years spend only on job c = 1.75 “cattle” years

The mean TWA dust estimates were 2.48 mg/m^3, 0.56 mg/m^3 and 0.59 mg/m^3 for pig, cattle and field work, respectively. For endotoxin the TWA estimates were 1107 EU/m3, 198.2 EU/m3and 49.4 EU/m3, respectively.

Then the type-specific cumulative exposure estimates for dust were calculated:

For pig work: CEpig = 2.48 * 6.75 = 16.74 (mg/m3)*years

For cattle work: CEcattle = 0.56*1.75 = 0.98 (mg/m3)*years

The cumulative exposure (CE) was computed throughout each individual's working period as the sum of the products of the TWA concentration (dust mg/m3, endotoxin EU/m3) and corresponding work duration (in years) per type of farm work.

CE = Σ(TWA-type specific concentration * number of farm-type specific years)

The overall cumulative exposure of dust was then estimated using the formula:

CE = 16.74 + 0.98 = 17.68 (mg/m3)*years.

Similarly CE was calculated for endotoxin exposure (EU/m3).

Topic of this thesis

Lung function is a predictor of morbidity and mortality in the general population. Organic dust is an occupational exposure that may affect change in lung function negatively. In order to explore if there is a causal relationship between organic dust exposure and excess decline in lung function, emphasis on longitudinal studies is needed and thorough assessments of exposure and outcome are crucial. The aetiology of pulmonary disease and lung function is, however, not only dependant on different external risk factors. Individual genetic variations and the functional state of the genes (epigenetics) are also of importance.

The overall aim of this PhD-thesis was to explore the association between exposure to organic dust and the long-term change in lung function. Furthermore, the association between level and change in lung function and genome-wide differential methylation signatures in blood DNA of discordant monozygotic twins was explored, in order to expand our knowledge and understanding of the pathophysiological processes underlying the impaired lung function.

This thesis contributes to the knowledge of external and internal risk factors for excess decline in lung function. We need to continue exploring the importance of exposure timing and the pathophysiological processes underlying lung function. This should be possible with the rapid technological developments of large scale genome/epigenome wide association studies that can be combined with epidemiological and functional analyses in the future.