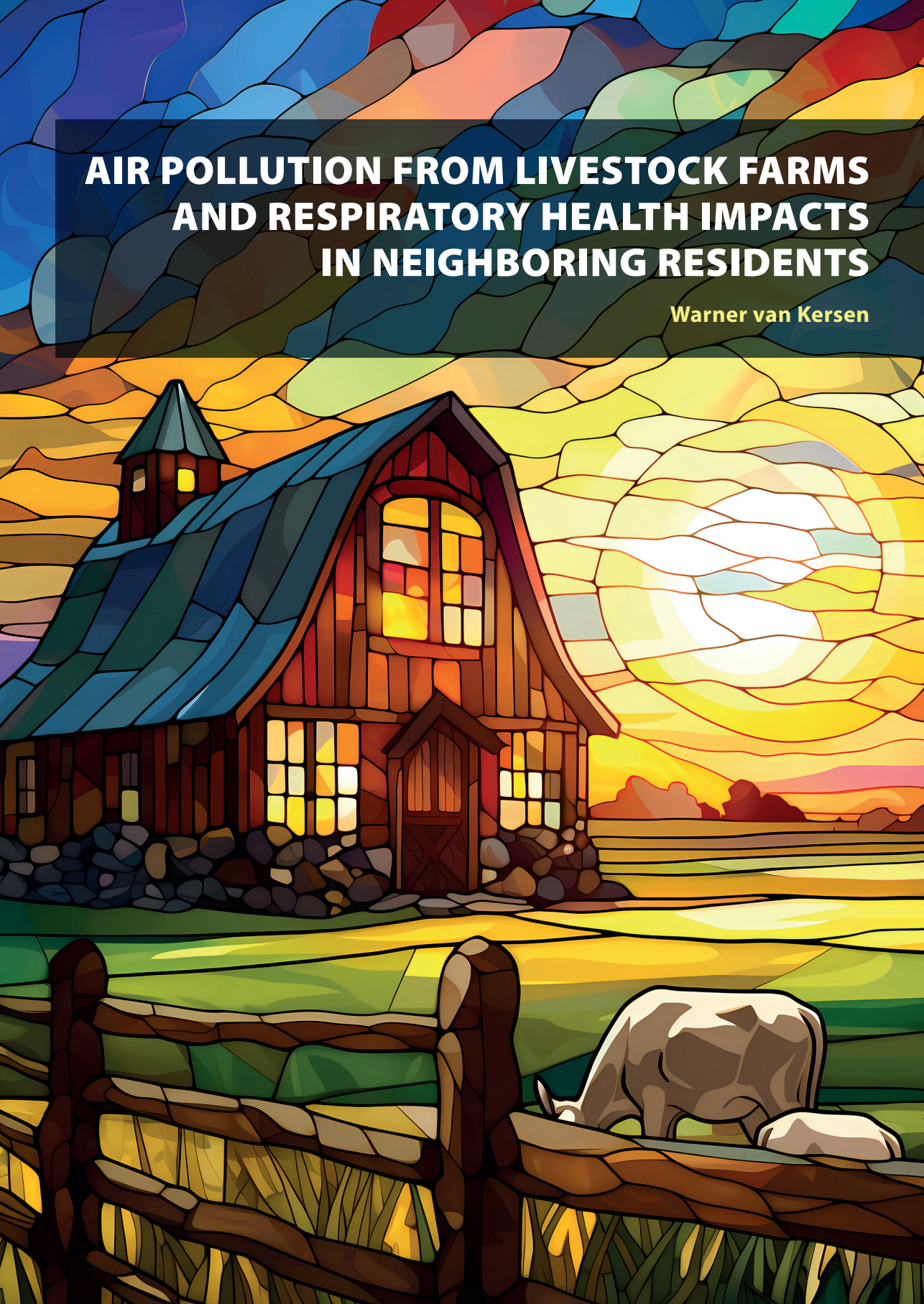
# AIR POLLUTION FROM LIVESTOCK FARMS AND RESPIRATORY HEALTH IMPACTS IN NEIGHBORING RESIDENTS

Warner van Kersen



# Air pollution from livestock farms and respiratory health impacts in neighboring residents

Warner van Kersen

Thesis, Utrecht University
**ISBN:** 978-90-393-7634-8
**Cover design:** Warner van Kersen
**Print & layout by:** Proefschrift all in one (AIO)

# Air pollution from livestock farms and respiratory health impacts in neighboring residents

**Luchtverontreiniging door veehouderijen en de impact op de respiratoire gezondheid van omwonenden**

(met een samenvatting in het Nederlands)

## Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 15 februari 2024 des middags te 4:15 uur

door

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geboren op 10 januari 1991 te Gorinchem

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Dit proefschrift werd mede mogelijk gemaakt met financiële steun van het Netherlands Centre for One Health (NCOH), het Longfonds, ZonMW, het Ministerie van Landbouw, Natuur en Voedselkwaliteit, en het Ministerie van Volksgezondheid, Welzijn en Sport.

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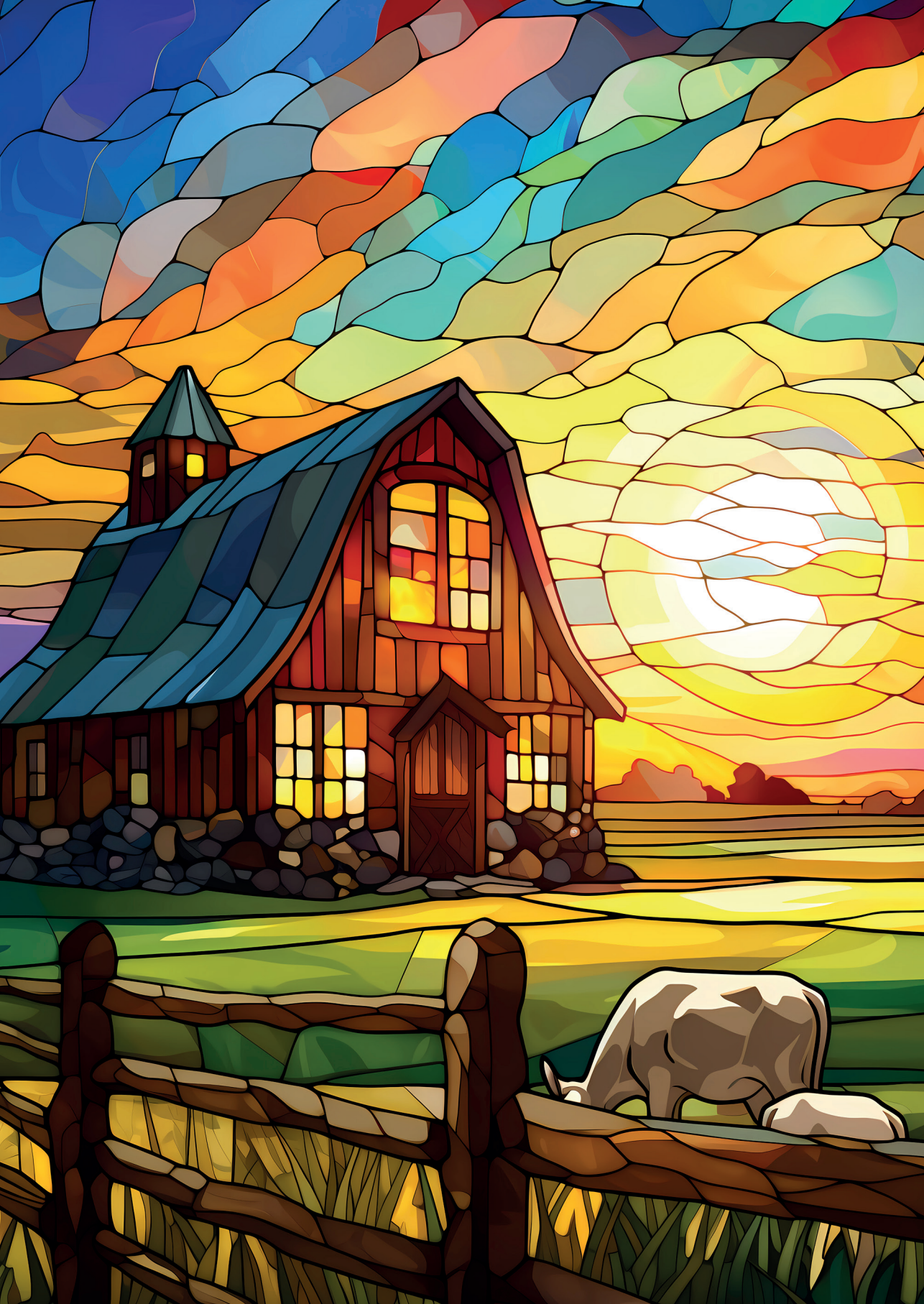

Chapter 1

# General Introduction

## Livestock farming and air pollution

Reports of the health effects of agricultural air pollutants in farmers date back to the 16th century.[1] Nevertheless, it took until the 20th century before occupational health impacts in farm workers became topic of scientific study. Livestock farmers were found to be at risk for respiratory diseases like asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), extrinsic allergic alveolitis (farmers' lung) and organic dust toxic syndrome.[2] Broader interest in farm emissions through the lens of public health is closely related to emerging health concerns (odor annoyance, particulate matter) following the rapid intensification of livestock farming in the decades after the Second World War. High food demand, advances in agricultural science (artificial fertilization, mechanization), import of feed crop (e.g. soy) and European policy aimed at efficient production supercharged industrialization of the Dutch livestock sector.[3,4] This led to a gradual shift over time towards fewer, bigger farms housing more animals. As a result, the modern Dutch livestock farm focusses on one animal species and is often further specialized in mass producing one type of product like dairy or eggs versus meat. To illustrate, the 10.7 million pigs in the Netherlands are housed in just 3,270 farms.[5] Additionally, the Dutch population (17.8 million) shares 41,543 $km^2$ with 88.4 million chickens, 3.7 million cows, 0.7 million sheep and 0.6 million goats.[5,6] Concentrated livestock industries housing large numbers of animals are a known source of air pollution.[7-9] Industrial livestock farming in population dense areas also occurs in other parts of Europe, the United States, South America and is becoming increasingly common in Asia and Africa.[10-11] This makes the health impact of livestock emissions a pressing topic for research.

Livestock farms emit a mixture of air pollutants that can roughly be divided into its gaseous and non-gaseous (particulate) constituents. Gaseous livestock farm emissions encompass products of the metabolism of the animals themselves ($CO_2$) or the microbiota of the digestive tract like ammonia ($NH_3$) or methane ($CH_4$).[8] Non-gaseous pollutants consist of small airborne particles, so called bio-aerosols, originating from the animals (hairs, feathers, dander) or their excretions like feces, urine and saliva.[12,13] Organic material required by the animals in the form of food or bedding is an additional source of bio-aerosols. Adding to the complexity of air pollution from livestock farms is the fact that gases emitted by livestock farms form secondary inorganic aerosols.[14] These particulates are reaction products of $NH_3$, with nitrogen- and sulfur oxides, form in the atmosphere over time and can be dispersed over large distances. Through this mechanism, livestock farm emissions contribute substantially to agricultural fine particulate matter pollution.[15] Most studies assessing respiratory health effects of particulate air pollutants focus on particles with an

aerodynamic diameter less than 10 µm or less than 2.5 µm ($PM_{10}$ and $PM_{2.5}$). Larger particles cannot penetrate the deepest (tracheobronchial and alveolar) regions of the airways and are thus less relevant for respiratory health.

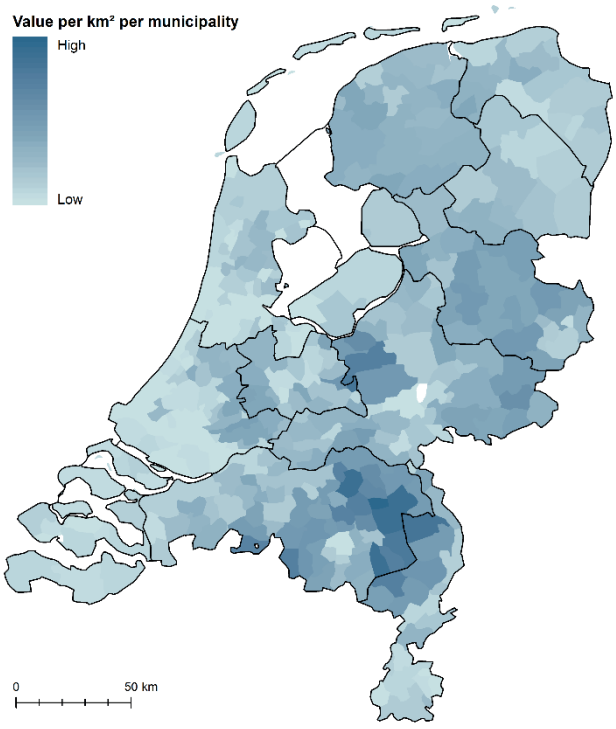


**Figure 1:** Farm density in the Netherlands expressed in standard profit values per km$^2$

*Note:* Figure based on agricultural data of 2012 obtained by the Regulations Service of the Dutch Ministry of Economic Affairs in combination with animals' standard profit values provided by Wageningen Economic Research, processed by the Dutch National Institute for Public Health (RIVM). Ben Bom (RIVM) is acknowledged for creating and providing this figure.

## Health effects of residential exposure to livestockfarming

Initial studies linking livestock exposure to health impacts in nearby residents used the large body of occupational health studies in livestock farmers as a stepping stone.[16] Health risks associated with occupational livestock exposure can roughly be described as infectious or non-infectious (e.g. $NH_3$). The same distinction can be made with regards to residential exposure to livestock emitted air pollutants. The

main infectious disease risk for nearby residents, is attributed to inhalation of bio-aerosols carrying pathogenic microorganisms, able to survive airborne transmission and capable of causing disease in humans after inhalation.[17] This includes 1) farm animal commensals, bacteria living in harmony with their host (e.g. *Escherichia coli*) that can opportunistically cause disease in humans; and 2) zoonotic pathogens that cause disease in humans and are communicable between animals and humans. Examples of the later include avian influenza virus and Q-fever caused by the bacterium *Coxiella burnetii*.[18,19] To lesser extent, compared to inhalation, ingestion of bio-aerosols by means of swallowing contaminated mucus from the upper respiratory track.[20] Dermal exposure, and subsequent hand-mouth transmission (e.g. fecal oral), form another possible exposure route for infectious agents associated with livestock.[21]

The far reaching impact of zoonotic outbreaks was shown in the 2007-2010 Q-fever epidemic in the Netherlands.[18] Based on the 4,000 reported and registered acute cases, the total number of infections was estimated to be more than 40,000. Individuals who experienced a *C. burnetii* infection are at risk of chronic Q-fever, a rare complication with a high mortality rate. Health risks associated with exposure to livestock commensals is related to anti-microbial resistance (AMR), evolved as a result of widespread use of antibiotics in livestock farming.[22] Once present, genes conferring AMR to bacteria can be transferred from one cell to the other by means of horizontal gene transfer.[23] This potentially leads to increasing numbers of bacteria causing difficult to treat infections due to limited treatment options.[24] DNA from livestock commensals and AMR genes have been detected in air samples up to 1200m away from livestock farms.[25] Additionally, living near farms has been associated with increased risk for carrying resistant bacteria (MRSA) in people not working on a farm.[26] There is, however, little evidence for aerial transmission,[27,28] leaving the exact routes of transmission unclear.

Compared to health risks of infectious origin, scientific interest in non-infectious health risks of residential exposure to livestock related air pollution is relatively new.[29] Examples include reports of airway obstruction in relation to the number of farms near the home address.[30,31] Lung function deficits related to elevated $NH_3$ concentrations in the air have also been reported.[31-33] $NH_3$ is presumed to be a marker for other air pollutants from livestock farms as ambient air levels are likely too low to cause acute respiratory effects. Additionally, living closer to livestock farms (compared to further away) is associated with increased symptoms (cough, wheezing) and exacerbations in people with chronic obstructive pulmonary disease (COPD).[34,35] Endotoxin, an inflammatory component of the cell wall of gram-negative

bacteria, is a plausible causative agent of such symptoms with a high toxic potential present in livestock emissions.[36] However, the exact causal pathways by which livestock emissions affect respiratory health are still poorly understood. Advances in molecular epidemiology (metagenomic sequencing) have enabled investigation of the respiratory microbiota and its potential role in the causal pathways connecting exposure to livestock farm emissions with associated respiratory problems. Important to note in this context is the increased pneumonia risk observed near goat and poultry farms,[37-39] which initially led to the hypothesis that the airway microbiota could play a role in the respiratory health impacts of livestock emissions. Endotoxin challenges in a mouse model have been shown to elicit an inflammatory response in the airways, potentially disrupting the airway microbiota, leaving them vulnerable to colonization by potentially harmful microorganisms.[40] Plausibility of the mechanistic pathway whereby livestock emissions modulate airway microbiota, was further supported by a study in hospitalized pneumonia patients. This study showed increased abundance of *Streptococcus pneumoniae* (a pathogen not related to livestock and known to thrive on inflamed mucosa) in patients living close to poultry farms compared to patients with no poultry farms near the home address.[41]

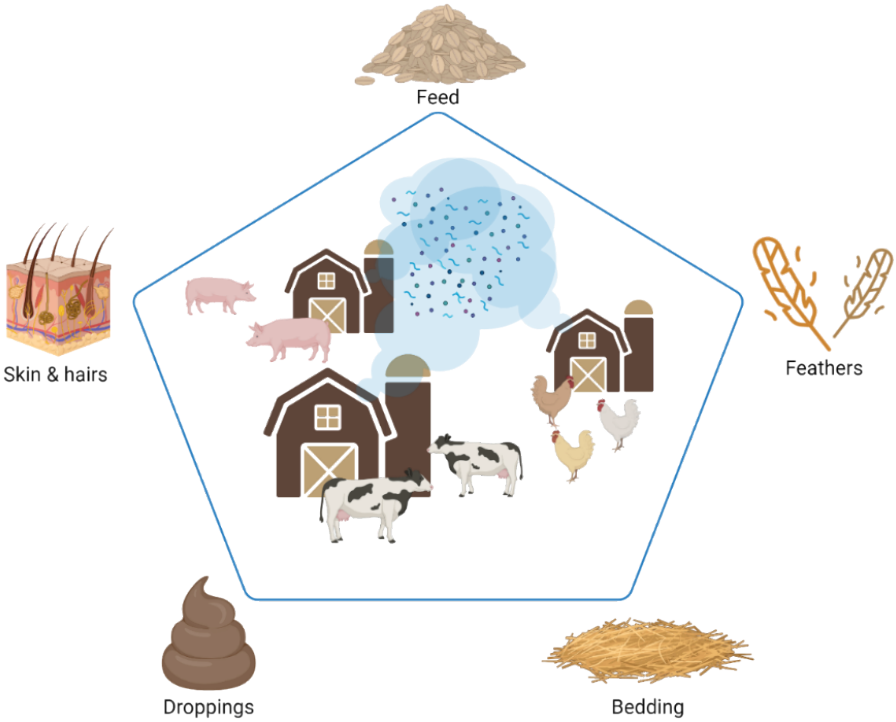


**Figure 2:** Main sources of primary particulate matter emissions from livestock farms

Besides links with adverse health outcomes, associations suggesting a protective effect of livestock exposure have also been reported. Long-term early-life farm exposure (e.g. living on a farm during childhood) is related to a lower risk for allergies, hypersensitivity characterized by production of antibodies called immunoglobulin E.[42] Additionally, people growing up on livestock farms less often develop allergic airway diseases (asthma, allergic rhinitis) compared with individuals without a farm childhood.[42,43] These protective associations were shown to persist later in life.[44-47] More importantly, similar associations have been found with residential livestock exposure. Prevalence of asthma, atopy and COPD was shown to be lower in people living close to livestock farms compared to further away.[30,34,48] Evidence regarding the underlying biology points towards the so called "old friends" hypothesis. This offers a potential explanation centered around exposure to non-pathogenic microbes (that evolved alongside primates), able to interact with the regulatory systems that balance the immune system and prevent overreaction.[49] Recently, endotoxin exposure was suggested to play a role in the protective association of residential livestock exposure with atopy and asthma.[50] In this relationship, however, endotoxin (a pathogen associated molecular pattern) is thought to be a marker for general non-pathogenic livestock-related microbial exposures or other microbial components like glucans or peptidoglycans. The bidirectional relationship of endotoxin exposure with respiratory health calls for research into the role of the airway microbiota in the associations between livestock exposure and human health.[51]

## Importance of elucidating mechanisms behind livestock related health effects

Increasing concerns among general practitioners and local organizations led to the first exploratory studies on health effects of neighboring residents of livestock farms in the Netherlands. While occupational health studies also sparked the interest in public health effects of livestock farming, the occupational and public health risks are far from interchangeable.[29] Occupational exposure to livestock emitted air pollution is incomparable to that in nearby residential areas where levels are much lower. On the other hand, nearby residents (including elderly, young children and people with chronic diseases) are potentially more vulnerable to livestock emissions compared to farmers who comprise a generally healthy working population. Most epidemiological studies investigating the health impact of residential livestock exposures, however, use exposure proxies like distance to the nearest farm or livestock odor, leaving the mechanisms underlying the associations poorly understood.[16] The resulting knowledge gap is not only detrimental for public health. The livestock sector itself

struggles as a result of the lack of knowledge. An example are policies like the 'Goat moratorium', installed in most Dutch provinces as of 2017, with the purpose of prohibiting expansion of goat farming as long as the mechanisms underlying the increased pneumonia risk near goat farms are unclear.[52] The association between pneumonia incidence and living in the vicinity of goat farms, leading to the goat moratorium, was first observed in 2007-2013. While this period overlapped with the Q-fever epidemic of 2007-2010, a later study confirmed the association in 2014-2016 after the epidemic had subsided.[39] Studies aiming to elucidate the underlying causes of the higher pneumonia incidence near goat farms, as well as the societal debate regarding the goat moratorium, are ongoing.

The societal unrest in relation to the environmental and public health impact of livestock farming in the Netherlands and other parts of the world,[53,54] further underline the need for epidemiological studies with advanced individual exposure assessment to elucidate the impact of livestock emitted air pollutants on respiratory health in nearby residents. Exposure assessment to air pollution from livestock farms is complex. Challenges in source attribution of general air pollutants like particulate matter, also emitted by traffic and industry, complicate investigation of dose-response relationships. Recent developments in methods for individual exposure assessment to livestock emissions have enabled further exploration of the impact of livestock attributed emissions on respiratory health and the biological mechanisms involved. Land-use regression and dispersion modelling are two examples of statistical and mathematical tools that have been shown to offer reliable ways to estimate livestock emitted endotoxin and $PM_{10}$.[55] Both methods use livestock characteristics of the surrounding area, dispersion modelling additionally takes into account atmospheric conditions to model exposure downwind of livestock farms. While modelling approaches rely heavily on the reliability of available geospatial data, alternatives (e.g. large scale measurement campaigns) are too costly. The answer lies in a combination of modeling supported by smaller scale validation studies.

## Objectives

The work in this dissertation aims to advance scientific knowledge regarding the respiratory health impact of air pollution from livestock farming in nearby residents. Objectives included:

- Assessing acute and long-term respiratory health impacts of livestock related air pollutants.
- Elucidating the impact of livestock related air pollution on natural lung function decline.
- Exploring the potential role of the airway microbiota composition in respiratory health impacts of livestock related air pollutants.

The research presented in this thesis is part of subsequent collaborative and multidisciplinary projects performed in the Netherlands. Starting in 2012, the VGO project (Dutch acronym of Livestock Farming and Neighboring Residents' Health) aimed at developing insights into the effects of livestock farm emissions in people who were not farmers themselves. The study area encompasses the Southeast of the Netherlands that is known for its large and intensive livestock industry. After an initial questionnaire conducted among 14,882 adults, a subset of 2,494 adults was medically examined in 2014 and 2015. Besides collection of a blood sample, nasal swab, and a fecal sample this examination included lung function testing (spirometry). Further subsets of the medically examined participants were enrolled in sub-studies of the subsequent VGO-2 project, aimed at specific sub-questions like acute effects of livestock emissions and the role of the airway microbiota. During VGO-3, aimed at finding the cause of the increased pneumonia risk found near goat farms, participants of the 2014/2015 medical examination were examined again between 2021 and 2022. This enabled a longitudinal analysis of the impact of air pollution from livestock farms.

The COVID-19 pandemic coincided with the fieldwork of VGO-3, offering a major challenge, but also the opportunity to investigate the impact of an unprecedented global health crisis. The potential role of livestock farms in the outbreak in the Netherlands, was shown when minks (fur animals) at multiple farms were found to be infected with the SARS-CoV-2 virus followed by mink-to-human infections.[56,57] Furthermore, chronic air pollution exposure has been linked to delayed and complicated recovery from COVID-19.[58] Studies assessing the role of air pollution on the transmission and severity of COVID-19 in the Netherlands are currently ongoing. The IMPACT initiative, a successful collaboration effort to employ existing

study populations to investigate the secondary impacts of the pandemic, gave an additional aim to this dissertation:

- Assessment of the impact of COVID-19 containment measures on health and wellbeing, with emphasis on effect modification by chronic disease and urbanization of the residential area.

## Thesis outline

Chapter 2 describes an investigation of the acute respiratory health effects of livestock-related $NH_3$ and $PM_{10}$ in a panel of 82 COPD patients. Chapter 3 describes differences in the respiratory microbiota of the upper respiratory tract between individuals with COPD, compared to control subjects without COPD, in relation to dispersion modelled livestock-related endotoxin and $PM_{10}$ exposure. This Chapter also includes an evaluation of the stability of the oropharyngeal microbiota within individuals over 12 weeks. Chapter 4 reports on the long-term effects of livestock emitted endotoxin and $PM_{10}$ on lung function decline over a seven year period in people living close to livestock farms. In Chapter 5, the impact of government measures to contain the COVID-19 outbreak in the Netherlands on health, health protective behavior and risk perception in the Netherlands are described. The Chapter focuses on differences between individuals with and without chronic disease and residents of urban compared to rural areas. In Chapter 6, the main findings of this dissertation are placed in broader context. The Chapter ends with a discussion regarding future research perspectives and an outlook on the future of livestock farming in relation to human health.

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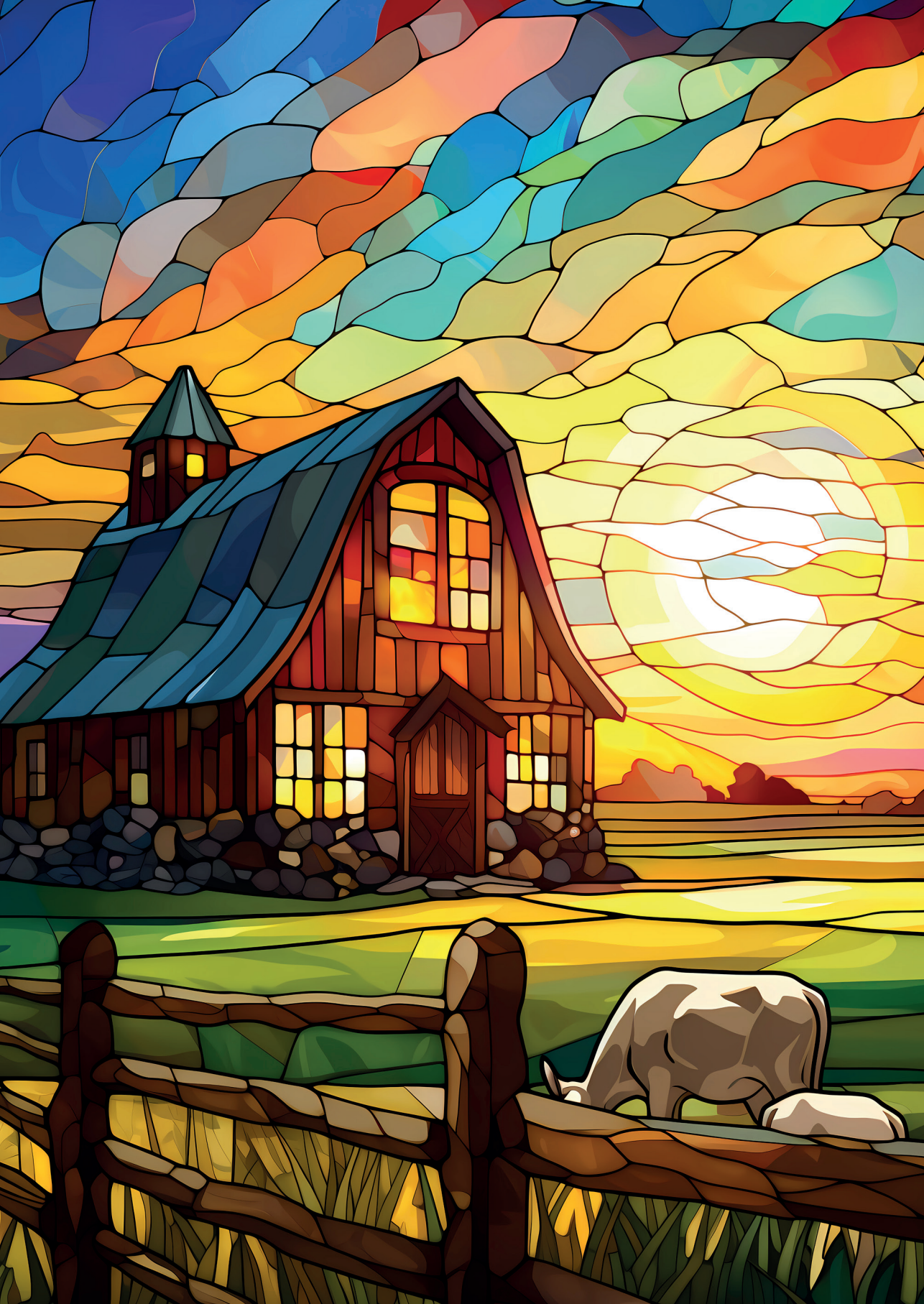

Chapter 2

# Acute respiratory effects of livestock-related air pollution in a panel of COPD patients

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Published in Environment International, March 2020
https://doi.org/10.1016/j.envint.2019.105426

## Abstract

Living close to livestock farms has been associated with increased symptoms in patients with chronic obstructive pulmonary disease (COPD). The causes of these effects are still poorly understood. This panel study attempts to assess the acute effects of livestock-related air pollution in patients with COPD living in an area with intensive livestock farming in the Netherlands.

Between February 2015 and July 2016, 82 participants took spirometry measurements twice daily (morning and evening) during a 3-month period, resulting in 12,672 $FEV_1$ and PEF records. Participants also kept a diary on respiratory symptoms as well as livestock-related odor annoyance. Daily average ammonia ($NH_3$, a proxy for livestock-related air pollution) and fine particulate matter ($PM_{10}$) levels were collected from monitoring stations in the area. Lung function was analyzed as decrements of >10% and >20% from their median as well as absolute values. Self-reported odor annoyance was analyzed as a dichotomous variable. All analyses were done using generalized estimated equations. We adjusted for humidity, temperature, linear trend, and took multiple testing into account.

We found an odds ratio of 1.14 95%CI [1.05;1.25] for decrements >20% in morning $FEV_1$ per interquartile range (12 μg/$m^3$) increase in $NH_3$ concentration (lag 2). Odor annoyance was negatively associated with evening PEF (-4.46 l/min 95%CI [-7.59; -1.33]). Sensitivity analyses showed a stronger effect in participants with worse baseline lung function. No associations with symptoms were found.

Our results show acute effects of livestock-related air pollution on lung function in COPD patients living in close proximity to livestock farms.



### Keywords

Environmental epidemiology, air pollution, livestock, COPD

## Introduction

Several studies on the effects of air pollution in patients with chronic obstructive pulmonary disease (COPD) have reported an increase in morbidity and mortality, emergency room visits, exacerbations and hospitalization rates.[1,2] These studies have mainly been conducted in COPD patients living in urban areas. However, in rural areas with intensive livestock farming, there is emerging evidence of health effects due to air pollution episodes, especially resulting from primary particulate matter and ammonia ($NH_3$) emissions as well as $NH_3$-related reaction products (secondary inorganic particles).[3] A recent study in the Netherlands showed that week-average $NH_3$ levels before lung function measurements were associated with lung function deficits in 2,308 nonfarming residents, suggesting that acute respiratory effects may occur due to temporally elevated $NH_3$ levels.[4]

Evidence for a relationship between livestock-related air pollutants and acute respiratory effects has also been found in multiple longitudinal panel studies amongst agricultural communities in the United States. In 2011, a study performed in North Carolina found that fine particulate matter with a diameter up to 2.5 µm ($PM_{2.5}$) was associated with a decline in the forced expiratory volume in one second ($FEV_1$) in healthy volunteers near swine feeding operations.[5] A study published in 2015 reported similar associations amongst asthmatic school-aged children in rural Washington State. Both increasing asthma morbidity and decrements in $FEV_1$ were associated with week average $PM_{2.5}$.[6] Later that year in the same area and cohort, an increase in $NH_3$ concentration was reported to be associated with decrements in $FEV_1$.[7] Besides effects from $PM_{2.5}$ and $NH_3$, associations between livestock odor annoyance and respiratory symptoms have also been reported.[5,8]

A cross-sectional German study has shown a lower $FEV_1$ in subjects exposed to higher annual (livestock farm emitted) $NH_3$ levels.[9] A similar spatial association between the number of stables in a 500m buffer around the home address and a lower $FEV_1$ was found in adults.[10] In several epidemiological and experimental studies, COPD patients appeared to be the most susceptible group for livestock farm exposures, showing increases in respiratory symptoms, exacerbations and inflammatory responses.[4,11-13]

To the best of our knowledge, the acute effects of livestock-related air pollution have not yet been investigated in patients with COPD, while one can hypothesize that the adverse respiratory effects described in healthy individuals will be more pronounced in this susceptible subgroup. The aim of this prospective panel study is therefore to assess the acute respiratory health effects of short-term exposure to $NH_3$, particulate

matter <10 μm in diameter ($PM_{10}$) and livestock farm odor in patients with COPD in a livestock-dense area in the Netherlands.

## Materials and Methods

### Study population and design

Study participants were selected from the cross-sectional Dutch 'Livestock farming and neighboring residents' health' study (VGO) population, of which the design and selection process have been described in detail.[11] Briefly, this general population sample was recruited from a questionnaire survey conducted among patients of 27 general practitioners. Out of the 14,882 participating adults (aged 18-70 years), 2,494 non-farming residents underwent medical examination in 2014-2015.[14] The medical examination included a lung function test and an extensive health questionnaire. Participants with COPD (n=213), who produced a good quality lung function test according to ATS/ERS criteria, were invited by mail to participate in this panel study, of which 117 agreed to participate (response 55%). Subjects who reported to be current smokers (n=27) were *a priori* excluded to avoid effect modification by tobacco smoke exposure. To investigate whether the effect of livestock-related air pollution differs for participants with different COPD definitions, we selected participants falling within one of the following three categories: 1) a post-bronchodilator (BD) measurement of $FEV_1$/FVC below the lower limit of normal or below 0.7 (Global Initiative for Chronic Obstructive Lung Disease); 2) a pre-BD measurement of $FEV_1$/FVC below 0.7 and wheezing, dyspnea or shortness of breath; 3) self-reported COPD, defined as a positive answer to the question: 'Have you ever been told by a doctor that you had COPD or emphysema?'.[14] Eight participants did not complete the study, or were excluded from data analysis because of current smoking at the time of the panel study. This resulted in a study population of 82 participants with COPD, see supplementary figure 1 for a flowchart of the selection procedure. The study protocol (no. 13/533) was approved by the Medical Ethical Committee of the University Medical Centre Utrecht. All participants signed informed consent.

### Data collection

Over a three month period, participants were asked to conduct morning and evening peak expiratory flow (PEF) and $FEV_1$ measurements, and to fill out an online diary on farm odor annoyance, medication use and respiratory symptoms (Appendix 1). Measurements were performed after waking or before going to bed in order to test the hypothesis that most exposure occurs overnight. Data collection started after an initial home visit by a fieldworker who explained the use of the spirometer (Asma-1

Monitor, Vitalograph, Buckingham, UK) and online diary. Instructions included taking measurements in an upright position and before taking medication. Participants were visited again after approximately six weeks to monitor their compliance, and one last time at the end of the study. The same fieldworker (M.O.) conducted all visits in this study. During each visit, an additional health questionnaire was completed and spirometry measurements were performed to monitor health status and spirometer use. The correlation between the mean $FEV_1$ measured by participants using the Asma-1 monitor and their baseline $FEV_1$ measured by spirometry during the earlier medical examination (according to GOLD initiative standards)[14] was found to be > 0.9. The data collection period lasted 491 days between February 15th 2015 and June 20th 2016. On average, individuals were observed in this study for 90 days (± 16) with a maximum of 20 participants enrolled simultaneously.

We distinguished morning and evening spirometry measurements based on a cut-off time at 13:00. Measurements between 00:00 and 01:59 were considered evening measurements that belonged to the previous day. Occasional measurements between 02:00 and 04:00 were evaluated individually based on the measurement pattern of that participant. Data completeness for the diary, morning and evening spirometry was 94.4%, 86.3% and 84.8%, respectively. The average time of measurement was 08:38 (± 01:22) in the morning and 20:59 (± 02:16) in the evening. In total, participants collected 12,673 good quality (best of three successful attempts) PEF and $FEV_1$ records.

## Air pollution exposure

Average daily ambient levels of $NH_3$ and $PM_{10}$ were obtained from the Dutch Air Quality Monitoring Network.[15] Daily levels were computed using the mean from two measurement stations. The distance between the stations and the participants' home addresses ranged from 2km to 40km with an average of 23km. Additionally, participants recorded daily livestock-related odor annoyance on a scale from 0 to 10 as a proxy for livestock exposure, with a higher score indicating more odor annoyance. We obtained data on daily ambient temperature and relative humidity from a weather station close to the study area, at the courtesy of the Royal Netherlands Meteorological Institute (KNMI).

## Statistical analysis

Relationships between morning and evening PEF and $FEV_1$, symptoms and (livestock-related) air pollution exposure were analyzed using generalized estimated equations (GEE) assuming a first order autoregressive (AR1) correlation structure. The AR1 assumption that correlation between observations decreases with time logically fits

our dataset with numerous observations per subject better than an exchangeable correlation structure. We included air pollution exposure from the same day (lag 0), the previous day (lag 1) or two days before (lag 2) the lung function measurements. Both single- and two-pollutant models were explored. Besides symptoms (wheezing, cold or flu, shortness of breath in rest) and absolute lung function values, decreases in PEF or $FEV_1$ values from an individual's median value greater than 10% and 20% were analyzed as more clinically relevant dichotomous response variables.[16] All models were adjusted for daily mean ambient temperature, relative humidity and day-in-study (linear trend). Sensitivity analyses were conducted to investigate the effect of the different COPD definitions as well as effects introduced by differences in active participants grouped by year in the study period (2015/2016). Model estimates are expressed per interquartile range increase (IQR) in $NH_3$ and $PM_{10}$. To limit the influence of differences in odor perception, odor annoyance scores were dichotomized before analysis. Therefore, the estimates for those models are expressed as changes on days with odor annoyance, compared to days without odor annoyance. All analyses were performed in R version 3.5.1 using the package Geepack.[17] Model p-values were corrected for multiple testing using the Benjamini–Hochberg procedure with a false discovery rate of 10%.[18]

## Results

### Study population

A detailed description of participant characteristics per COPD definition is given in Table 1. Participants were on average 61.4 years old and 43% was female. Ex-smokers made up 70% of the study population with an average smoking history of 13.1 pack-years. Forty-three participants had COPD according to definition 1 (a post-bronchodilator (BD) measurement of $FEV_1$/FVC below the lower limit of normal or below 0.7). Categories 2 (a pre-BD measurement of $FEV_1$/FVC below 0.7 and wheezing, dyspnea or shortness of breath) and 3 (self-reported) each held 22 and 17 participants, respectively. Participants had on average 10 livestock farms within 1km from their homes and the average distance to the nearest farm was 437m. The study area is characterized by a large number of dairy, pig, and poultry farms as previously described in detail.[14]

### Air pollution exposure and odor annoyance

Figure 1A shows the daily mean $NH_3$ and $PM_{10}$ concentrations over the study period. The interquartile ranges for $NH_3$ and $PM_{10}$ were 12.0 and 11.3 $\mu g/m^3$, respectively. A steep increase in $NH_3$ levels was observed in the Spring of 2016. The proportion

of reported odor annoyance is highest in the first half of the entire study period (Figure 1). This is probably explained by the lower mean number of farms within 1km from the home address amongst participants active in 2016 compared to those in 2015 (-5 farms; 95% CI [-9; -1]).

## Air pollution and lung function

The most consistent associations between air pollutant levels and lung function were found for $NH_3$ levels (lag 2) and $FEV_1$ (Table 2). The models for morning $FEV_1$ decrements (dichotomous) in relation to an IQR increase in $NH_3$ (lag 2) showed statistically significant odds ratios (OR) 1.06; 95% confidence interval (95%CI) [1.00;1.13] for a 10% decline and 1.14; 95% CI [1.05;1.25] for a >20% decline (Figure 2). The association between $NH_3$ (lag 2) and >20% decrements in morning $FEV_1$ remained significant after correction for multiple testing (Supplementary Table 2). The models for $NH_3$ (lag 2) and evening $FEV_1$ decrements >10% followed the same trend with an OR of 1.07, 95%CI [1.01,1.13], while the OR for a >20% decrement was 0.89, 95%CI [0.88,1.10]. No associations with dichotomized PEF decrements where observed.

For analyses of lung function as a continuous variable, a weak negative association was seen between $NH_3$ (lag 2) and morning $FEV_1$ (β=-3.1ml, 95% CI [-6.9,0.6]) (Table 2). Lag 2 $NH_3$ also showed weak negative associations with evening $FEV_1$ and both PEF variables. Lag 0 and Lag 1 $NH_3$ showed weak effects in both directions. No associations were found between $NH_3$ levels and respiratory symptoms (Supplementary Figure 2). A similar but weaker pattern was found for $PM_{10}$. An OR of 1.11 95%CI [1.01;1.23] was found for decrements of >10% in morning PEF with an IQR increase in lag0 $PM_{10}$ (Figure 2). For all lags, $PM_{10}$ showed effects in both directions across the lung function variables. $PM_{10}$ (Lag 1) showed a negative association with evening $FEV_1$ (β=-4.8 ml, 95% CI [-9.5,-0.2]) (Table 2). None of the associations with $PM_{10}$ were significant after adjustment for multiple testing. Little difference was seen between single- and two-pollutant models (Table 2). $PM_{10}$ showed no effects on symptoms (Supplementary Figure 2).

Models with odor annoyance as an exposure proxy revealed an association between odor annoyance (Lag 0) and evening PEF (β=-4.5 l/min 95%CI [-7.6,-1.3]), which remained statistically significant after adjustment for multiple testing (Table 2). No relation was found between symptoms and odor annoyance.

## Sensitivity analyses

Models with an interaction term between exposure and COPD definition revealed that the described effects are the strongest in participants with COPD definition

1. Participants with COPD definition 1 show an OR of 1.23 95%CI [1.12,1.36] for decrements in $FEV_1$ >20% per interquartile range increase of $NH_3$ (lag 2). For the same relationship, participants with COPD definition 2 and 3 show OR of 0.98 95%CI [0.93,1.03] and 1.13 95%CI [1.01,1.29] respectively (Supplementary Figure 3). Results for the stratified analysis of odor annoyance and evening PEF are shown in Supplementary Table 3. A sensitivity analysis, using models stratified over study year, showed that the association between lung function and $NH_3$ (lag 2) is strongest in 2016. Similarly, the effect of $PM_{10}$ (lag 1) on evening $FEV_1$ was slightly more pronounced in 2016 compared to 2015. (Supplementary Table 1).

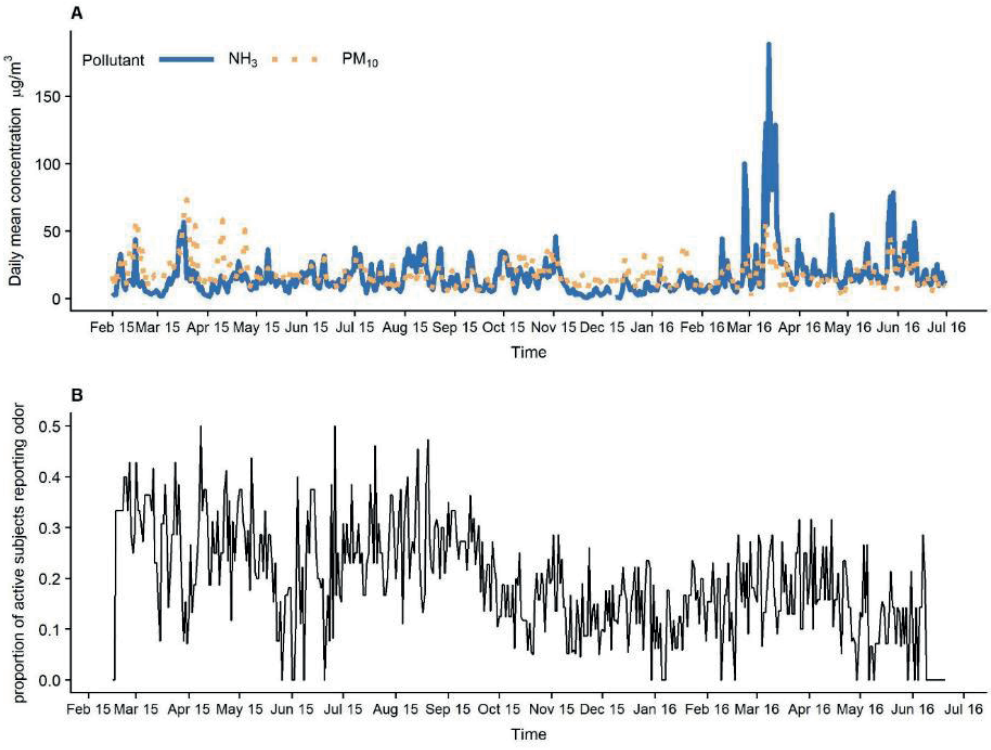


**Figure 1: A:** Daily mean NH3 and PM10 concentration over the study period (February 2015-June 2016); **B:** The proportion of active subjects reporting livestock odor over the study period. Participants collected data for 90 days with a maximum of 20 active participants at a time

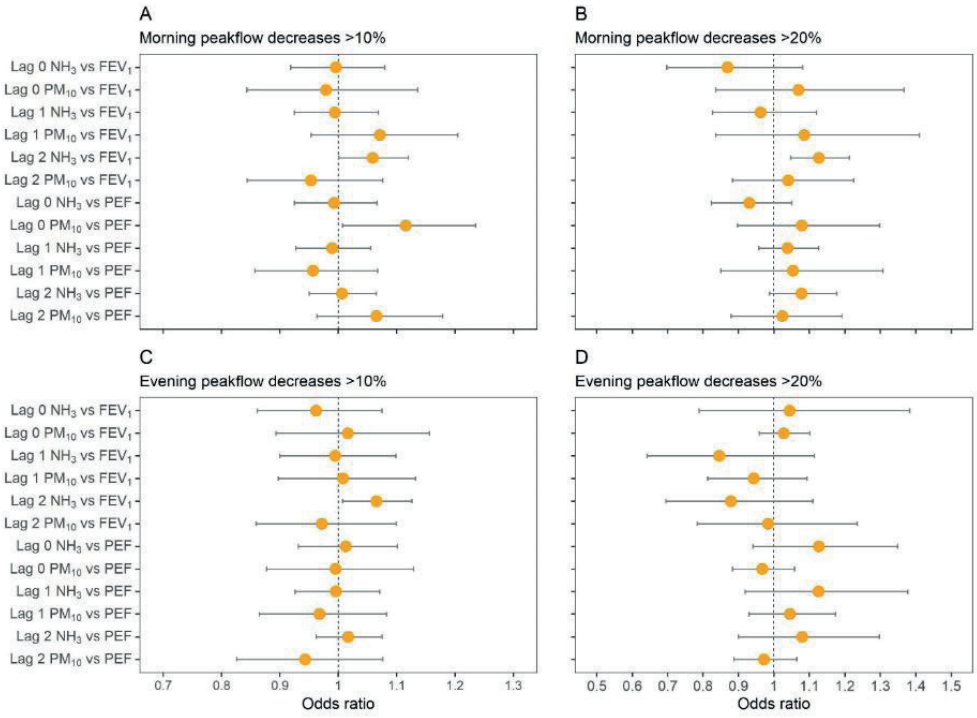


**Figure 2:** Effects of an IQR increase in air pollutant levels on (**A**) morning peak flow decreases >10% from median; (**B**) morning peak flow decreases >20% from median; (**C**) evening peak flow decreases >10% from median; and (**D**) evening peak flow decreases >20% from median, expressed as odds ratio and 95% confidence interval

**Table 1:** Study population characteristics per COPD definition

| | COPD Definition | | |
|---|---|---|---|
| **Characteristics** | **1** | **2** | **3** |
| n | 43 | 22 | 17 |
| Age, yr | 60.8 ± 9.7 | 63.5 ± 5.4 | 60.4 ± 7.4 |
| Female | 15 (34.9) | 9 (40.9) | 11 (64.7) |
| BMI* | 27.3 ± 5.3 | 28.0 ± 3.7 | 26.1 ± 4.0 |
| Former smoker | 32 (74.4) | 15 (68.2) | 10 (58.8) |
| Pack-years† | 15.5 ± 15.2 | 9.0 ± 10.5 | 12.2 ± 15.3 |
| Education level | | | |
| Low | 10 (23.3) | 5 (22.7) | 5 (29.4) |
| Medium | 24 (55.8) | 10 (45.5) | 4 (23.5) |
| High | 9 (20.9) | 7 (31.8) | 8 (47.1) |
| Lung function characteristics | | | |
| Pre-BD measurement % predicted | | | |
| $FEV_1$ | 71.3 ± 17.4 | 80.8 ± 12.4 | 100.7 ± 14.0 |
| FVC | 96.9 ± 17.5 | 96.1 ± 15.8 | 105.8 ± 15.2 |
| $FEV_1$/FVC | 72.8 ± 10.0 | 84.0 ± 6.1 | 95.0 ± 7.3 |
| Post-BD measurement % predicted | | | |
| $FEV_1$ | 75.7 ± 17.4 | 87.7 ± 13.4 | 105.8 ± 12.5 |
| FVC | 99.0 ± 16.0 | 99.5 ± 17.3 | 108.4 ± 14.1 |
| $FEV_1$/FVC | 75.8 ± 10.7 | 88.1 ± 4.6 | 97.6 ± 8.3 |
| Coughing most days | 14 (32.6) | 7 (33.3) | 10 (58.8) |
| Phlegm most days | 11 (26.8) | 6 (30.0) | 9 (52.9) |
| Wheezing most days | 21 (48.8) | 19 (86.4) | 8 (47.1) |
| Nasal allergies | 14 (32.6) | 12 (54.5) | 7 (41.2) |
| Farms within 1km from home address | 12 ± 8 | 11 ± 7 | 10 ± 6 |
| Distance to closest farm, m | 429 ± 251 | 393 ± 226 | 512 ± 347 |

*Definition of abbreviations:* BD=bronchodilator; BMI=body mass index; COPD=chronic obstructive pulmonary disease; $FEV_1$=forced expiratory volume in 1 second; FVC=forced vital capacity; PEF=peak expiratory flow. Data are presented as mean ± SD or n (%). Education levels: low—lower secondary school or less; medium—intermediate vocational education or upper secondary school; high—higher vocational education or university. *BMI=mass(kg)/(height (m))$^2$. †Mean pack-years for former smokers. COPD definitions: 1—a post-BD measurement of $FEV_1$/FVC below the lower limit of normal or below 0.7 (Global Initiative for Chronic Obstructive Lung Disease); 2— a pre-BD measurement of $FEV_1$/FVC below 0.7 and wheezing, dyspnea or shortness of breath; 3—self reported, defined as a positive answer to the question: 'Have you ever been told by a doctor that you had chronic obstructive pulmonary disease or emphysema?'[14]. Participants in group 2 did not meet criteria for group 1, participants in group 3 did not meet criteria for group 1 and 2.

**Table 2.** Associations between air pollutant levels, livestock odor annoyance and lung function

| Model | $FEV_1$ morning (ml) | | | $FEV_1$ evening (ml) | | | PEF morning (l/min) | | | PEF evening (l/min) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Estimate | 95% CI | q-value | Estimate | 95% CI | q-value | Estimate | 95% CI | q value | Estimate | 95% CI | q-value |
| **single pollutant (per IQR)** | | | | | | | | | | | | |
| Lag 0 $NH_3$ | 0.88 | -3.34 ; 5.10 | 0.91 | 0.70 | -2.74 ; 4.14 | 0.82 | -0.49 | -1.39 ; 0.41 | 0.56 | 0.45 | -0.46 ; 1.36 | 0.61 |
| Lag 0 $PM_{10}$ | -3.12 | -8.04 ; 1.79 | 0.76 | 0.92 | -4.86 ; 6.70 | 0.82 | -1.17 | -2.38 ; 0.04 | 0.41 | -0.61 | -2.05 ; 0.83 | 0.61 |
| Lag 1 $NH_3$ | 0.05 | -2.58 ; 2.69 | 0.97 | -1.84 | -6.42 ; 2.74 | 0.82 | -0.34 | -1.06 ; 0.37 | 0.56 | -0.45 | -1.49 ; 0.58 | 0.61 |
| Lag 1 $PM_{10}$ | 1.73 | -3.14 ; 6.60 | 0.91 | -4.88 | -9.55 ; -0.21 | 0.49 | 1.04 | -0.31 ; 2.38 | 0.41 | 0.97 | -0.16 ; 2.11 | 0.56 |
| Lag 2 $NH_3$ | -3.14 | -6.86 ; 0.59 | 0.76 | -0.89 | -4.07 ; 2.30 | 0.82 | -0.29 | -1.36 ; 0.77 | 0.79 | -0.16 | -0.92 ; 0.59 | 0.74 |
| Lag 2 $PM_{10}$ | 0.75 | -4.01 ; 5.50 | 0.91 | 1.89 | -4.28 ; 8.05 | 0.82 | -0.01 | -1.44 ; 1.42 | 0.99 | 0.26 | -1.24 ; 1.75 | 0.74 |
| **multiple pollutant (per IQR)** | | | | | | | | | | | | |
| Lag 0 $NH_3$ | 1.05 | -3.41 ; 5.52 | 0.76 | 0.68 | -3.39 ; 4.75 | 0.82 | -0.41 | -1.32 ; 0.50 | 0.41 | 0.62 | -0.44 ; 1.68 | 0.61 |
| Lag 0 $PM_{10}$ | -3.09 | -8.41 ; 2.22 | 0.76 | -0.19 | -7.32 ; 6.94 | 0.82 | -1.01 | -2.35 ; 0.33 | 0.41 | -0.88 | -2.53 ; 0.76 | 0.61 |
| Lag 1 $NH_3$ | -0.10 | -2.83 ; 2.63 | 0.91 | -1.52 | -7.39 ; 4.35 | 0.82 | -0.49 | -1.21 ; 0.23 | 0.41 | -0.83 | -2.06 ; 0.39 | 0.51 |
| Lag 1 $PM_{10}$ | 0.85 | -4.14 ; 5.84 | 0.91 | -3.75 | -9.12 ; 1.62 | 0.82 | 1.29 | -0.16 ; 2.74 | 0.41 | 1.36 | 0.04 ; 2.68 | 0.51 |
| Lag 2 $NH_3$ | -3.24 | -7.42 ; 0.93 | 0.76 | -0.94 | -4.57 ; 2.69 | 0.82 | -0.17 | -1.21 ; 0.86 | 0.89 | -0.26 | -1.03 ; 0.51 | 0.68 |
| Lag 2 $PM_{10}$ | 2.32 | -3.32 ; 7.95 | 0.76 | 2.41 | -3.77 ; 8.60 | 0.82 | -0.02 | -1.55 ; 1.51 | 0.89 | 0.40 | -1.09 ; 1.90 | 0.68 |
| **Odor annoyance (yes/no)** | | | | | | | | | | | | |
| Lag 0 | -6.45 | -18.9 ; 6.00 | 0.76 | -2.68 | -15.49 ;10.13 | 0.82 | 1.04 | -2.61 ; 4.70 | 0.77 | -4.46 | -7.59 ; -1.33 | 0.06 |
| Lag 1 | 0.26 | -10.76 ; 11.27 | 0.96 | 5.35 | -9.71 ; 20.40 | 0.76 | -0.86 | -3.18 ; 1.45 | 0.76 | 1.84 | -1.59 ; 5.26 | 0.76 |
| Lag 2 | 3.65 | -7.10 ; 14.40 | 0.76 | -1.40 | -15.56 ; 12.76 | 0.92 | 1.10 | -1.80 ; 3.99 | 0.76 | 1.84 | -1.64 ; 5.33 | 0.76 |

## Discussion

Our results show acute effects of livestock-related air pollution on lung function of COPD patients. The described associations with $NH_3$ are more pronounced compared to those with $PM_{10}$, which suggests that livestock farm emissions are the driver of the effect. The OR (1.14, 95%CI [1.05;1.25]) we found for the effect of an IQR increase in $NH_3$-levels (lag 2) on decrements in $FEV_1$ of >20% from the individual median, supports the findings of a recent cross-sectional study conducted in the same area amongst 2,308 adults. In that study, a 25 μg/m$^3$ increase in week-average $NH_3$ was associated with a 2.22% lower $FEV_1$.[4] Similarly, two longitudinal panel studies in the United States conducted amongst children with asthma reported acute effects of exposure to $NH_3$ and $PM_{2.5}$ on $FEV_1$.[6,7] Temporal effects of farm-related pollutants on both $FEV_1$ and self-reported respiratory symptoms were also reported by a third American panel study amongst 101 healthy adults.[5]

An earlier study in the Netherlands showed that a closer residential distance to one or more livestock farms was associated with wheezing among COPD patients.[11] Two American studies also found associations between residential proximity to livestock farms and self-reported respiratory health.[19,20] The association we found between self-reported odor annoyance and a lower evening PEF is another indication for acute effects of livestock farm emissions on lung function. Another Dutch study has shown a positive relationship between modelled odor exposure (based on the presence of livestock farms) and reported odor annoyance, as well as an increase in odor annoyance over the last decade.[21] A Danish study reported an association between residential exposure to $NH_3$ and increased respiratory symptoms, mediated by odor annoyance.[22] Reported odor annoyance did not increase with $NH_3$ in our study. This is likely due to other (e.g. sulfur) compounds that also contribute to livestock odor.[23] The odor threshold for $NH_3$ lies between 5 and 53 ppm.[3] The maximum daily mean $NH_3$ level measured at the central monitor during the study period was 188.6 μg/m$^3$ or 0.27 ppm, making it unlikely to be registered as odor annoyance. The steep increase in $NH_3$ levels measured in the spring of 2016 seems to coincide with the period in which farmers apply most of their manure on agricultural fields. The absence of an increase of similar magnitude in 2015 could be explained by differences in atmospheric conditions. We did not find significant associations between $PM_{10}$ and lung function. In a meta-analysis of panel studies on acute effects of (urban) air pollution among patients with COPD, Bloemsma et al. found a small overall effect of $PM_{10}$ on $FEV_1$ (–3.38 mL, 95%CI –6.39 to –0.37) with considerable heterogeneity of the outcomes.[24]

At the core of its design a panel study has repeated measurements on individuals at fixed short time intervals. Its strengths lie in detailed individual participants' data as well as the ability to control for (unmeasured) confounders that are stable over time, because every individual acts as her/his own control. Furthermore the traditional alternative of the registry-based time series does not detect specific health outcomes like (subclinical) changes in lung function and symptoms.[25] Our study has a few limitations. First, as in most panel studies, measurement error due to the use of central site monitoring is inevitable. The relatively large population size and long duration of our panel study precluded measuring daily personal air pollutant exposure. However, the correlation between the two monitoring sites was moderate to high ($NH_3$: 0.53 and $PM_{10}$: 0.90) and in a panel design one models fluctuations over time instead of spatial differences between participants. Therefore, the impact on the associations is deemed to be low. Selection bias could have influenced the associations with odor annoyance as participants might have joined the study based on their view on livestock farming. However, only 12 out of 82 (14.6%) participants reported that they attribute their health complaints to livestock farms. A recent study, conducted in the population from which our participants were selected, showed that there was no association between lung function and attitude toward livestock farming.[26] The mean effects of day-to-day variations in air pollutant concentrations on lung function tend to be small as seen in similar studies.[24] We do however see a more pronounced effect on large decrements in lung function indicating that there is an especially vulnerable group within our population. Based on the stratified analysis according to COPD definition, participants with COPD defined as fixed airway obstruction showed the strongest association with $NH_3$ exposure. Given that these participants already have an irreversible airway obstruction, a further >20% decrement in lung function can be clinically relevant, although we did not detect an increase in reported symptoms. Similar observations were made for the relationship between odor annoyance and evening PEF (Supplementary Table 3). A final point to address is the representativeness of the COPD patients selected from a general population sample. We used different operational definitions of COPD to classify the, mainly mild, COPD patients.[14] COPD is a heterogeneous condition, and, especially in older patients a substantial overlap exists between COPD and asthma making for a complex diagnosis.[27] A priori, we hypothesized that the effect of exposure is unlikely to be differential for the different chronic lung disease phenotypes.

Despite livestock farming being indicated as the driver, the exact mechanisms behind the observed associations remain elusive. At the concentrations measured during the present study, it is unlikely that $NH_3$ is directly responsible for the observed effect. However, the fact that the described associations with $NH_3$ are more pronounced

compared to those with $PM_{10}$ does suggests that livestock farm emissions are the driver of the effect. A possible explanation is that both $NH_3$ and odor annoyance act as proxies for a complex mixture of air pollutants. The described effect of $NH_3$ could also be caused by $NH_3$-related secondary inorganic particles. It was previously found that $NH_3$ accounts for more than half of the secondary inorganic particles that make up the $PM_{10}$ fraction in the study area.[28] A recent study in China found that reducing livestock $NH_3$ emissions would be highly effective at reducing these secondary particles.[29]

## Conclusion

Our longitudinal panel study has shown acute effects of livestock-related air pollutants on the lung function of COPD patients. Our results indicate that the effects of $NH_3$ exposure are more pronounced in patients with fixed airway obstruction. Our results add to a growing body of evidence showing that agricultural sources of air pollution are of public health relevance.

## Acknowledgements

The authors are grateful to Dominique Hamerlijnck and Betty Frankemölle (patient representatives of the Lung Foundation Netherlands) for their invaluable input on data collection and communication materials. We thank Eef van Otterloo for data management and design of the online diary, and 'Stichting Informatie Voorziening Zorg' (Trusted Third Party) for ensuring participants' privacy.

## Funding

This study was supported by a grant from the Lung Foundation Netherlands (Grant number: 3.2.11.022).

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Chapter 2

# Supplementary Materials

# Appendix 1

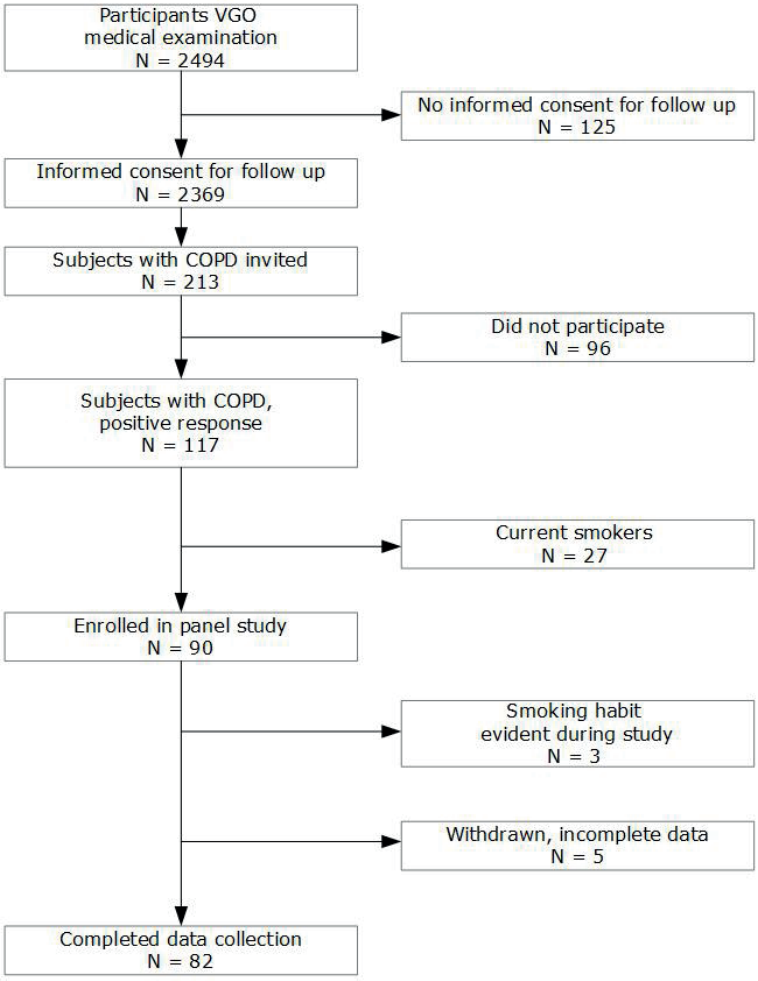
## Content of the diary kept by participants

1. Did you complete the peak flow measurements? (0 = no 1= yes)
   a. Morning
   b. Evening

2. How much time did you spend away from home? (cumulative)
3. Did anyone smoke in your presence? (0 = no 1= yes)
4. Which number between 0 and 10 best describes the severity of livestock odor in and around your home? (0 = no odor, 10 = extreme odor)
5. Note the time at which you took additional respiratory medication and the number of puffs administered.
   a. First time
   b. Second time
   c. Third time

6. Did you suffer from the following complaints today?
   (0=no, 1=mild, 2=moderate/severe)
   a. Waking up with breathing problems
   b. Sleep problems
   c. Shortness of breath in rest
   d. Shortness of breath after exertion
   e. Wheezing
   f. Coughing
   g. Producing / coughing up phlegm
   h. Sore throat
   i. Eye irritation
   j. Nasal complaints (sneezing, irritation, stuffy nose)
   k. Cold or flu
   l. Skin problems
   m. Unusual tiredness
   n. Headache
   o. Nausea
   p. Feeling fearful, nervous or tense
   q. Feeling depressed

# Supplementary Tables and Figures




**Supplementary figure 1:** Flowchart of the study population

**Supplementary Table 1:** Associations between air pollutant levels and lung function stratified by year, original unstratified estimates included

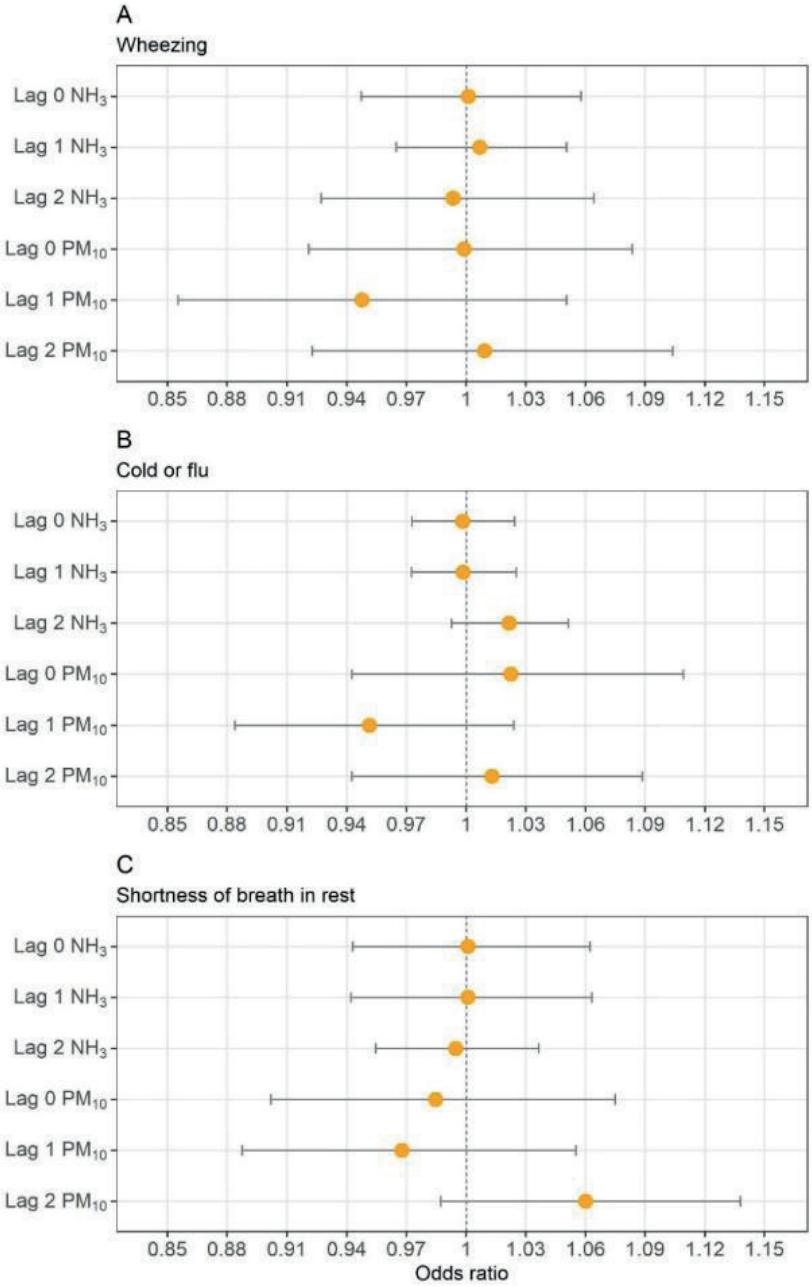
| | FEV1 morning (ml) | | | FEV 1 evening (ml) | | | PEF morning (l/min) | | | PEF evening (l/min) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Estimate** | **95% CI** | **q-value** | **Estimate** | **95% CI** | **q-value** | **Estimate** | **95% CI** | **q-value** | **Estimate** | **95% CI** | **q-value** |
| **lag 0 (per IQR)** | | | | | | | | | | | | |
| *Original* $NH_3$ | 0.88 | -3.34 ; 5.10 | 0.91 | 0.70 | -2.74 ; 4.14 | 0.82 | -0.49 | -1.39 ; 0.41 | 0.56 | 0.45 | -0.46 ; 1.36 | 0.61 |
| 2015 $NH_3$ | -2.19 | -7.13 ; 2.74 | 0.74 | 0.28 | -4.49 ; 5.04 | 0.98 | -0.24 | -1.58 ; 1.1 | 0.87 | -0.26 | -1.31 ; 0.79 | 0.85 |
| 2016 $NH_3$ | 2.39 | -3.38 ; 8.16 | 0.74 | 0.91 | -3.67 ; 5.48 | 0.98 | -0.61 | -1.79 ; 0.58 | 0.68 | 0.84 | -0.39 ; 2.07 | 0.85 |
| *Original* $PM_{10}$ | -3.12 | -8.04 ; 1.79 | 0.76 | 0.92 | -4.86 ; 6.70 | 0.82 | -1.17 | -2.38 ; 0.04 | 0.41 | -0.61 | -2.05 ; 0.83 | 0.61 |
| 2015 $PM_{10}$ | -0.99 | -6.54 ; 4.56 | 0.79 | -0.08 | -6.04 ; 5.87 | 0.98 | -1.10 | -2.47 ; 0.27 | 0.68 | -0.47 | -2.1 ; 1.15 | 0.85 |
| 2016 $PM_{10}$ | -7.91 | -17.71 ; 1.88 | 0.68 | 2.80 | -8.86 ; 14.46 | 0.98 | -1.33 | -3.9 ; 1.24 | 0.68 | -0.92 | -3.75 ; 1.9 | 0.85 |
| **lag 1 (per IQR)** | | | | | | | | | | | | |
| *Original* $NH_3$ | 0.05 | -2.58 ; 2.69 | 0.97 | -1.84 | -6.42 ; 2.74 | 0.82 | -0.34 | -1.06 ; 0.37 | 0.56 | -0.45 | -1.49 ; 0.58 | 0.61 |
| 2015 $NH_3$ | 2.07 | -1.57 ; 5.71 | 0.74 | 0.16 | -4.86 ; 5.19 | 0.98 | -0.80 | -2 ; 0.41 | 0.68 | -0.37 | -1.69 ; 0.95 | 0.85 |
| 2016 $NH_3$ | -1.03 | -4.41 ; 2.36 | 0.74 | -2.93 | -9.37 ; 3.51 | 0.92 | -0.11 | -0.99 ; 0.78 | 0.89 | -0.50 | -1.93 ; 0.92 | 0.85 |
| *Original* $PM_{10}$ | 1.73 | -3.14 ; 6.60 | 0.91 | -4.88 | -9.55 ; -0.21 | 0.49 | 1.04 | -0.31 ; 2.38 | 0.41 | 0.97 | -0.16 ; 2.11 | 0.56 |
| 2015 $PM_{10}$ | 1.44 | -4.22 ; 7.1 | 0.74 | -3.79 | -9.51 ; 1.93 | 0.77 | 0.09 | -1.49 ; 1.67 | 0.91 | 1.19 | -0.23 ; 2.6 | 0.85 |
| 2016 $PM_{10}$ | 2.46 | -6.98 ; 11.9 | 0.74 | -7.57 | -15.36 ; 0.23 | 0.68 | 3.34 | 1.05 ; 5.64 | 0.05 | 0.45 | -1.41 ; 2.3 | 0.85 |
| **lag 2 (per IQR)** | | | | | | | | | | | | |
| *Original* $NH_3$ | -3.14 | -6.86 ; 0.59 | 0.76 | -0.89 | -4.07 ; 2.30 | 0.82 | -0.29 | -1.36 ; 0.77 | 0.79 | -0.16 | -0.92 ; 0.59 | 0.74 |
| 2015 $NH_3$ | 0.56 | -4.43 ; 5.55 | 0.83 | -3.33 | -8.34 ; 1.69 | 0.77 | 0.31 | -0.94 ; 1.55 | 0.84 | -0.34 | -1.65 ; 0.96 | 0.85 |
| 2016 $NH_3$ | -5.10 | -9.85 ; -0.35 | 0.43 | 0.43 | -3.47 ; 4.32 | 0.98 | -0.65 | -2.15 ; 0.85 | 0.68 | -0.08 | -1.03 ; 0.88 | 0.96 |
| *Original* $PM_{10}$ | 0.75 | -4.01 ; 5.50 | 0.91 | 1.89 | -4.28 ; 8.05 | 0.82 | -0.01 | -1.44 ; 1.42 | 0.99 | 0.26 | -1.24 ; 1.75 | 0.74 |
| 2015 $PM_{10}$ | 1.97 | -3.81 ; 7.75 | 0.74 | 2.90 | -3.63 ; 9.43 | 0.92 | 0.58 | -1.01 ; 2.17 | 0.71 | 0.34 | -1.59 ; 2.27 | 0.88 |
| 2016 $PM_{10}$ | -3.39 | -11.51 ; 4.74 | 0.74 | -0.30 | -14.23 ; 13.62 | 0.98 | -1.55 | -4.94 ; 1.84 | 0.68 | 0.06 | -2.33 ; 2.45 | 0.96 |

Estimates are expressed as changes in $FEV_1$/PEF per IQR increase in pollutant concentration. Sample size is 82. The IQRs for $NH_3$ and $PM_{10}$ were 12.0 and 11.3 $\mu g/m^3$ respectively. Models are corrected for ambient temperature, humidity and linear trend. The q-value describes the false discovery rate according the Benjamini-Hochberg procedure, a q-value < 0.10 is considered statistically significant.

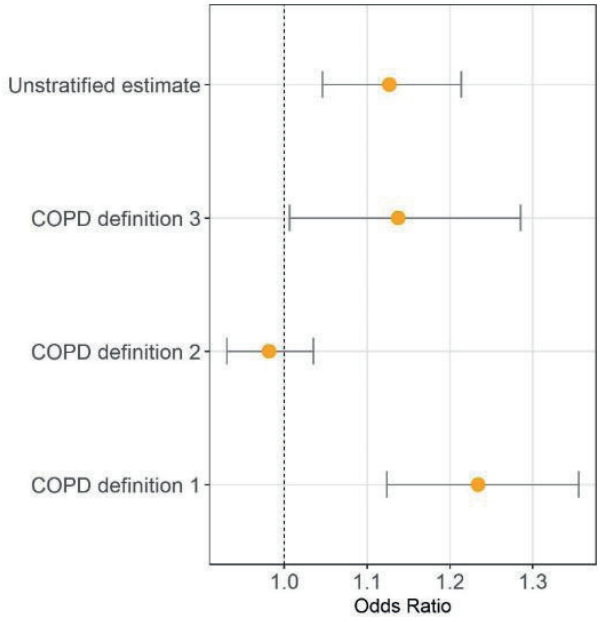
**Supplementary Table 2:** Relationships between livestock-related air pollutants and lung function decrements >10% and >20% from the individual median

| | | Morning decrements >10% | | | Morning decrements >20% | | | Evening decrements >10% | | | Evening decrements > 20% | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Model (per IQR)** | **OR** | **95% CI** | **q-value** | **OR** | **95% CI** | **q-value** | **OR** | **95% CI** | **q-value** | **OR** | **95% CI** | **q-value** |
| **lag 0** | $NH_3$ vs FEV1 | 1.00 | 0.92 ; 1.08 | 0.93 | 0.87 | 0.70 ; 1.08 | 0.70 | 0.96 | 0.86 ; 1.07 | 0.94 | 0.97 | 0.89 ; 1.06 | 0.64 |
| | $NH_3$ vs PEF | 0.99 | 0.92 ; 1.07 | 0.93 | 0.93 | 0.82 ; 1.05 | 0.70 | 1.01 | 0.93 ; 1.10 | 0.94 | 0.98 | 0.78 ; 1.23 | 0.88 |
| | $PM_{10}$ vs FEV1 | 0.98 | 0.84 ; 1.14 | 0.93 | 1.07 | 0.84 ; 1.37 | 0.70 | 1.02 | 0.89 ; 1.16 | 0.94 | 1.08 | 0.90 ; 1.30 | 0.62 |
| | $PM_{10}$ vs PEF | 1.12 | 1.01 ; 1.24 | 0.29 | 1.08 | 0.90 ; 1.30 | 0.70 | 1.00 | 0.88 ; 1.13 | 0.94 | 0.88 | 0.7 ; 1.11 | 0.62 |
| **lag 1** | $NH_3$ vs FEV1 | 0.99 | 0.93 ; 1.07 | 0.93 | 0.96 | 0.83 ; 1.12 | 0.70 | 0.99 | 0.90 ; 1.10 | 0.94 | 1.04 | 0.93 ; 1.17 | 0.62 |
| | $NH_3$ vs PEF | 0.99 | 0.93 ; 1.06 | 0.93 | 1.04 | 0.96 ; 1.13 | 0.70 | 1.00 | 0.93 ; 1.07 | 0.94 | 0.94 | 0.81 ; 1.09 | 0.62 |
| | $PM_{10}$ vs FEV1 | 1.07 | 0.95 ; 1.20 | 0.74 | 1.09 | 0.84 ; 1.41 | 0.70 | 1.01 | 0.90 ; 1.13 | 0.94 | 1.13 | 0.92 ; 1.38 | 0.62 |
| | $PM_{10}$ vs PEF | 0.96 | 0.86 ; 1.07 | 0.88 | 1.05 | 0.85 ; 1.31 | 0.70 | 0.97 | 0.86 ; 1.08 | 0.94 | 0.85 | 0.64 ; 1.11 | 0.62 |
| **lag 2** | $NH_3$ vs FEV1 | 1.06 | 1.00 ; 1.12 | 0.29 | 1.14 | 1.05 ; 1.21 | 0.02 | 1.07 | 1.01 ; 1.13 | 0.30 | 0.97 | 0.88 ; 1.06 | 0.62 |
| | $NH_3$ vs PEF | 1.01 | 0.95 ; 1.07 | 0.93 | 1.08 | 0.99 ; 1.18 | 0.56 | 1.02 | 0.96 ; 1.08 | 0.94 | 1.03 | 0.96 ; 1.10 | 0.62 |
| | $PM_{10}$ vs FEV1 | 0.95 | 0.84 ; 1.08 | 0.88 | 1.04 | 0.88 ; 1.22 | 0.70 | 0.97 | 0.86 ; 1.10 | 0.94 | 1.13 | 0.94 ; 1.35 | 0.62 |
| | $PM_{10}$ vs PEF | 1.07 | 0.96 ; 1.18 | 0.74 | 1.02 | 0.88 ; 1.19 | 0.77 | 0.94 | 0.83 ; 1.08 | 0.94 | 1.04 | 0.79 ; 1.38 | 0.83 |

Estimates are given as odds ratios per IQR increase in pollutant concentration. Sample size is 82. The IQRs for $NH_3$ and $PM_{10}$ were 12.0 and 11.3 µg/m$^3$ respectively. Models are corrected for ambient temperature, humidity and linear trend. The q-value describes the false discovery rate according the Benjamini-Hochberg procedure, a q-value < 0.10 is considered statistically significant.



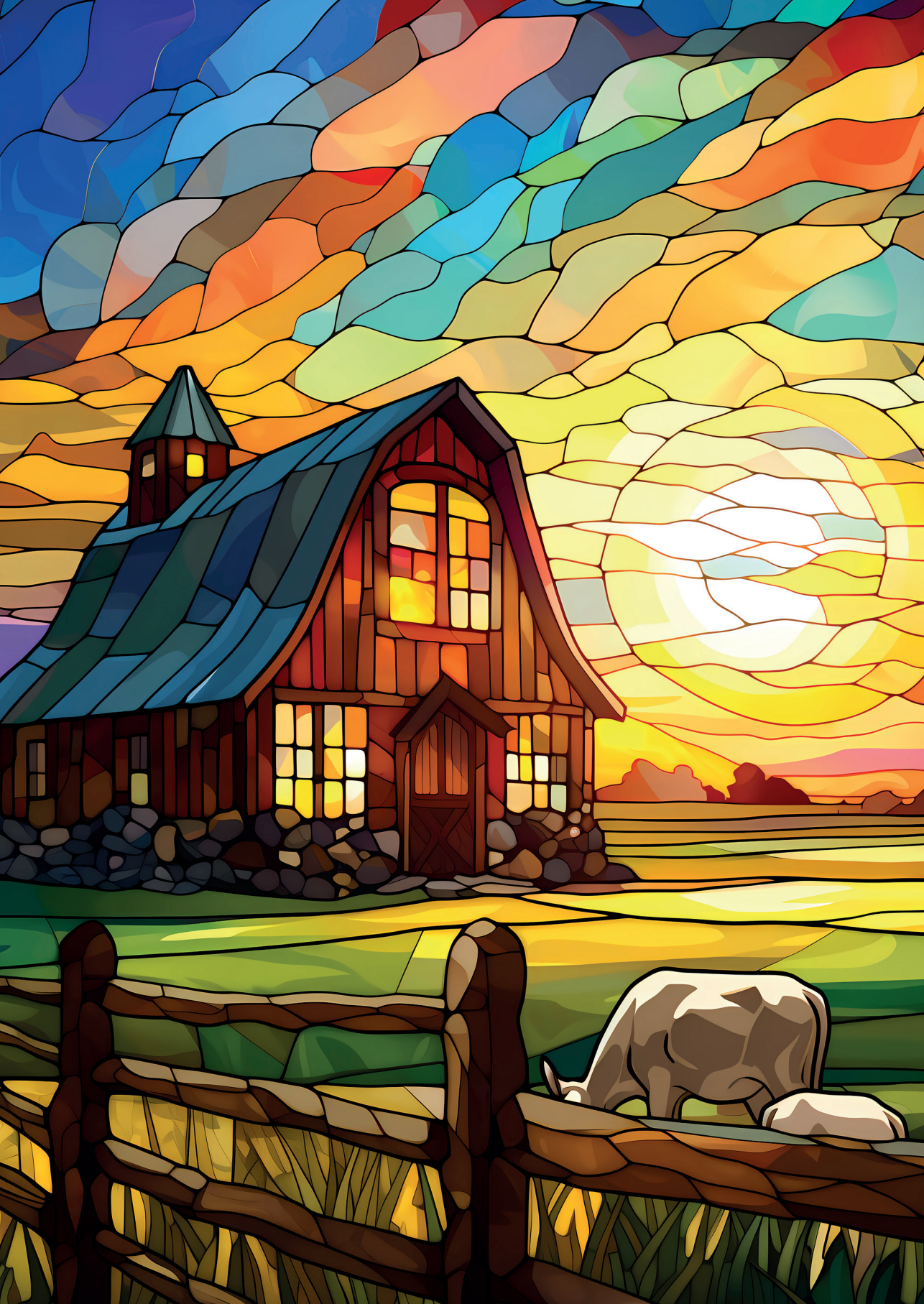

**Supplementary Figure 2:** Effects of an interquartile range increase in air pollutant levels on (**A**) wheezing; (**B**) cold and flu; and (**C**) shortness of breath in rest




**Supplementary Figure 3:** Odds ratios and 95% CI for decrements in FEV1 > 20% for an IQR increase in NH3 per COPD definition

**Supplementary Table 3:** Relationship between odor annoyance and evening PEF stratified by COPD definition

| Odor annoyance vs. evening PEF | N participants | Estimate (l/min) | 95% CI |
|---|---|---|---|
| Unstratified | 82 | -4.46 | -7.59 ; -1.33 |
| COPD definition 1 | 43 | -6.55 | -10.47 ; -2.63 |
| COPD definition 2 | 22 | -0.28 | -6.49 ; 5.92 |
| COPD definition 3 | 17 | -4.10 | -9.88 ; 1.69 |



Chapter 3

# Air pollution from livestock farms and the oropharyngeal microbiome of COPD patients and controls

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Published in Environment International, November 2022
https://doi.org/10.1016/j.envint.2022.107497

## Abstract

Air pollution from livestock farms is known to affect respiratory health of patients with chronic obstructive pulmonary disease (COPD). The mechanisms behind this relationship, however, remain poorly understood. We hypothesise that air pollutants could influence respiratory health through modulation of the airway microbiome. Therefore, we studied associations between air pollution exposure and the oropharyngeal microbiota (OPM) composition of COPD patients and controls in a livestock-dense area.

Oropharyngeal swabs were collected from 99 community-based (mostly mild) COPD cases and 184 controls (baseline), and after 6 and 12 weeks. Participants were non-smokers or former smokers. Annual average livestock-related outdoor air pollution at the home address was predicted using dispersion modelling. OPM composition was analysed using 16S rRNA-based sequencing in all baseline samples and 6-week and 12-week repeated samples of 20 randomly selected subjects (n=323 samples). A random selection of negative control swabs, taken every sampling day, were also included in the downstream analysis.

Both farm-emitted endotoxin and $PM_{10}$ levels were associated with increased OPM richness in COPD patients ($p<0.05$) but not in controls. COPD case-control status was not associated with community structure, while correcting for known confounders (multivariate PERMANOVA $p>0.05$). However, members of the genus *Streptococcus* were more abundant in COPD patients (Benjamini-Hochberg adjusted $p<0.01$). Moderate correlation was found between ordinations of 20 subjects analysed at 0, 6, and 12 weeks (Procrustes $r=0.52$ to $0.66$; $p<0.05$; Principal coordinate analysis of Bray-Curtis dissimilarity), indicating that the OPM is relatively stable over a 12 week period and that a single sample sufficiently represents the OPM.

Air pollution from livestock farms is associated with OPM richness of COPD patients, suggesting that the OPM of COPD patients is susceptible to alterations induced by exposure to air pollutants.

## Introduction

Living in livestock dense areas has been associated with health effects in epidemiological studies worldwide. Particularly livestock-related air pollution at the residential level is suggested to be relevant for public health.[1-17] Adverse health effects reported in relation to livestock farm emissions such as ammonia ($NH_3$) and particulate matter (PM) include lung function deficits, as well as increased respiratory symptoms like coughing and wheezing.[2,9,13] Several studies have linked increased ambient $NH_3$ levels with lung function deficits in non-farming residents.[2,17,18] People with chronic obstructive pulmonary disease (COPD) were found to be especially vulnerable to livestock-related $NH_3$ levels.[17] Similarly, increased respiratory symptoms in COPD patients living near livestock farms have been reported.[4] Recently, exposure to livestock farm emitted PM was found to be associated with respiratory health effects, indicating endotoxin as a plausible etiologic agent.[19-21] However, mechanisms behind adverse respiratory health effects in COPD patients associated with livestock farm emissions remain poorly understood. A biological mechanism that could play a role is alteration of the airway microbiota composition. Livestock operations are a potential source of microbes and air pollutants, both of which could act on the microbial composition of the airways.



The predominant determinants of the lung microbiota are thought to be explained by immigration and elimination processes of bacteria from the upper respiratory tract (URT).[22,23] In contrast, the microbiota of diseased lungs is suggested to be determined by regional growth conditions like nutrient availability, competition and activation of host inflammatory cells. While disruptions of the airway microbiota have mainly been linked to severe disease, signs of functional distortion have been shown in the microbiota of mild COPD patients while the comunity composition remained indistinguishable from healthy volunteers. Indicating that the airway microbiota may be relevant in the earlier stages of COPD as well.[22] Therefore, studying changes in the airway microbiota in relation to the living environment, especially in patients with a respiratory condition, could help to explain the associations between environmental exposures and respiratory health. A similar mechanism of dysbiosis in airway microbiota was suggested to play a role by studies in the Netherlands and United States where exposure to air pollution from livestock farms was associated with an increased risk of pneumonia.[24,25]

The goal of this explorative study is to investigate whether the oropharyngeal microbiota (OPM) microbiota of COPD patients differs from controls, and whether residential exposure to air pollution from livestock farms is associated with microbial

community composition. We performed 16S rRNA gene amplicon sequencing of oropharyngeal samples from 99 COPD patients and 184 healthy controls living in an area with a high density of intensive livestock (mainly poultry, pig, cattle, goat and mink) farms in the Netherlands. In analysing the resulting OPM compositions, we were particularly interested in differences associated with residential exposure to livestock farm-emitted endotoxin and particulate matter with a nominal aerodynamic diameter of 10μm or less ($PM_{10}$). Lastly, we analyzed reproducibility of the individual OPM composition by sequencing samples collected repeatedly from the same individuals over a 12-week period.

## Materials and Methods

### Study population and design

Study participants were selected from 2,369 participants of the cross-sectional Dutch Livestock Farming and Neighbouring Residents' Health study (VGO) population, of which the design, enrollment, and medical examination have been described.[3,4] The selection procedure for the present case-control study is shown in a flowchart (Figure 1). First, all COPD patients were selected by a lung function specialist, based on their spirometry values and curves. COPD was defined as a post-bronchodilator (BD) measurement of $FEV_1$/FVC below the lower limits of normal. We also invited subjects if they had a pre- or post- BD $FEV_1$/FVC below 0.7 in combination with at least one self-reported respiratory symptom (wheeze, shortness of breath) or if they reported doctor-diagnosed COPD. Current smokers were excluded. Control subjects were randomly selected from all non-smoking, non-asthmatic and non-COPD subjects with normal lung function. Both cases and controls were excluded post-enrollment when a smoking habit became apparent during home visits. The study protocol (no. 13/533) was approved by the Medical Ethical Committee of the University Medical Centre Utrecht. All participants signed informed consent.

### Sampling procedure

Between February 2015 and July 2016, during home visits, oropharyngeal samples were collected from 99 COPD cases and 184 controls. In addition to the baseline (t0) sample, participants were sampled again after 6 (t1) and 12 weeks (t2). Samples were collected using Copan Eswabs and stored on ice in 1 ml liquid Amies Medium (483CE, Copan Diagnostics Inc., CA) during transport. DNA extraction and sequencing was done for all baseline (t0) samples, and for a random selection of t1 and t2 samples from 10 COPD cases and 10 controls. A random selection of field blanks (air swabs),

taken every sampling day, together with unused swabs and laboratory controls was included in the downstream analysis.

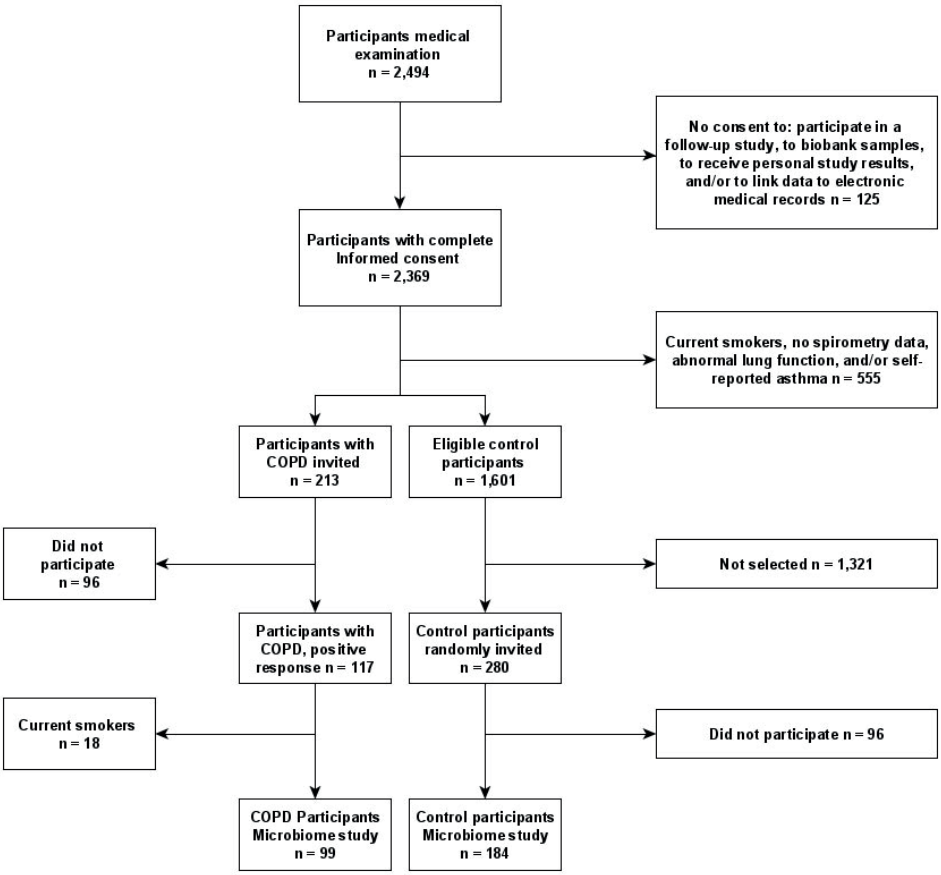


**Figure 1:** Flowchart of the study population of 99 COPD cases and 184 control subjects

## DNA Extraction, 16S rRNA gene amplification and sequencing

The DNA isolation procedure was performed as previously described and can be found in the supplementary methods.[26] A 469-bp (base pair) amplicon, encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene, was amplified and sequenced using the Illumina MiSeq Reagent Kit v3 (600-cycle) on an Illumina MiSeq instrument according to Fadrosh *et al*.[27] More details can be found in supplementary methods.

## Bioinformatics

Sequencing primers and heterogeneity spacers were removed using cutadapt version 2.8. [28] Output sequences were processed in R version 4.0.2 using the dada2 package version 1.16, resolving the sequences into amplicon sequence variants (ASVs).[29,30] For the sequence filtering step, forward and backward reads were truncated to high-quality regions at 200 and 250 base pairs respectively. After inspection of the read quality profiles, the maximum expected errors (maxEE) was set to 3. Taxonomy was assigned using the Silva reference database and the naïve Bayesian classifier, version 138.[31] Species level annotation was confirmed using BLASTnt against the REFSEQ targeted loci database at the National Center for Biotechnology Information.[32] Further filtering steps are described in the supplemental methods.

## Data analysis

Associations involving beta-diversity, measured by Bray-Curtis dissimilarity, were visualized using Principal Coordinates Analysis (PCoA) and tested by multivariable PERMANOVA. To explore which taxa drive the overall compositional differences between COPD cases and controls, or individuals with high or low livestock-emitted endotoxin concentrations (tertiles T3 vs. T1),[20] differential abundance analysis was performed at ASV, genus, family and phylum level using DESeq2 version 1.28.1 and MaAsLin2 version 1.2.0 (see supplemental methods).[33,34]

Differences in alpha-diversity between COPD cases and controls were assessed using linear models. As response variables, species richness and the Shannon diversity index were used. Explanatory variables included case-control status, sex, age (continuous), education level (low, medium, high), lung medication (yes/no), antibiotic use (yes/no), COPD GOLD stage, former smoker (yes/no) and season (Winter Dec-Feb, Spring Mar-May, Summer Jun-Aug, fall Sep-Nov). To assess relations between OPM composition and livestock exposure, we included presence of a poultry farm within 1km of the home address,[25] and annual-average concentrations of livestock-emitted $PM_{10}$ and endotoxin at home addresses (predicted by dispersion modelling; continuous variables) .[20] Relationships were first explored in univariable analyses. Consecutively, predictors that yielded p-values <0.2 were used in multivariable models to identify independent drivers while correcting for known confounders. To determine whether a single sample is representative for an individual's OPM and if its composition can be reproduced over a short period of time, multiple robustness analyses were performed as described in the supplemental methods.

# Results

## Study population

A detailed overview of participant characteristics, stratified by cases and controls, can be found in Table 1. Overall, 48.1% of the participants were female, and mean age was 58.2 years. Cases were found to be slightly older with a mean age of 61.4 years compared to 56.5 years in controls (p<0.001). Cases differed from controls in terms of smoking history with a mean difference of 6.1 pack-years (p<0.001). The majority of cases were classified as mild COPD patients with 45 (45.5%) and 30 (30.3%) participants assigned to GOLD stage 1 and 2 respectively. The remaining 24 (24.2%) cases were classified with GOLD stage 0. One third (33.3%) of the cases used lung medication during the sampling period. Besides mono- or combination therapies with inhaled corticosteroids, bèta 2-sympathicomimetics and parasympatholytics, monotherapy with oral leukotriene antagonists also occurred.



**Table 1:** Study population characteristics of COPD cases and healthy controls sampled for oropharyngeal microbiota analysis

| | Case (N=99) | Control (N=184) | Overall (N=283) | p-value [a] |
|---|---|---|---|---|
| Sex, female | 42 (42.4%) | 94 (51.1%) | 136 (48.1%) | 0.205 |
| Age (y) | 61.4 [28.9, 71.8] | 56.5 [28.7, 71.7] | 58.2 [28.7, 71.8] | <0.001 |
| BMI* | 26.8 [18.1, 48.9] | 27.3 [17.2, 48.1] | 27.1 [17.2, 48.9] | 0.368 |
| Education level | | | | |
| low | 24 (24.2%) | 40 (21.7%) | 64 (22.6%) | 0.738 |
| medium | 48 (48.5%) | 86 (46.7%) | 134 (47.3%) | |
| high | 27 (27.3%) | 58 (31.5%) | 85 (30%) | |
| Pack-years of cigarettes smoked† | 13.9 [0, 54.6] | 7.74 [0, 127] | 9.91 [0, 127] | 0.001 |
| COPD grade, GOLD | | | | |
| 0 | 24 (24.2%) | 184 100%) | 208 (73.5%) | <0.001 |
| 1 | 45 (45.5%) | 0 (0.%) | 45 (15.9%) | |
| 2 | 30 (30.3%) | 0 (0%) | 30 (10.6%) | |
| Pre-BD measurements | | | | |
| $FEV_1$ (l) | 2.51 [0.88, 4.62] | 3.23 [1.49, 5.12] | 2.98 [0.88, 5.12] | <0.001 |
| FVC (l) | 4.00 [1.66, 8.02] | 4.16 [2.09, 7.21] | 4.10 [1.66, 8.02] | 0.236 |
| $FEV_1$/FVC (l) | 0.63 [0.26, 0.90] | 0.78 [0.66, 0.92] | 0.73 [0.29, 0.92] | <0.001 |
| Post BD measurements | | | | |
| $FEV_1$ (l) | 2.67 [1.03, 4.79] | 3.34 [2.26, 5.39] | 3.11 [1.03, 5.39] | <0.001 |
| FVC (l) | 4.10 [1.90, 7.87] | 4.17 [2.61, 7.16] | 4.15 [1.90, 7.87] | 0.616 |
| $FEV_1$/FVC | 0.65 [0.31, 0.92] | 0.80 [0.71, 0.90] | 0.75 [0.31, 0.92] | <0.001 |
| Uses lung medication | 33 (33.3%) | 1 (0.5%) | 34 (12.0%) | <0.001 |

**Table 1:** Continued

| | Case (N=99) | Control (N=184) | Overall (N=283) | p-value [a] |
|---|---|---|---|---|
| Atopy | 30 (30.3%) | 51 (27.7%) | 81 (28.6%) | 0.711 |
| Childhood on farm | 36 (36.4%) | 59 (32.1%) | 95 (33.6%) | 0.592 |
| Respiratory symptoms during sampling | 20 (20.2%) | 48 (26.1%) | 68 (24.0%) | 0.337 |
| Antibiotic use within 4 weeks prior to sampling | 13 (13.1%) | 5 (2.7%) | 18 (6.4%) | 0.002 |
| Residential exposure to livestock farm emitted endotoxin (EU/$m^3$)[ŦŦ] | 0.23 [0.032, 1.26] | 0.25 [0.032, 0.93] | 0.24 [0.032, 1.26] | 0.428 |
| Residential exposure to livestock farm emitted $PM_{10}$ (µg/$m^3$) [Ŧ] | 0.29 [0.041, 1.22] | 0.30 [0.036, 1.07] | 0.30 [0.036, 1.22] | 0.482 |
| N farms within 500m, tertiles | | | | |
| no farms | 33 (33.3%) | 62 (33.7%) | 95 (33.6%) | 0.984 |
| 1 or 2 farms | 36 (36.4%) | 65 (35.3%) | 101 (35.7%) | |
| >2 farms | 30 (30.3%) | 57 (31%) | 87 (30.7%) | |
| N farms within 1000m, 50% quantiles | | | | |
| 0-16 farms | 87 (87.9%) | 164 (89.1%) | 251 (88.7%) | 0.904 |
| >16 farms at 1km | 12 (12.1%) | 20 (10.9%) | 32 (11.3%) | |
| Poultry exposure | | | | |
| nearest poultry at >1km | 41 (41.4%) | 76 (41.3%) | 117 (41.3%) | 1 |
| poultry farm within 1km | 58 (58.6%) | 108 (58.7%) | 166 (58.7%) | |

Data are presented as mean [range] or n (%). Definition of abbreviations: BD=bronchodilator; COPD=chronic obstructive pulmonary disease; EU: endotoxin unit; $FEV_1$=forced expiratory volume in 1 second; FVC=forced vital capacity; Education levels: low—lower secondary school or less; medium—intermediate vocational education or upper secondary school; high—higher vocational education or university. *BMI=mass(kg)/(height (m))$^2$. †Mean pack-years for former smokers. [Ŧ] Annual average concentration at the home address estimated by dispersion modelling. [a] Two sample t-test or $Chi^2$ test across cases and controls.

## Sequencing

Approximately 5,411,000 reads were generated with an average of 14,000 reads per sample, ranging from 430 to 54,000. Processing the raw data resulted in 2,705 ASVs. Compositional analyses were performed using 1,092 taxa that remained after filtering, as detailed in the supplemental methods.

## Oropharyngeal microbiota composition

An overview of the taxonomic composition at family level can be found in Figure 2. To summarise, relative abundance was dominated by Streptococcaceae (mean 47%; range 5-91%), Veillonellaceae (mean 16%; range 0-44%) and Prevotellaceae (mean 10%; range 0-37%). A comparison of the top 10 most abundant genera per sample type (including negative controls) can be seen in Supplementary Figure 1. We explored whether the OPM composition differed between COPD cases and controls in a multivariable analysis adjusting for age, gender, smoking history, season and antibiotic use (Supplementary Table 1). No compositional differences were shown in this analysis related to case-control status (PERMANOVA p=0.26, $R^2$=0.004) or livestock-emitted endotoxin (PERMANOVA p=0.31, $R^2$=0.012). Community composition differed slightly between males and females (PERMANOVA p<0.01, $R^2$=0.007). Season (PERMANOVA p=0.07;$R^2$=0.014) and farm childhood (PERMANOVA p=0.09;$R^2$=0.005) were found to have a borderline significant effect.

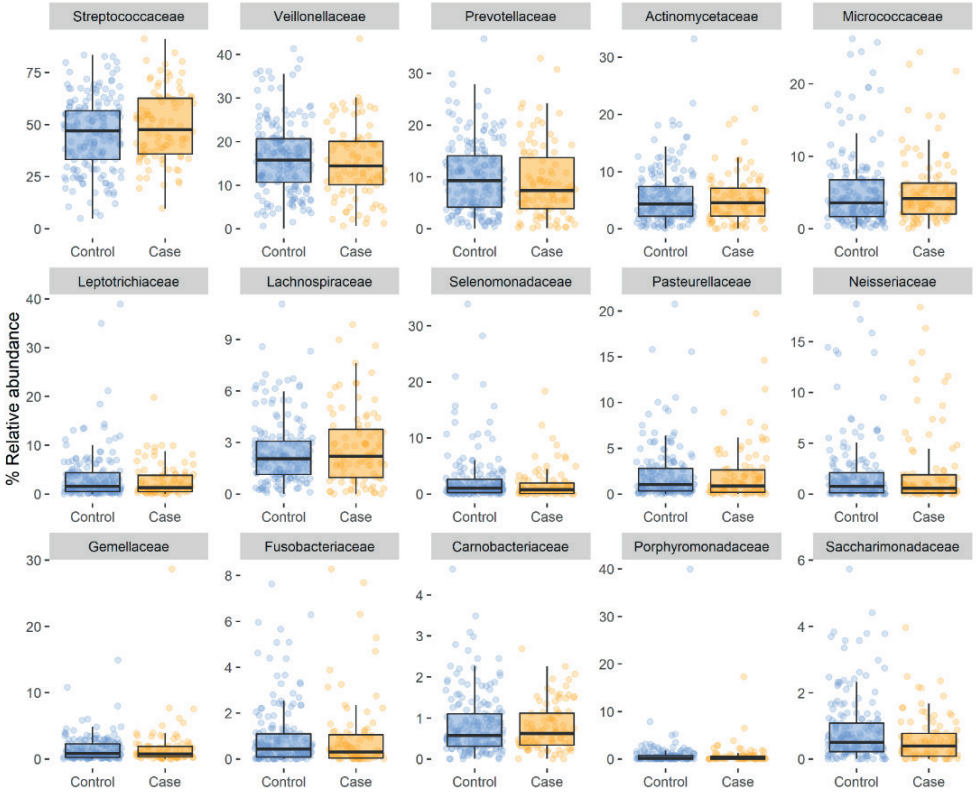


**Figure 2:** Relative abundances at family level, showing taxa accounting for at least 0.5% (mean relative abundance) of the oropharyngeal microbiota

## Differential abundance analysis

As the compositional analysis revealed little biological variation, we chose to focus on the more abundant taxa in the differential abundance analysis. To this end, we removed taxa which accounted for less than 0.1% relative abundance in less than 25% of the samples. The remaining dataset consisted of 74 ASVs belonging to 26 genera, 19 families and 7 phyla. Using DESeq2, we found that the genus *Streptococcus* was significantly more abundant in COPD cases compared to controls (log2 fold change [LFC]=0.68; Benjamini Hochberg [BH] adjusted p=0.001). This association was confirmed by testing for differences in normalized counts using the Kruskal-Wallis test (Figure 3). At species level, *Streptococcus salivarius*, *Streptococcus parasanguinis* and another undefined *Streptococcus* species were associated with COPD, although not statistically significant (Supplementary Figure 2). In comparison to DESeq2, MaAsLin2 did not return any significant associations. It did, however, show a similar, but statistically non-significant association between *Streptococcus* abundanceand COPD (Supplementary Table 2 β=0.03, BH adjusted p=0.38). Differential abundance analysis in relation to livestock-related endotoxin exposure revealed slight differences in Firmicutes and Actinobacteriota abundance between low and high exposed individuals (Supplementary Figure 3). However, these associations were no longer statistically significant after adjusting for multiple testing.

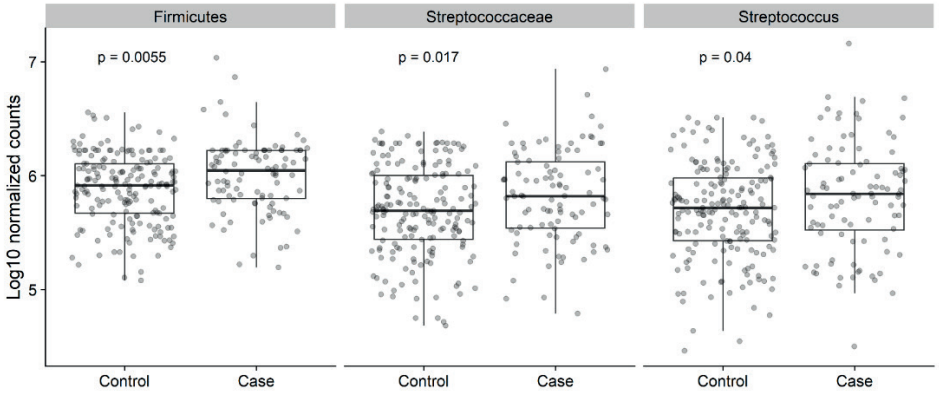


**Figure 3:** DESeq2 normalized counts of differentially abundant taxa at phylum (LFC=0.47), family (LFC=0.53) and genus level (LFC=0.68) between COPD cases and controls. P-values calculated using the Kruskal-Walis test

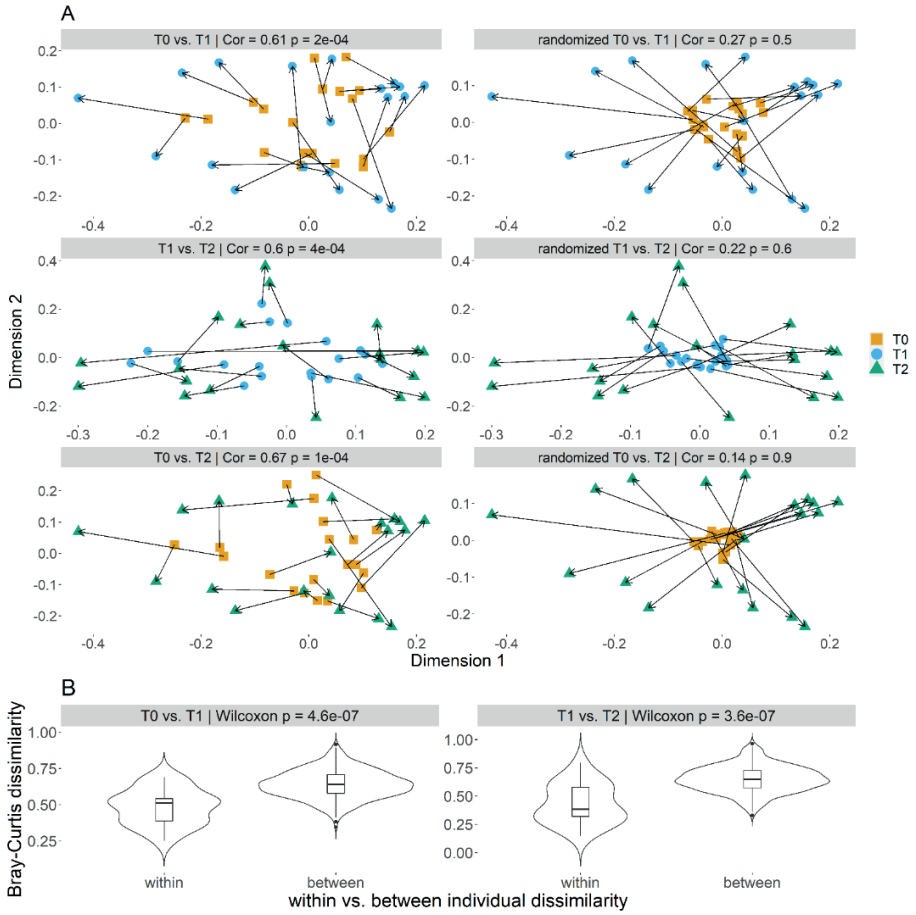


**Figure 4** **A**: Procrustes errors comparing ordinations (PCoA – Bray-Curtis) of the 20 individuals that had their oropharyngeal microbiota sampled at t0, t1 (after 6 wks) and t2 (after 12 wks). **B:** Within vs. between individual Bray-Curtis distances over timepoints

## Alpha-diversity

Differences in within-subject diversity were analysed using data rarefied at 5,000 sequences per sample, maintaining 177 controls and 94 cases in the dataset (96% of all samples). Rarefaction curves of all samples can be found in Supplementary Figure 4. We used multivariable linear models, adjusting for sex, smoking history, education level, season, and antibiotic use, after variable selection with univariable models. Univariable analysis of observed richness and Shannon indices did not show an association with COPD status (Supplementary Table 3). Likewise, in the multivariable linear model, COPD status, gender, smoking history, and antibiotic use were not associated with alpha diversity (Table 2). In the multivariable analysis, medium education level (vs.

low) was associated with both higher richness (β=6.63;p=0.05) and increased Shannon diversity (β=0.21;p=0.01). Shannon diversity appeared higher in samples taken during summer (Jun-Aug) compared to winter (Dec-Feb)( β=0.17;p=0.06). Similarly, we found an association between residential exposure to livestock-emitted endotoxin and increased richness (β=4.91; p=0.06). When stratifying the model for COPD case-control status, the positive association of residential exposure to livestock-emitted endotoxin with richness is shown to be driven by the COPD cases (Supplementary Table 4, β=10.02;p=0.02). Residential exposure to livestock-emitted $PM_{10}$ showed a similar relationship with increased richness (Supplementary Table 5, β=5.58;p=0.08) and COPD status (Supplementary Table 6, β=13.11;p=0.01).

## Oropharyngeal microbial community stability over time

Procrustes analysis of 20 randomly selected subjects sampled at 0, 6, and 12 weeks showed moderate but significant within-individual correlation (r=0.50-0.66, p<0.05). This correlation was lost when the sample identifiers were randomized, resulting in a between-individual comparison (r=0.15-0.23; p>0.6; Figure 4A). Repeating the analysis with randomized sample identifiers over 1,000 Monte Carlo iterations showed that this was not due to chance. Likewise, comparing Bray-Curtis dissimilarity of the repeated samples within and between individuals revealed significantly higher between (vs. within) individual dissimilarity (Figure 4B). Therefore, we could conclude that the individual OPM community is relatively stable, at least over a 12-week period.

**Table 2:** Determinants of alpha-diversity indices of the oropharyngeal microbiota of COPD cases and controls in a multivariable linear model

| | Richness | | | Shannon diversity | | |
|---|---|---|---|---|---|---|
| **Variable** | **β** | **95% CI** | **p value** | **β** | **95% CI** | **p value** |
| COPD case vs. control | -2.20 | -8.1:3.7 | 0.46 | -0.041 | -0.18:0.1 | 0.57 |
| Gender female vs. male | -0.76 | -6.11:4.6 | 0.78 | -0.002 | -0.13:0.13 | 0.97 |
| Education level | | | | | | |
| Medium vs. low | 6.63 | -0.09:13.35 | 0.05 | 0.21 | 0.04:0.37 | 0.01 |
| High vs. low | 0.78 | -6.56:8.11 | 0.84 | 0.10 | -0.08:0.28 | 0.27 |
| Smoking history former vs. never | -0.35 | -5.87:5.17 | 0.90 | -0.05 | -0.18:0.08 | 0.46 |
| Residential exposure to livestock farm emitted endotoxin (EU/m$^3$)[Ŧ] | 4.91 | -0.1:9.92 | 0.06 | 0.081 | -0.04: 0.2 | 0.19 |
| Season | | | | | | |
| Spring (Mar-May) vs. winter (Dec-Feb) | 0.15 | -6.96:7.25 | 0.97 | 0.09 | -0.08:0.26 | 0.29 |
| Summer (Jun-Aug) vs. winter | 4.10 | -3.02:11.21 | 0.26 | 0.17 | -0.01:0.34 | 0.06 |
| Fall (Sep-Nov) vs. winter | -7.16 | -17.23:2.9 | 0.16 | -0.16 | -0.4:0.08 | 0.19 |
| Antibiotics within 4 weeks prior to sampling | -2.65 | -13.47:8.18 | 0.63 | 0.16 | -0.1:0.42 | 0.23 |

[Ŧ] Annual average concentration at the home address by dispersion modelling, scaled to the 10-90$^{th}$ percentile range. EU: endotoxin unit.

## Discussion

The aim of our study was to investigate whether the URT microbiota composition of community-based COPD patients differed from adults without COPD living in the same geographic region. Subsequently, we explored the potential role of residential exposure to livestock-emitted air pollution in shaping the OPM in subjects with and without COPD. We found evidence suggesting that residential exposure to livestock-emitted endotoxin and $PM_{10}$ is associated with increased species richness in COPD patients. While the overall OPM composition did not differ between mild and non-exacerbating COPD cases and subjects with normal lung function and no diagnosis of asthma or COPD, *Streptococcus spp.* was more abundant in the OPM of COPD patients. However, the exploratory nature of our epidemiological study precludes further conclusions about direction of associations, causality, or underlying mechanisms.

We did not find differences in overall bacterial community composition related to COPD status or livestock exposure. This might be explained by the relative

stability of the OPM compared to that of the nasopharynx which is known to be more susceptible to environmental exposures like pig farming.[35] A study in Iowa (United States) reported significantly higher alpha-diversity in the nasal microbiome of 33 livestock workers compared to 26 non-livestock workers, though this was not observed for the oropharyngeal microbiome.[36] Likewise, the OPM community structure in our study did not differ between former and never smokers. Although previous studies reported differences in URT microbiota due to smoking,[37,38] these studies included current smokers which were excluded in the present study.

Differential abundance analysis revealed that three ASV's, annotated to the genus *Streptococcus*, were more highly represented in subjects with COPD, compared to the controls. A BLAST search revealed that these belonged to *Streptococcus salivarius*, *Streptococcus parasanguinis* and an undefined *Streptococcus spp*. *Streptococcus* (especially *Streptococcus pneumoniae*) has been associated with COPD exacerbations and disease severity.[39,40] In addition both *S. salivarius* and S. *parasanguinis* are opportunistic pathogens and have been associated with COPD and smoking.[41] In an endotoxin induced lung inflammation mouse model the lung microbiota shifted towards endogenous opportunistic pathogens, suggesting a immunological mechanism behind our observed results.[42] Thus, studying OPM may help to provide an explanation why COPD patients living in a livestock-dense area are at increased risk of wheezing, exacerbations and a lower lung function.[4,43] This, however, needs to be confirmed by longitudinal studies. Likewise, further experimental studies are needed to elucidate which taxa of the OPM are susceptible to air pollution.

We report increased richness related to residential exposure to livestock farm-emitted endotoxin and $PM_{10}$, but only among COPD patients. We speculate that the airways of COPD patients are more susceptible to irritation by air pollutants, resulting in an environment that promotes bacterial growth. The similarity between the effect of endotoxin and $PM_{10}$, is explained by the fact that endotoxin was modeled as a fraction of the livestock farm-emitted $PM_{10}$. Our results suggest that the impact of mild COPD and residential livestock exposure on the microbial community of the oropharynx is subtle. However, it has been shown that in mild COPD patients the largely normal microbiome may still be distorted due to subtle changes in the presence or absence in rare key members and is therefore less able to react to and mediate changes in host inflammation.[22] These subtle changes in microbial community of COPD patients are different from changes in other respiratory diseases. Notable changes in the respiratory microbiota have been detected early on in asthma, whereas in COPD distortion of the microbiota is mainly associated with advanced disease.[22] While advanced COPD has been associated with a shift in microbiota composition from

Bacteroidetes to Proteobacteria, a phylum known for its opportunistic pathogenic members like *Pseudomonas* and *Haemophilus*, shifts towards a Firmicutes dominant community have also been reported.[22] In combination with our reported association between mild COPD and increased Firmicutes abundance this suggest that changes in the respiratory microbiome in COPD patients occur more gradually than previously thought. This is also supported by a recent multi-omic meta-analysis which showed that Proteobacteria, Actinobacteria, and Firmicutes are main contributors to the biosynthesis of pro-inflammatory agents in COPD patients.[34]



Limitations of the present study include its exploratory nature. While this is one of the largest microbiome studies performed in COPD patients, further increasing the sample size might lead to identifying more associations, especially those with smaller effect sizes. The mainly mild, population-based COPD patients we included, probably do not show as much variation in their OPM compared to healthy controls as one might expect in more severe patients recruited from a clinical setting. Furthermore, given that the microbiota of diseased lungs is thought to be determined by regional growth conditions, traditional sampling techniques might be preferred. However, it has been shown that oropharyngeal swabs are an adequate proxy for traditional samples like sputum when studying COPD.[44] In addition, oropharyngeal swabs are easier to standardize and less intrusive compared to sputum sampling. Lastly, the striking difference in output between DESeq2 and MaAsLin2 illustrates that these results should be carefully interpreted. We argue that the negative binomial model performed by DESeq2, compared to the linear model used by MaAsLin2, theoretically fits our case-control design best. More importantly, differences in abundance between groups were verified using a Kruskal-Wallis test on normalized counts.

Strengths of this study include the use of validated models that predict livestock-related endotoxin levels at the home address as a measure for livestock exposure of microbial origin.[20] Further, our analysis of repeated samples confirmed stability of individual microbiota profiles within a 3-month period, although we were unable to identify clear predictors that explain the between-subjects variability in overall community composition. Lastly, the use of multiple negative and community controls enabled us to minimise the influence of contamination which is a notorious source of bias when working with low-biomass samples.

In conclusion, we showed a relationship between residential exposure to livestock-related endotoxin and $PM_{10}$ and OPM richness of COPD patients, suggesting that OPM of COPD patients is susceptible to alterations induced by exposure to air pollutants. While we did not find community structural differences in the OPM of

mild COPD patients versus controls, results do suggest increased *Streptococcus spp.* abundance in COPD patients. The implications and the underlying mechanisms of these associations offer multiple angles for future research regarding health effects from air pollution.

## Acknowledgements

We are grateful to Marieke Oldenwening,who performed the fieldwork, and Gerdit Greve and Betty Jongerius-Gortemaker for technical assistance (Institute for Risk Assessment Sciences, Utrecht University).

## Data availability

Availability of data and materials Raw sequence data were submitted into the Sequence Read Archive (SRA) at the NCBI under accession number PRJNA810336. The phyloseq object is available at 10.5281/zenodo.6303131.

## Support statement

Data collection for this study was supported by a grant from the Lung Foundation Netherlands (Grant number: 3.2.11.022). Sequencing costs were covered by internal funding of the Institute of Risk Assessment Sciences, Utrecht University and the University Medical Center Utrecht.

## Conflict of interest statement

The authors declare no conflict of interest

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Chapter 3s

# Supplementary Materials

# Supplementary Methods

### *DNA extraction*

The extraction procedure was performed as previously described.[1]For DNA extraction 650µl 0.1mm zirconium beads (Biospec Products USA, cat # 11079101Z) in lysis buffer (LGC Genomics, cat# NAP40012) was combined in Eppendorf tubes with 550µl Phenol in Tris (Fisher Scientific, cat # 10001173) and 200µl sample or lysis buffer for blanks. After two homogenization steps using a mini bead beater 24 (BioSpec Products USA) for 2 minutes at level 5, with a cool down step on ice in between and after, the tubes were centrifuged for 10 minutes at room temperature with 4500 RCF. The clear supernatant, approximately 720µl, was transferred to new 2ml tubes already containing 10µl magnetic beads (LGC Genomics, cat # NAP40137) and 1300µl binding buffer (LGC Genomics, cat # NAP40102). These tubes were then incubated in a mixing machine at room temperature. After a ten second spin down the tubes were put in a magnetic separator for one minute. The supernatant was removed and 200µl wash buffer 1 (LGC Genomics, cat # NAP40181) was added. After homogenization, the tubes were incubated in a shaker at room temperature for 5 minutes. Following another 10 second spin down, the tubes were put back in a magnetic separator for one minute. After removal of the supernatant, wash buffer 2 (LGC Genomics, cat # NAP40211) was added, contents of the tubes were homogenized and incubated in a shaker at room temperature for 5 minutes. Following a third spin down and another minute in the magnetic separator the supernatant was removed and the magnetic beads were dried by placing the tubes at 55˚C in a thermal heating block for 20 minutes. The magnetic beads where then resuspended in 50µl elution buffer (LGC Genomics, cat # NAP40241) and incubated for 15 minutes at 55˚C. After a spin down of ten seconds and a minute in a magnetic separator the supernatant was pipetted into new 1.5ml Eppendorf tubes and stored at -20˚C for further processing.

### *16S rRNA gene amplification and sequencing*

A 469-bp (base pair) amplicon, encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene, was amplified and sequenced according to Fadrosh *et al*.[2] For PCR preparation, Phusion High-Fidelity PCR Master Mix with HF Buffer was used. From each DNA isolate, 5 µL was used per PCR reaction. The PCR protocol consisted of the following: 98˚C 60s; followed by 98˚C 15s; 58˚C 15s; 72˚C 20s (30x); 72˚C 60s; 4˚C hold. PCR products were checked on 2% agarose gel, negative PCRs were repeated. Library preparation was performed with the Nextera XT DNA prep kit, followed by sequencing on an Illumina MiSeq using a 600-cycle (PE300) Reagent Kit v3 (Illumina San Diego, California, United States).

***Bioinformatics***

In total, 1,533 taxa that did not reach a confident level of detection (>0.1% abundant in 1% of the samples) were removed.[3] Additional filtering was performed removing 17 taxa classified as mitochondria, chloroplasts, Archaea, Eukaryota and taxa with no kingdom level annotation. Contaminant ASVs were identified and removed using the decontam package (version 1.8.0) which uses data from the negative controls, as well as DNA concentrations derived from the qPCR, to identify contaminants.[4] Comparing the relative abundance of the top 10 most abundant taxa in the different sample types and controls showed minimal contamination. In total, 63 taxa were removed as they were flagged as contaminants by decontam. These mainly involved genera that have previously been described as laboratory contaminants.[5]

## Data analysis



***Differential abundance methods***

Two differential abundance (DA) algorithms were used to provide a sense of robustness for analytical choices, as different DA tools have been reported to produce highly variable results.[6] Input taxonomic count data for both methods was additionally filtered, maintaining taxa that account for at least 0.1% of the counts in at least 25% of the samples. Both DESeq2 and MaAsLin2 were used with default settings.

***Oropharyngeal microbial community stability over time***

To gain more insight into how well our samples represent an individual's oropharyngeal microbial community, we analysed the repeated samples of 20 randomly selected subjects. First, a Procrustes analysis was performed on ordinations (PCoA, Bray-Curtis dissimilarity) of repeated samples from 20 randomly selected individuals. The Procrustes correlations between paired ordinations of timepoints (t0, t1, t2) were compared with the correlations between the original ordinations and the same ordination with randomized sample names, resulting in an unpaired comparison. In addition, Bray-Curtis dissimilarities of both paired and unrelated repeated samples were calculated to compare within-person dissimilarity with between-person dissimilarity over time.

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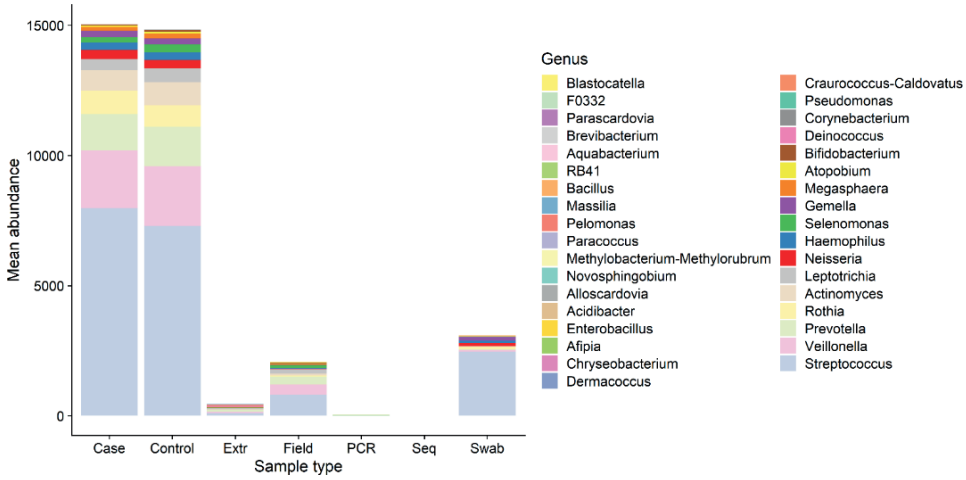
# Supplementary Tables and Figures

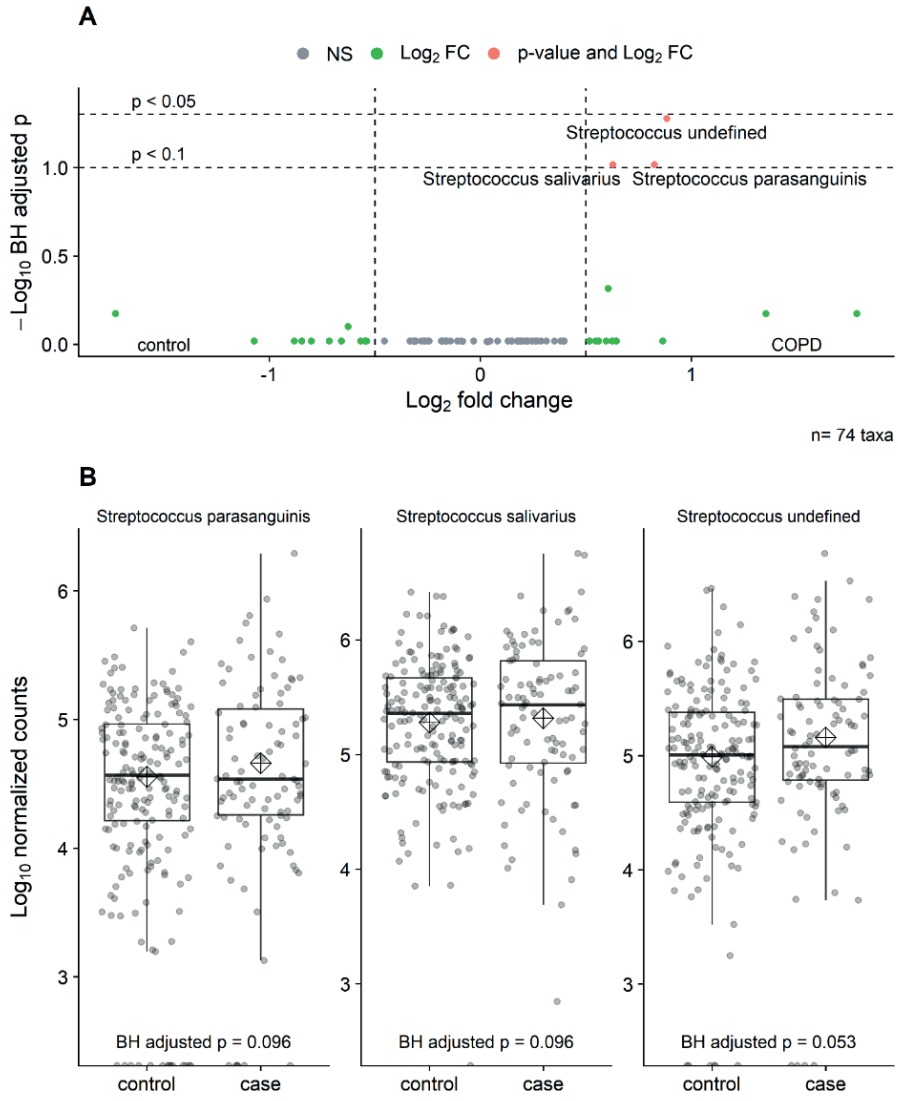
**Supplementary Table 1:** Multivariable PERMANOVA comparing oropharyngeal microbiome composition (PCoA Bray-Curtis) between COPD cases and healthy controls

| Variable | p - value | $R^2$ |
|---|---|---|
| Season* | 0.07 | 0.014 |
| Residential exposure to livestock farm emitted endotoxin (quartiles EU/m³)ŦŦ | 0.31 | 0.012 |
| Age (quartiles) | 0.82 | 0.009 |
| Gender | 0.007 | 0.007 |
| Childhood on farm | 0.09 | 0.005 |
| COPD case vs. control | 0.26 | 0.004 |
| Lung medication yes vs. no | 0.28 | 0.004 |
| Poultry farm within 1km | 0.31 | 0.004 |
| Current symptoms | 0.70 | 0.003 |
| Smoking history former vs. never | 0.61 | 0.003 |
| Antibiotics within 4 weeks prior to sampling | 0.62 | 0.003 |

* Seasons defined as: Winter (Dec-Feb), Spring (Mar-May), Summer (Jun-Aug), Fall (October Oct-Nov) ŦŦ Annual average concentration at the home address by dispersion modelling. Beta dispersion p-value was >0.15 for all variables.



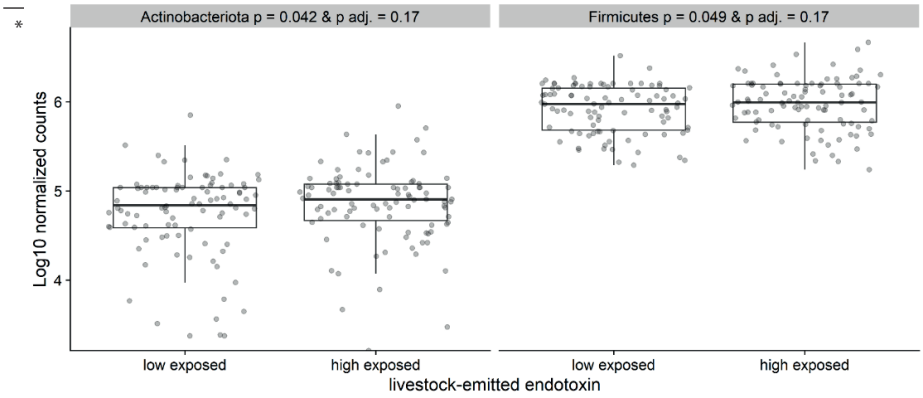

**Supplementary Figure 1:** Mean abundance of the top 35 (combined top 10) most abundant genera per sample type. Comparing COPD cases and controls with extraction controls (Extr), unused swabs (Swab), unused swabs opened during fieldwork (Field), PCR and sequencing (Seq) negative controls



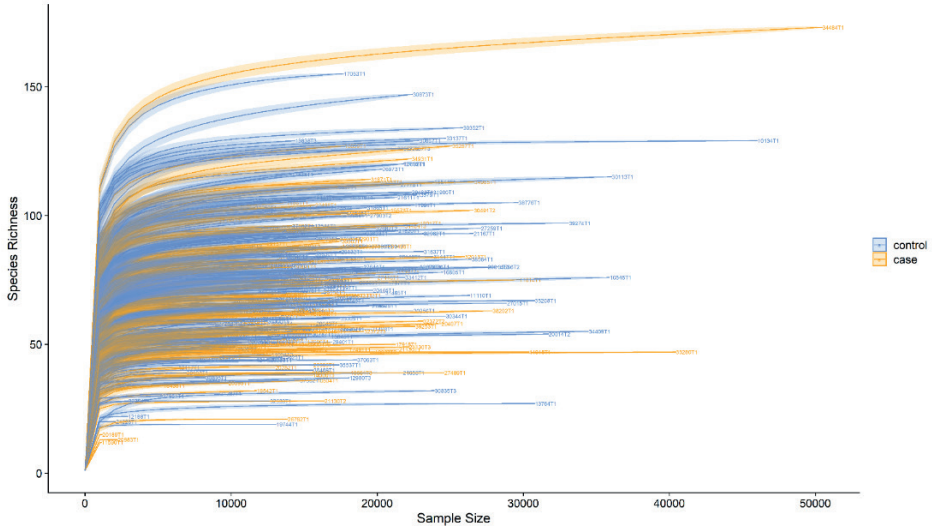

**Supplementary Figure 2: A:** Volcano plot depicting DESeq2 differential abundance results of 74 oropharyngeal taxa. **B:** Differences in log transformed normalized counts between COPD cases and controls, showing taxa with Benjamini Hochberg adjusted p<0.1 and a diamond for the mean

**Supplementary Table 2:** Differentially abundant taxa in the oropharyngeal microbiome of COPD cases and controls by MaAsLin2 at genus, family and phylum level

| | β* | p-value | BH adjusted p[Ŧ] |
|---|---|---|---|
| Genus | | | |
| *TM7x* | -0.22 | 0.06 | 0.38 |
| *Leptotrichia* | -0.19 | 0.10 | 0.38 |
| *Haemophilus* | -0.17 | 0.11 | 0.38 |
| *Streptococcus* | 0.03 | 0.13 | 0.38 |
| *Campylobacter* | -0.11 | 0.15 | 0.38 |
| *Atopobium* | -0.09 | 0.31 | 0.67 |
| *Prevotella* | -0.06 | 0.38 | 0.70 |
| *Fusobacterium* | -0.08 | 0.46 | 0.73 |
| *Lachnoanaerobaculum* | 0.06 | 0.51 | 0.73 |
| *Gemella* | -0.03 | 0.74 | 0.92 |
| *Veillonella* | -0.01 | 0.87 | 0.92 |
| *Granulicatella* | -0.01 | 0.89 | 0.92 |
| *Oribacterium* | -0.01 | 0.92 | 0.92 |
| Family | | | |
| *Saccharimonadaceae* | -0.22 | 0.06 | 0.35 |
| *Leptotrichiaceae* | -0.19 | 0.10 | 0.35 |
| *Pasteurellaceae* | -0.17 | 0.11 | 0.35 |
| *Streptococcaceae* | 0.03 | 0.13 | 0.35 |
| *Campylobacteraceae* | -0.11 | 0.15 | 0.35 |
| *Atopobiaceae* | -0.09 | 0.31 | 0.62 |
| *Prevotellaceae* | -0.06 | 0.38 | 0.65 |
| *Fusobacteriaceae* | -0.08 | 0.46 | 0.69 |
| *Lachnospiraceae* | -0.04 | 0.66 | 0.89 |
| *Gemellaceae* | -0.03 | 0.74 | 0.89 |
| *Veillonellaceae* | -0.01 | 0.87 | 0.89 |
| *Carnobacteriaceae* | -0.01 | 0.89 | 0.89 |
| Phylum | | | |
| *Patescibacteria* | -0.22 | 0.06 | 0.24 |
| *Firmicutes* | 0.01 | 0.07 | 0.24 |
| *Proteobacteria* | -0.17 | 0.11 | 0.25 |
| *Campilobacterota* | -0.11 | 0.15 | 0.26 |
| *Fusobacteriota* | -0.12 | 0.26 | 0.36 |
| *Actinobacteriota* | -0.09 | 0.31 | 0.36 |
| *Bacteroidota* | -0.06 | 0.38 | 0.38 |




**Supplementary figure 3:** DESeq2 normalized counts of differentially abundant taxa at phylum level between high and low (1st vs 3rd tertile) residential exposure to livestock-emitted endotoxin




**Supplementary figure 4:** Rarefaction plot of all samples colored by COPD case-control status

**Supplementary Table 3:** Univariable linear model estimates for alpha-diversity of oropharyngeal microbiome expressed as richness and Shannon index, corrected for case-control status

| Variable | Richness | | Shannon | |
|---|---|---|---|---|
| | β | p value | β | p value |
| COPD case vs. control | -4.36 | 0.12 | -0.09 | 0.20 |
| Age quartiles | | | | |
| 2nd vs. 1st quartile | -4.01 | 0.28 | -0.03 | 0.72 |
| 3rd vs. 1st quartile | -1.50 | 0.70 | 0.03 | 0.74 |
| 4th vs. 1st quartile | 1.77 | 0.65 | 0.10 | 0.28 |
| Education level | | | | |
| Medium vs. low | 5.54 | 0.10 | 0.18 | 0.03 |
| High vs. low | 0.03 | 0.99 | 0.09 | 0.31 |
| Gender female vs. male | -0.54 | 0.84 | 0.01 | 0.83 |
| Anti-inflammatory medication yes vs. no | -1.07 | 0.82 | -0.02 | 0.88 |
| Poultry <1km yes vs. no | 2.61 | 0.34 | -0.09 | 0.17 |
| Residential exposure to livestock farm emitted endotoxin ($EU/m^3$)[Ŧ] | 4.45 | 0.08 | 0.06 | 0.33 |
| Residential exposure to livestock farm emitted $PM_{10}$ ($\mu g/m^3$) [Ŧ] | 4.40 | 0.15 | 0.03 | 0.70 |
| Season | | | | |
| Fall (Sep-Nov) | -6.65 | 0.19 | -0.17 | 0.17 |
| Spring (Mar-May) | -1.05 | 0.77 | 0.06 | 0.47 |
| Summer (Jun-Aug) | 3.80 | 0.29 | 0.14 | 0.10 |
| Smoking history former vs. never | 0.12 | 0.97 | -0.02 | 0.76 |
| Antibiotics in the last 4 weeks yes vs no | -3.39 | 0.54 | 0.15 | 0.26 |

[Ŧ] Annual average concentration at the home address by dispersion modelling, scaled to the 10-90$^{th}$ percentile range.

**Supplementary Table 4:** Multivariable regression model for oropharyngeal microbiome alpha diversity stratified by COPD case-control status and including endotoxin exposure

| | Observed richness | | | | | | Shannon diversity | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | COPD case | | | Control | | | COPD case | | | Control | | |
| **Variable** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** |
| Gender female vs. male | 0.97 | 0.84 | -8.48 : 10.42 | -1.18 | 0.73 | -8 : 5.64 | 0.03 | 0.78 | -0.21 : 0.28 | -0.01 | 0.89 | -0.17 : 0.15 |
| Smoking history former vs. never | 4.36 | 0.4 | -5.98 : 14.7 | -2.79 | 0.41 | -9.52 : 3.94 | 0.02 | 0.88 | -0.25 : 0.29 | -0.09 | 0.25 | -0.25 : 0.06 |
| Education level | | | | | | | | | | | | |
| Medium vs. low | 11.41 | 0.05 | 0.1 : 22.73 | 4.66 | 0.29 | -3.97 : 13.29 | 0.29 | 0.06 | -0.01 : 0.58 | 0.17 | 0.09 | -0.03 : 0.37 |
| High vs. low | 5.87 | 0.36 | -6.92 : 18.67 | -1.12 | 0.81 | -10.43 : 8.19 | 0.17 | 0.3 | -0.16 : 0.5 | 0.08 | 0.47 | -0.14 : 0.3 |
| Residential exposure to livestock farm emitted endotoxin ($EU/m^3$)[₸] | 10.02 | 0.02 | 1.84 : 18.21 | 1.96 | 0.55 | -4.57 : 8.49 | 0.16 | 0.14 | -0.05 : 0.37 | 0.04 | 0.59 | -0.11 : 0.19 |
| Season | | | | | | | | | | | | |
| Fall (Sep-Nov) | 4.97 | 0.43 | -7.46 : 17.39 | -2.78 | 0.54 | -11.7 : 6.15 | 0.21 | 0.19 | -0.11 : 0.53 | 0.02 | 0.84 | -0.19 : 0.23 |
| Spring (Mar-May) | 5.56 | 0.41 | -7.68 : 18.8 | 2.41 | 0.59 | -6.34 : 11.15 | 0.27 | 0.11 | -0.07 : 0.62 | 0.1 | 0.32 | -0.1 : 0.31 |
| Summer (Jun-Aug) | -4.21 | 0.54 | -17.87 : 9.44 | -11.76 | 0.15 | -27.8 : 4.28 | -0.15 | 0.4 | -0.5 : 0.2 | -0.13 | 0.49 | -0.5 : 0.24 |
| Antibiotics in the last 4 weeks yes vs no | 0.88 | 0.9 | -12.38 : 14.14 | -11.19 | 0.27 | -31.22 : 8.84 | 0.2 | 0.26 | -0.15 : 0.54 | 0.04 | 0.86 | -0.42 : 0.51 |

[₸] Annual average concentration at the home address by dispersion modelling, scaled to the 10-90th percentile range.

**Supplementary Table 5:** Multivariable linear model for oropharyngeal microbiome alpha-diversity, including livestock-emitted $PM_{10}$

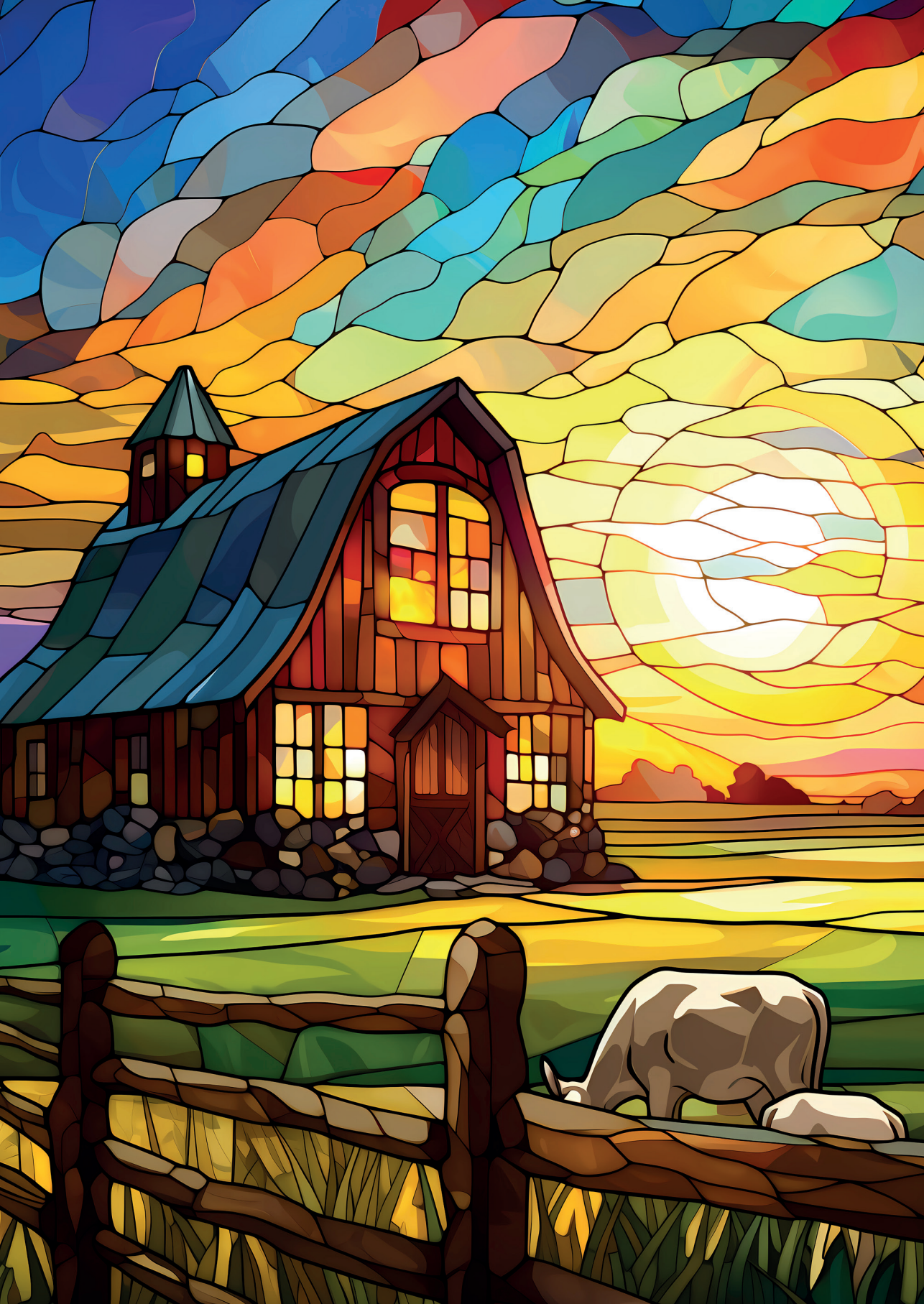
| | Richness | | | Shannon | | |
|---|---|---|---|---|---|---|
| **Variable** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** |
| COPD case vs. control | -2.29 | 0.45 | -8.2:3.62 | -0.043 | 0.55 | -0.19:0.1 |
| Gender female vs. male | -0.74 | 0.79 | -6.1:4.62 | -0.002 | 0.98 | -0.13:0.13 |
| Education level | | | | | | |
| Medium vs. low | 6.71 | 0.05 | -0.03:13.45 | 0.21 | 0.01 | 0.04:0.37 |
| High vs. low | 0.83 | 0.82 | -6.51:8.17 | 0.10 | 0.26 | -0.08:0.28 |
| Smoking history former vs. never | -0.71 | 0.80 | -6.24:4.82 | -0.06 | 0.42 | -0.19:0.08 |
| Residential exposure to livestock farm emitted $PM_{10}$ ($\mu g/m^3$) ₸ | 5.58 | 0.08 | -0.59: 11.74 | 0.08 | 0.29 | -0.07:0.23 |
| Season | | | | | | |
| Spring (Mar-May) vs. winter (Dec-Feb) | 1.02 | 0.78 | -6.23:8.27 | 0.10 | 0.24 | -0.07:0.28 |
| Summer (Jun-Aug) vs. winter | 5.81 | 0.12 | -1.47:13.08 | 0.19 | 0.03 | 0.02:0.36 |
| Fall (Sep-Nov) vs. winter | -5.45 | 0.29 | -15.6:4.7 | -0.13 | 0.28 | -0.38:0.11 |
| Antibiotics within 4 weeks prior to sampling | -2.50 | 0.65 | -13.36:8.36 | 0.16 | 0.23 | -0.1:0.42 |

₸ Annual average concentration at the home address by dispersion modelling, scaled to the 10-90th percentile range.

**Supplementary Table 6**: Multivariable regression model for oropharyngeal microbiome alpha diversity stratified by COPD case-control status and including $PM_{10}$ exposure

| | Observed richness | | | | | | Shannon diversity | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | COPD case | | | Control | | | COPD case | | | Control | | |
| **Variable** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** | **β** | **p value** | **95%CI** |
| Gender female vs. male | 0.4 | 0.93 | -9.03 : 9.82 | -1.08 | 0.76 | -7.89 : 5.74 | 0.03 | 0.83 | -0.22 : 0.27 | -0.01 | 0.91 | -0.17 : 0.15 |
| Smoking history former vs. never | 3.96 | 0.45 | -6.34 : 14.26 | -2.9 | 0.4 | -9.66 : 3.86 | 0.01 | 0.92 | -0.25 : 0.28 | -0.09 | 0.24 | -0.25 : 0.06 |
| Education level | | | | | | | | | | | | |
| Medium vs. low | 11.25 | 0.05 | 0 : 22.5 | 4.67 | 0.29 | -4 : 13.33 | 0.28 | 0.06 | -0.02 : 0.57 | 0.17 | 0.09 | -0.03 : 0.38 |
| High vs. low | 4.81 | 0.45 | -7.91 : 17.53 | -0.99 | 0.83 | -10.3 : 8.32 | 0.16 | 0.35 | -0.17 : 0.49 | 0.08 | 0.45 | -0.13 : 0.3 |
| Residential exposure to livestock farm emitted $PM_{10}$ ($\mu g/m^3$) [Ŧ] | 13.11 | 0.01 | 2.91 : 23.3 | 1.42 | 0.72 | -6.49 : 9.34 | 0.17 | 0.2 | -0.09 : 0.44 | 0.04 | 0.69 | -0.15 : 0.22 |
| Season | | | | | | | | | | | | |
| Fall (Sep-Nov) | 7.38 | 0.25 | -5.29 : 20.04 | -2.66 | 0.56 | -11.75 : 6.42 | 0.24 | 0.15 | -0.09 : 0.57 | 0.03 | 0.81 | -0.19 : 0.24 |
| Spring (Mar-May) | 9.44 | 0.16 | -3.82 : 22.7 | 2.83 | 0.54 | -6.19 : 11.84 | 0.33 | 0.06 | -0.02 : 0.67 | 0.11 | 0.28 | -0.1 : 0.32 |
| Summer (Jun-Aug) | -0.06 | 0.99 | -13.55 : 13.42 | -11.5 | 0.17 | -27.81 : 4.8 | -0.09 | 0.61 | -0.44 : 0.26 | -0.12 | 0.52 | -0.5 : 0.26 |
| Antibiotics in the last 4 weeks yes vs no | 1.71 | 0.8 | -11.58 : 14.99 | -11.3 | 0.27 | -31.37 : 8.76 | 0.2 | 0.25 | -0.14 : 0.55 | 0.04 | 0.87 | -0.43 : 0.51 |

[Ŧ] Annual average concentration at the home address by dispersion modelling, scaled to the 10-90th percentile range.



Chapter 4

# Air pollution from livestock farms and lung function decline in neighboring residents: a 7-year follow up study

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*In preparation*

# Abstract

## Introduction

Longitudinal studies investigating air pollution from livestock farms and respiratory health effects in neighboring residents are lacking. The aim of this study was to assess the relationship between residential livestock farm exposures and lung function decline over a 7-year period in people living in livestock dense areas.

## Methods

Spirometry was performed in 2014/2015 and 2021/2022 for 847 adults (28-80 years). We analyzed the annual rate of change in $FEV_1$, FVC, $FEV_1$/FVC, PEF and MMEF in relation to the annual-average livestock-emitted endotoxin concentration and $PM_{10}$ at the home address, which was predicted by dispersion modelling at baseline. Data analysis was performed using generalized additive models with a non-linear term for endotoxin or $PM_{10}$ exposure. Models were adjusted for age, sex, height, BMI, education level, smoking history, atopy and growing up on a livestock farm.

## Results

Endotoxin and $PM_{10}$ exposure were not associated with annual rate of change in lung function ($p>0.05$). Subjects with a farm childhood had larger annual decreases in $FEV_1$ (-5.63 ml/y, $p=0.018$) and MMEF (-11.15 ml/s per year, $p=0.032$), compared to those who did not grow up on a farm. Models stratified for atopy showed that the association between accelerated $FEV_1$ decline and farm childhood was more pronounced in individuals without atopy.

## Conclusion

Early life farm exposures were associated with accelerated lung-function decline in non-atopic adults. Longitudinal studies with more observations per individual are needed to assess the impact of livestock-related air pollution in rural populations during early life and adulthood.

## Introduction

Ambient air pollution has been associated with adverse effects on lung function throughout the course of human life.[1] An example is the acceleration of the natural process of lung function decline occurring from early adulthood onwards. In adults, longitudinal studies found concentrations of ambient pollutants as well as proxies of exposure (e.g. distance to the nearest road) to be associated with accelerated lung function decline.[2-5] An increasing number of studies focusing on air pollution from livestock farms indicated lung function deficits in nearby residents,[6-12] but most evidence comes from cross-sectional research or stems from panel studies with limited follow-up time. As a result, the long-term effects of exposure to air pollutants from livestock farms on the rate of natural lung function decline remain poorly understood. Livestock farms emit complex mixtures of gaseous pollutants and particulate matter (PM). This primary PM is distinctly different from traffic related PM; it has an organic composition and contains high levels of microorganisms including their components such as endotoxin, a Gram-negative bacterial cell wall component that has a high toxic potential.[13,14] Ammonia ($NH_3$) is an irritant gas that is formed from manure and emitted by livestock farms into the atmosphere. There, as an important precursor for secondary inorganic aerosols (SIA), it contributes greatly to ambient PM with an aerodynamic diameter of less than 2.5 µm ($PM_{2.5}$), which can be transported over long distances.[15]

Rural health studies reported negative relationships of annual and week average ambient $NH_3$ concentrations, in Germany and the Netherlands respectively, with lung function parameters such as the forced expiratory volume in the first second ($FEV_1$).[6,7] In the United States, panel studies in children with asthma and adults showed similar negative associations between $FEV_1$ and exposure to $NH_3$ and $PM_{2.5}$.[8,10,11] Recently, a cross-sectional study in Dutch adolescents reported a negative relationship between $FEV_1$ and modelled residential exposure to livestock farm emitted particulate matter ≤10 µm ($PM_{10}$).[16] In the same study, shorter distances to livestock farms and more farms within 3km of the home address were associated with lower $FEV_1$. A study in Germany reported a 7% lower $FEV_1$ in adults with more than twelve stables within 500m of the home address compared with five or fewer stables.[17] In comparison, In Dutch adults, the number of livestock farms within 1000m of the home address was associated with a lower maximum mid-expiratory flow (MMEF).[6]

Besides associations with worse respiratory health, protective associations with livestock exposure have also been reported. Children growing up on farms have been found to be less likely to develop atopy and allergic disease.[18-20] Later studies

showed that this association persists into adulthood.[21] Living in close proximity of livestock farms has also been associated with a lower prevalence of atopy,[22] after controlling for a farm childhood, suggesting that the protective farm effect could extend into non-farming adults. Recently, a negative association between livestock farm emission concentrations and atopy in Dutch adults was reported.[23] It has been hypothesized that early exposure to allergens on the farm may favour immune tolerance and protection against subsequent development of atopic diseases.[24] Maturation of the microbiome has also been suggested to play a role in the observed negative associations of childhood environmental exposures with allergic disease.[25]

The aim of the present study is to investigate the relationship between air pollution from livestock farms and long-term changes in lung function in a general, non-farming, rural population in the Netherlands. This population was examined by spirometry for the first time in 2014-2015,[6] and followed-up in 2021-2022. We hypothesise that air pollution from livestock farms is associated with an acceleration in the natural decline in lung function. To test this hypothesis, the rate of change in lung function over the follow-up period was analysed in relation to long-term residential exposure to livestock farming emitted $PM_{10}$ and endotoxin. As many inhabitants of the study area grew up on a livestock farm, the role of early-life livestock exposure in lung function decline was also evaluated.

## Materials and Methods

### Study population and design

The present prospective cohort study describes a 7-year follow-up between medical examinations of participants from the Livestock Farming and Neighboring Residents' Health research program (Dutch: Veehouderij en Gezondheid Omwonenden (VGO)).[6] The original design and recruitment of the VGO cohort has been described in detail before.[9,26] Briefly, in 2012, 14,882 individuals completed a survey among patients (aged 18-70 years) of 21 general practitioners in a livestock dense area in the southeast of the Netherlands. Subsequently, a medical examination was conducted between March 2014 and February 2015 in 2,494 subjects that agreed to participate and were not working or living on a farm. Of this study population, 2,369 participants had signed informed consent to be contacted for future research. These participants were invited for follow-up by mail, with maximally two reminders, between September 2020 and May 2022. In total, 969 participants (response rate 41%) underwent a follow-up examination during a home visit between August 2021 and July 2022. A flow chart of the study population can be found in Figure 1. Home

visits were performed by trained fieldworkers who self-tested for COVID-19 before starting their workday. Participants were phoned by the fieldworker prior to the visit, which was rescheduled in case of (suspected) COVID-19 on either side.

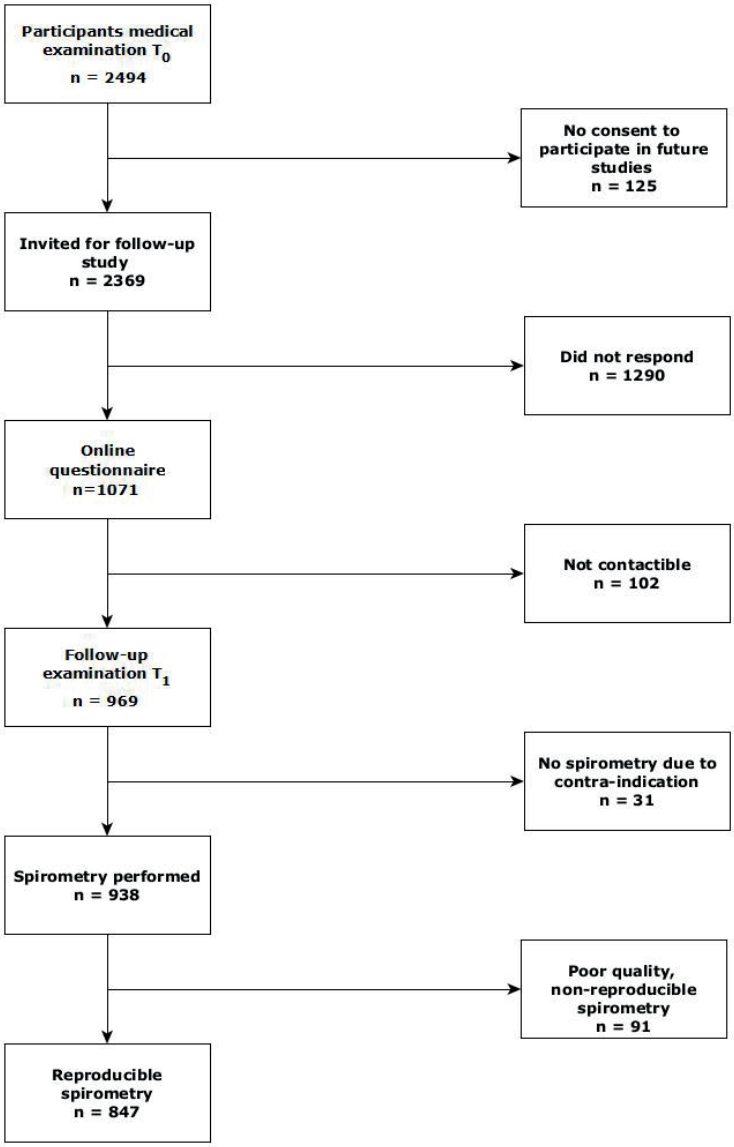


**Figure 1:** Flowchart of the study population

## Respiratory health assessment

Both at baseline ($T_0$=2014-15) and at 7-year follow-up ($T_1$=2021-22), population characteristics were collected by questionnaires. At both examinations, pre-bronchodilator $FEV_1$ (l),FVC (l), $FEV_1$/FVC, MMEF (l/s) and Peak Expiratory Flow (PEF, l/s) were measured by spirometry and expressed as both continuous variables and percent-predicted values using the GLI reference equations.[27] Only reproducible spirometry measurements that conform to ERS/ATS standards were included in the analysis.[28] Additional details can be found in the supplementary methods. We calculated the annual rate of change in lung function parameters between examinations as the difference between $T_1$ and $T_0$ spirometry values, divided by the time period between $T_0$ and $T_1$ dates (e.g. 7.2 years).[2] New onset airway obstruction was defined as $T_0$ FEV1/FVC>0.7 and $T_1$ FEV1/FVC<0.7, based on the global initiative for chronic lung disease (GOLD stage 1 and higher).[29] Self-reported doctor diagnosed asthma, chronic cough, chronic phlegm and wheezing were collected via (ECRHS-III based) questionnaire as dichotomous respiratory health measures (supplementary methods). Additionally, atopy was defined as one or both of 1) elevated levels of specific IgE antibodies (>0.35 U/ml) to at least one common allergen (cat, dog, grass, house dust mite) and 2) total IgE exceeding 100 IU/ml assessed by ELISA in serum samples collected at $T_0$.[22,30] All participants gave written informed consent and the study protocols of the initial (13/533) and present study (19-536/D) were approved by the Medical Ethical Committee of the University Medical Centre Utrecht.

## Exposure assessment

### *Air pollution from livestock farms*

Long-term individual exposure to livestock emissions was defined as the baseline annual-average livestock-related $PM_{10}$ (μg/$m^3$) and endotoxin (EU/$m^3$) concentrations at the home address, predicted by dispersion modelling as previously described.[23] Briefly, to model dispersion of air pollutants from a farm to the surroundings, a Gaussian plume model was used incorporating the Netherlands New National Model. The model used farm-type specific PM emission and PM size distribution characteristics as well as meteorological conditions and terrain roughness to estimate PM dispersion. Endotoxin emission and dispersion was modelled by including farm-type specific endotoxin content per PM size fraction. Residential exposure was estimated by applying dispersion modelling to individual barns within 10km of each residential address. Unrealistically high values in residential exposures, resulting from an erroneous overlap in geocoordinates of home addresses and farms, were winsorized to the 99.5 percentile in the downstream analysis. Additionally, as an exposure proxy, distance of the home address to the nearest farm was included

which was based on provincial livestock data of 2015 (https://veehouderijen.igoview.nl). Lastly, average ambient $NH_3$ concentrations (μg/m$^3$) in the week prior to spirometry at $T_0$ and $T_1$ were calculated as a proxy for short term exposure to livestock emissions. This was done using hourly observations in the study area, obtained from the national air quality monitoring network (www.luchtmeetnet.nl).

***Early life and other farm exposures***

Information on early life and other farm exposures were extracted from questionnaire data from closed questions (yes/no) about having lived on a farm childhood, performing farm jobs during childhood, and whether or not farms were visited in the past year.

## Data analysis

Data was analyzed in R studio with R version 4.2.1.[31] We first explored associations between residential exposure to livestock farming emitted endotoxin and $PM_{10}$ with the annual rate of change in each lung function parameter between observations. Since other studies of agricultural exposures and respiratory health measures have reported non-linear associations,[6,9,22,23] we ran penalized regression splines by means of the GAM function from the mgcv-package (version 1.8-42) using the (default) "thin plate" basis as well as the number of knots set to 3. Additionally, associations between residential exposure to livestock farming emitted endotoxin and $PM_{10}$ concentrations and the odds of new onset airway obstruction, and self-reported asthma, wheeze and chronic cough were investigated using logistic regression. All models were adjusted for age, sex, smoking history (current, ever, never), height, BMI, education level (low, intermediate, high), atopy, farm childhood and farm jobs during childhood. The shape of the associations in combination with smooth term 95% confidence intervals were assessed to determine relationships between livestock exposure and lung function parameters. To investigate associations between baseline lung function and covariates used to adjust the regression splines, we ran a linear model for baseline lung function.

***Secondary analysis***

To evaluate the role of atopic predisposition on the association between agricultural exposures and lung function, we analyzed exposure-response relationships among individuals with and without atopy separately in a subgroup analysis. To distinguish between childhood and current livestock exposures, we ran secondary models additionally adjusting for distance to nearest farm (m) and farm visits (yes/no). As spirometry measurements were performed during the COVID-19 pandemic in 2021 and 2022, and COVID-19 infection could have an impact on lung function, we

also ran models additionally adjusting for self-reported COVID-19 within 8 weeks of the measurement (yes/no). Differences in baseline characteristics between participants who did or did not partake in the follow-up examination were explored in a linear model. The potential impact of selection bias was further explored by assessing whether associations between $NH_3$ exposure and lung function were similar in responders and non-responders by including an interaction term for baseline $NH_3$ concentrations and follow-up participation. We investigated whether variability introduced by short-term effects of livestock air pollution exposure at both timepoints influenced our results. To this end, we ran models additionally adjusting for the difference between follow-up and baseline in $NH_3$ concentration in the week prior to spirometry.

# Results

## Study population

A total of 969 respondents (response rate 41%) were visited for follow-up examination between August 2021 and July 2022, of which 847 participants performed a reproducible lung function test. General characteristics of the study participants can be found in table 1. On average, 7.42 years had passed between baseline and follow-up examination. Participants were on average 63.3 years old, had a BMI of 27.2, tended to be intermediate (46.7%) or higher (37.1) educated and 52.5% were female. About half were former smokers (49.7%) with only 40 current smokers (4.7%). Just under a third of the participants were atopic (29.7%) and 311 (36.7%) reported having experienced a COVID-19 infection within 8 weeks before the follow-up examination. More than half (55.6%) worked on a farm during childhood and over a third (35.6%) grew up on a livestock farm. The average decrease in $FEV_1$ was 26 ml/year (sd 32.3 ml/year) and in FVC was 14.1 ml/year (sd 37.8 ml/year). Descriptive statistics of spirometry results at both examinations, used to calculate the annual rate of change, can be found in supplementary table S1. One out of eight (n=106, 12.5%) participants, without airway obstruction ($FEV_1$/FVC<0.7) at baseline, had developed obstruction at the time of the follow-up examination (supplementary table S2). Asthma (self-reported doctor diagnosed) was reported by 60 (7.1%) participants, chronic daily cough by 144 (17.2%), chronic daily phlegm by 120 (14.3%) and wheezing in the past year by 90 (10.8%). The distribution of exposure to livestock farming emitted endotoxin and $PM_{10}$ at participants' home addresses can be found in figure 2. Concentrations of both air pollutants were highly correlated (Pearson's r=0.79, p<0.001).

Individuals with a farm childhood typically lived closer to livestock farms compared to those without (p<0.001, Supplementary table S3). Additionally, those with a farm childhood were more likely to report farm visits in the past year (60.7% vs 45.2%, p<0.001). Performing farm jobs during childhood was highly prevalent among those growing up on a farm (89.3% vs 36.8%, p<0.001). However, even in those who did not live on a farm during childhood, 36.8% reported working on a farm during childhood. Characteristics of participants who agreed to the follow-up examination, compared to those who did not, can be found in supplementary table S4. Compared to non-responders, the population followed-up had a higher percentage of males (49.6% vs 42.6%) and tended to be higher educated (35.8% vs 26.7%). Baseline lung function was slightly better in respondents, with a higher %-predicted $FEV_1$ (100% vs 98.3%), FVC (103% vs 102%) and MMEF (96.6% vs 92.5%). A linear model for baseline spirometry with an interaction term for average $NH_3$concentration in the week prior to lung function testing and response to follow-up, found no difference in the association between $NH_3$ exposure and lung function between responders and non-responders (Supplementary table S5). The covariate model (without air pollution exposure) for baseline lung function showed that living on a livestock farm during childhood was associated with a higher MMEF (174.86 ml/s, p=0.01, supplementary table S8) but not $FEV_1$ (41.47, p=0.23) at baseline.



## Lung function decline and livestock-related air pollutants

Regression splines used to assess the relationship between air pollutants and annual rate of change in lung function parameters can be found in figure 3. No clear associations between decline rate in $FEV_1$, FVC, $FEV_1$/FVC and PEF are observed in relation to residential exposure to endotoxin. Increased endotoxin exposure (up to the 90$^{th}$ percentile) appears to be associated with accelerated MMEF decline based on the shape of the spline but this is not statistically discernable from zero (p=0.26). Similar weak associations between annual rate of change in lung function parameters and livestock-related $PM_{10}$ are shown. Confidence intervals are wide and rug plots show fewer observations in the higher range of exposure.

The covariate model (without air pollution exposure) that was used to adjust the regression splines can be found in supplementary table S9. Living on a livestock farm in childhood was associated with accelerated annual decline in $FEV_1$ (Figure 4, -5.15 ml, p=0.03) and MMEF (-12.04 ml/s, p=0.02). Likewise, former smokers had an accelerated annual decline in $FEV_1$ (-6.20 ml p=0.01) and MMEF (11.36 ml/s, p=0.02) compared to never smokers. Adjusting models for distance to the nearest farm, farm visits, performing farm jobs during childhood, or COVID-19 within 8 weeks of the follow-up examination did not significantly alter the observed relationships

between farm childhood and lung function (supplementary table S6). A sensitivity analysis performed in participants with and without atopy, showed the association of farm childhood with accelerated $FEV_1$ and MMEF decline was most pronounced in individuals without atopy (supplementary table S7).

**Table 1:** VGO study participant characteristics at 7-year follow-up

| | **Overall (N=847)** |
|---|---|
| **Age (y)** | 63.3 (10.0) |
| **Female** | 445 (52.5%) |
| **Height (cm)** | 170 (9.03) |
| **BMI (kg/m$^2$)** | 27.2 (4.33) |
| **Education level** | |
| low | 136 (16.2%) |
| intermediate | 392 (46.7%) |
| high | 312 (37.1%) |
| **Early-life livestock exposure** | |
| no | 343 (40.7%) |
| childhood job at farm | 200 (23.7%) |
| farm childhood* | 300 (35.6%) |
| **Smoking status** | |
| never | 386 (45.6%) |
| current | 40 (4.72%) |
| former | 421 (49.7%) |
| **Atopy** | 247 (29.7%) |
| **Self-reported COVID-19** | 311 (36.7%) |
| **Time between examinations (y)** | 7.41 (0.382) |
| **Lung function annual rate of change** | |
| Δ $FEV_1$ (ml/y) | -26.4 (32.3) |
| Δ FVC (ml/y) | -14.1 (37.8) |
| Δ $FEV_1$/FVC*100% (% per year) | -0.33% (0.49%) |
| Δ PEF (ml/sec per year) | -61.2 (143) |
| Δ MMEF (ml/sec per year) | -60.7 (68.8) |
| **Livestock exposure** | |
| Endotoxin (EU/m$^3$)$^\dagger$ | 0.246 (0.161) |
| $PM_{10}$ (µg/m$^3$) $^\dagger$ | 0.311 (0.177) |
| Distance to nearest farm (m) | 424 (252) |

Data are presented as mean (SD) or n (%). Education levels: low = lower secondary school or less; intermediate = intermediate vocational education or upper secondary school; high = higher education or university. *growing up on a farm with or without performing farm jobs. $^\dagger$ Baseline annual average concentration at the home address by dispersion modeling.

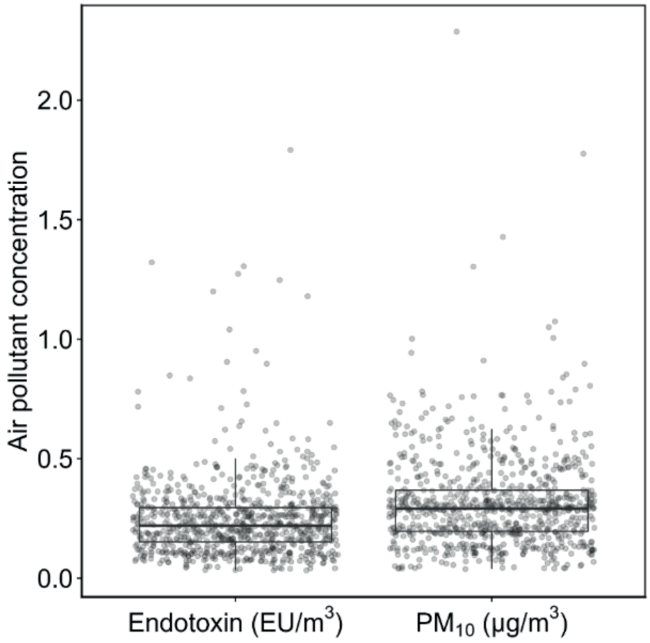


**Figure 2:** Baseline annual-average exposure to livestock farming emitted endotoxin at the home address estimated by dispersion modelling

## Dichotomous respiratory health outcomes and livestock-related air pollutants

We found that residential exposure to livestock farming emitted endotoxin was associated with a lower odds of developing airway obstruction in the period between baseline and follow-up examinations (supplementary figure S1, OR=0.85, 95%CI=0.71,0.99). A similar association was found for $PM_{10}$ (OR=0.86, 95%CI=0.73,1.01). Associations between endotoxin, $PM_{10}$ and self-reported doctor diagnosed asthma, chronic cough, chronic phlegm and wheezing were close to the null and not statistically discernable. Both asthma and new onset airway obstruction were less common in participants who grew up on a livestock farm compared to this who did not. However, this association was not statistically discernable ($p>0.05$).

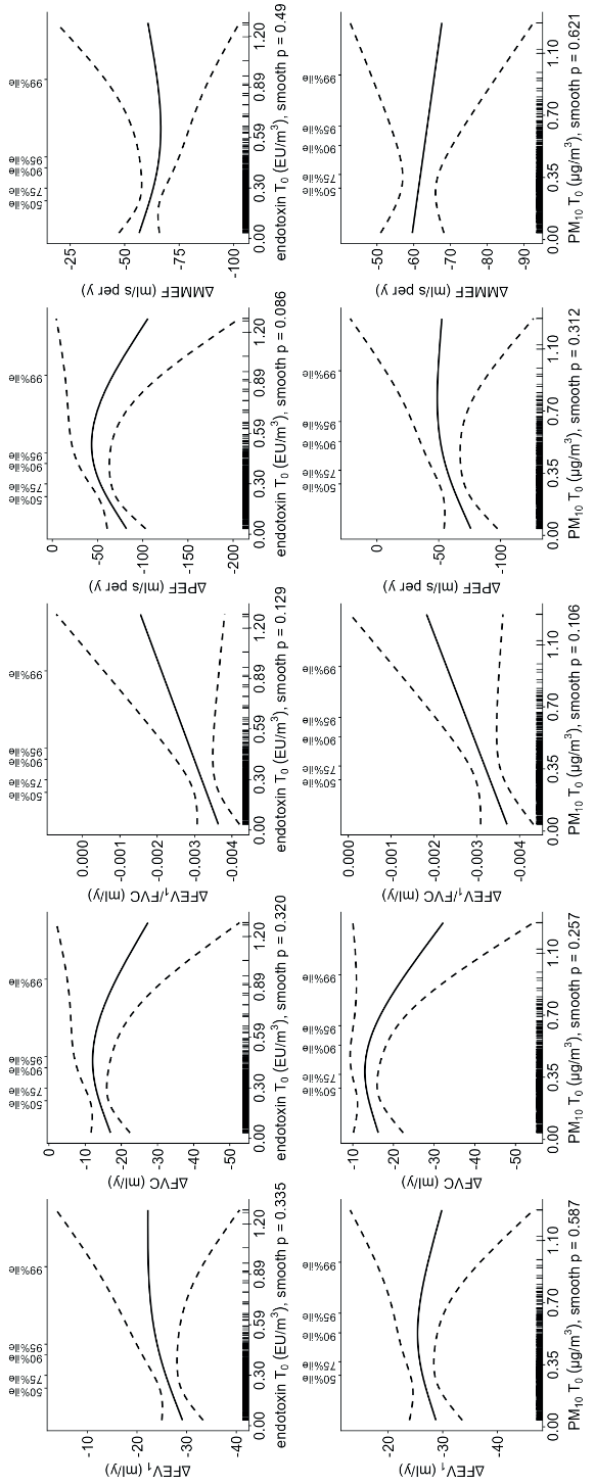


**Figure 3:** Splines of associations between annual rate of change in lung function parameters and livestock-related endotoxin and $PM_{10}$ concentrations predicted by dispersion modelling

*Note.* Number of knots was set to 3. Dashed lines indicate 95% confidence intervals. Associations were adjusted for age, sex, height, BMI, education level, smoking history, atopy, farm childhood. Rug plot shown on lower x-axis, percentiles shown on upper x-axis. Predicted livestock emitted air pollutant concentrations at residential addresses were winsorized to 99.5 percentile.

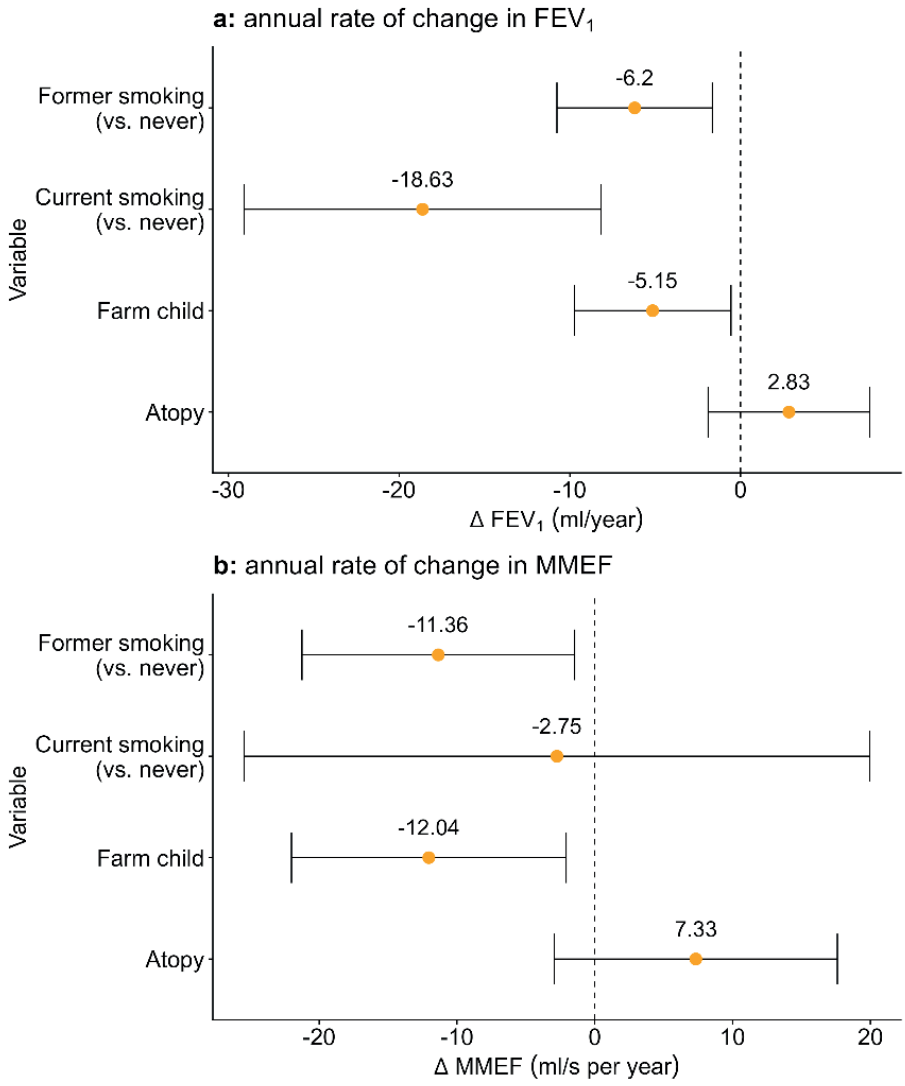


**Figure 4:** Covariate generalized additive model results, used to adjust air pollutant regression splines, estimating the impact of covariates on **a:** annual rate of change in $FEV_1$ and; **b:** annual rate of change in MMEF over seven years of follow-up

*Note:* models were additionally adjusted for age, sex, height, BMI and education level. The full covariate model can be found in supplementary table S1.

## Discussion

This study describes associations between long term residential exposure to air pollution from livestock farms and the rate of lung function decline over a period of seven years. No evidence was found for a relationship between residential exposure to livestock farming emitted endotoxin or $PM_{10}$ and the rate of decline in lung function parameters. Our results did indicate that growing up on a livestock farm is associated with accelerated lung function decline later in life, especially among nonatopic adults.

Our results show that living on a livestock farm during childhood is associated with accelerated $FEV_1$ and MMEF decline later in life. This finding is surprising in light of the well documented protective associations of childhood farm exposure with asthma and allergic sensitization that have been suggested to persist in adults.[32-35] An explanation for these protective associations has been sought in the hygiene hypothesis or similar 'old friends' hypothesis, revolving around microorganisms that coevolved with humans by embedding themselves (or their products) as regulatory inducers of immunologic pathways.[36] Lack of exposure to these organisms (loss of 'old friends') is thought to decrease the immune system's ability to modulate its response to allergens, causing it to overreact more often. In the present study, participants with atopic sensitization had a less pronounced association between early-life livestock exposure and lung function decline than those without. In a study with 10,201 adults from 14 European countries, growing up on a farm was associated with higher $FEV_1$ in women.[37] It has been argued that this apparent beneficial effect of a farm childhood on lung function could be driven by the protective effect of childhood farm exposure on atopic sensitization.[38] Separate models for participants with and without atopy, revealed that our observed associations between childhood farm exposure and lung function decline was more pronounced in individuals without atopy. Analysis of baseline lung function showed that those who grew up on a farm had higher lung function as an adult at baseline ($T_0$). This indicates that the relationship we found, between farm childhood and lung function decline, is not due to stunted lung function development during childhood and adolescence but accelerated decline later in life. Our results suggest that there might be a detrimental side to early life livestock exposure that emerges later in life. This is in line with a recent study from Finland that reported childhood farm exposure to be associated with late asthma diagnosis in adults aged 40-69 years.[39] In addition, a recent Canadian study reported an association between doing routine chores with large farm animals and increased asthma prevalence in farm and rural dwelling children.[40] This suggest that the impact of early-life exposure to non-infectious livestock emissions cannot only be described as healthy and beneficial.

Considering the limitations of the present study, it would be premature to conclude that livestock emitted air pollutants have no impact on the natural rate of lung function decline in nearby residents. The fact that we did not find an association between livestock related air-pollution and lung function decline can partially be explained by a lack of statistical power. Enrollment and subsequent fieldwork for the present study took place during the COVID-19 pandemic, which is likely to have resulted in a lower number of respondents (response rate 41%) and selection bias towards relatively healthy individuals who felt comfortable with a home visit relatively soon after lockdown measures were lifted in the Netherlands. This is supported by the non-response analysis, showing that participants of both baseline and follow-up examinations had a better baseline lung function, compared to participants who only underwent the baseline examination. This suggests that participants with worse baseline lung function tended to be lost to follow-up, which is partly explained by fewer smokers participating. As a result, estimates for the lung function rate of change are presumed to be underestimated considering the total study population at baseline. Selective loss to follow-up (or attrition) is a known concern in longitudinal lung function studies, especially in older adults like the present cohort.[5] However, the previously reported negative association between week prior $NH_3$ and baseline lung function was not found to differ between responders and non-responders.[6] This shows that effects of livestock emissions could also be detected in this sub-group with better lung function, suggesting that selection bias did not influence our results. Another limitation is that the follow-up examinations took place during 2021-2022, overlapping with the COVID-19 pandemic. However, adjusting our models for testing COVID-19 positive within 8 weeks of the home visit did not significantly alter our results. Strengths of our study include the use of high quality spirometry measurements. Apart from a software update, the exact same spirometers were used during baseline and follow-up, limiting potential bias introduced by equipment changes, a known issue of longitudinal lung function studies.[41] Additionally, the dispersion modelled livestock farm emitted endotoxin and $PM_{10}$ concentrations at the home address offered more detailed residential exposure assessment compared to previously used crude exposure proxies.[6]

Prior research on the effects of livestock-related air pollution on lung function, using data from the present cohort at baseline, has not yielded consistent findings.[6,23] The study by Borlée et al., focusing on short term exposure, found that week average $NH_3$ concentrations (in the week before examination) were negatively associated with $FEV_1$ and MMEF.[6] In the present study, focusing on long-term effects of air pollutants on lung function decline, short-term air pollutant exposure could have confounded our results. Our sensitivity analysis, additionally adjusting for the difference in week

prior $NH_3$ concentrations between examinations, showed that the influence of short-term exposure on our results was negligible. The same study by Borlée et al., reported a negative association between the number of livestock farms within 1km of the home address and MMEF. In the sensitivity analysis of the present study, no association with this exposure proxy was found and no effect on our results was observed. The study by De Rooij et al, also using the same baseline observations, found that annual average endotoxin concentrations, but not $PM_{10}$,tended to be associated with lower FVC.[23] In addition, a recent cross-sectional study in a different Dutch cohort consisting of adolescents reported an association between long term livestock-related $PM_{10}$ exposure and lower $FEV_1$.[16] While the body of evidence from cross-sectional studies is convincing that short-term and long-term residential exposure to livestock-related air pollution is associated with lower lung function, the relationship of air pollution from livestock farms with long-term changes in lung function remains unclear.

Earlier literature on, mainly traffic-related, air pollution and lung function shows a similar pattern but evidence on longitudinal effects is emerging.[5] In 2015 the ESCAPE study reported a cross-sectional association between traffic-related air pollution and lower lung function in adults living in multiple cities across Europe.[4] At the same time, no evidence for a relationship with longitudinal change in lung function was found. Later that year, however, a study from the United States reported an association of $PM_{2.5}$ with lower $FEV_1$ as well as greater annual $FEV_1$ decline in adults.[2] A more recent study in the United States investigated relationships between long-term ambient air pollution exposure with change in occurrence of emphysema and lung function in adults.[42] Higher baseline ozone ($O_3$), nitrogen oxide ($NO_x$), $PM_{2.5}$and black carbon concentrations at the home address were associated with a greater increase in emphysema incidence. Only $O_3$ concentrations at baseline, and during follow-up, were associated with increased $FEV_1$ decline per 10 years.

Our results on dichotomous respiratory health outcomes showed that residential exposure to livestock-emitted endotoxin is suggested to be associated with decreased odds for developing airway obstruction between baseline and follow-up. Similarly, prevalence of COPD has been shown to be lower in close proximity to livestock farms in the same area.[9] The same study, however, reported increased respiratory symptoms in COPD patients living close to livestock farms. Another study has found that individuals with overlapping diagnosis of asthma and COPD also experience increased symptom burden.[43] Important to note in this context is the multitude of definitions for COPD used in literature. Prevalence and risk factors of COPD have been shown to vary depending on the operational definition.[26] In

the present study, airway obstruction was assessed solely by spirometry which is thought to lead to an overestimation of clinical COPD which should incorporate other indicators like respiratory symptoms, and risk factors such as smoking, family history, and occupational history.[44] For epidemiological purposes, with no intention to treat, relying on spirometry alone has been found acceptable.[26] In addition, in our study with limited power adding further stratification for COPD grades (e.g. incorporating symptoms) would not be meaningful.

In conclusion, our results indicate that early life exposure to livestock farming is associated with accelerated lung function decline later in life. Given that livestock industries are increasingly situated near densely populated areas across the globe, this calls for future longitudinal studies assessing the impact of livestock-related air pollution in rural populations during early life and in adulthood.



## Funding

This study was part of the Livestock Farming and Neighboring Residents' Health study(VGO) that was funded by the Ministry of Health, Welfare and Sport and the Ministry of Agriculture, Nature and Food Quality of The Netherlands, and supported by a grant from ZonMW (TOP project 91217040) and the research programme of the Netherlands Centre for One Health (www.ncoh.nl).

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Chapter 4s

# Supplementary Materials

# Supplementary methods

## Questionnaire

1. What is your sex?

□ Male □ Female

2. What is your birthdate?

Day month year

3. Have you had wheezing or whistling in your chest at any time in the last 12 months?

□ Yes □ No

4. Have you ever had asthma?

□ Yes □ No (if no, continue with question 8)

*If yes*, ...

5. Was the asthma confirmed by a doctor?

□ Yes □ No

6. Do you cough nearly daily, for as long as three months per year?

□ Yes □ No

7. Do you produce phlegm nearly daily, for as long as three months per year?

□ Yes □ No

8. Have you had pneumonia in the past 3 years?

□ Yes □ No

9. As a child (until 18 years old), did you live on a farm with animals?

□ Yes □ No

10. As a child (up to the age of 18), did you carry out one or more of the following activities on a farm? (multiple answers possible)

□ Animal care with intensive animal contact
□ Working with manure
□ Working with straw / hay / grass silage / animal feed
□ Crop care
□ None of the above

11. Have you visited a farm in the past 12 months? (multiple answers possible)

□ Yes, visiting family
□ Yes, for work
□ Yes, to buy produce (e.g. vegetables, fruit, eggs or meat)
□ Yes, a petting zoo
□ Yes, for another reason
□ No



## Spirometry

During both examinations pre-bronchodilator (BD) spirometry was conducted according to ERS/ATS standards.[1] Pre-BD spirometry measures the lung function of the participant without any effect of lung medication. Participants stopped using inhalers and oral lung medication 4 and 8 hours prior to the examination. The EasyOne Spirometer (NDD Medical Technologies, Inc.) was used which measures flow and volume by ultra-sound transit time. In an effort to increase the quality of the spirometry data, we attempted to obtain four acceptable spirograms per participant. In addition, an expert reviewed the quality of all lung function curves in the NDD software. In the quality review process, the three best curves were ranked manually based on predefined ERS criteria.[1] Additional exclusions were made after inspecting scatterplots of $FEV_1$ and FVC at baseline and follow-up in combination with questionnaire data. One participant with standout lower $FEV_1$ and FVC at follow-up (compared to baseline) was excluded due to bruised ribs during follow-up examination. In addition, 5 participants were excluded as the data suggested that the follow-up examination was not performed by the same individual as at baseline (e.g. different birthdate with an abnormal increase in lung function). Only data from

participants with a spirometry quality grade C (at least two reproducible curves within 200ml) or higher were used in the downstream analysis (Figure 1).

Particulate matter air pollution, as well as airborne endotoxin exposure, is associated with decreases in parameters of both large airways and small airways.[2,3] Large airway function is assessed with parameters: Forced Expiratory Volume in 1 second (FEV1), Forced Vital Capacity (FVC) and the ratio between these two (FEV1/FVC), small airway function is assessed with Peak Expiratory Flow (PEF) and Maximum Mid-Expiratory Flow (MMEF).

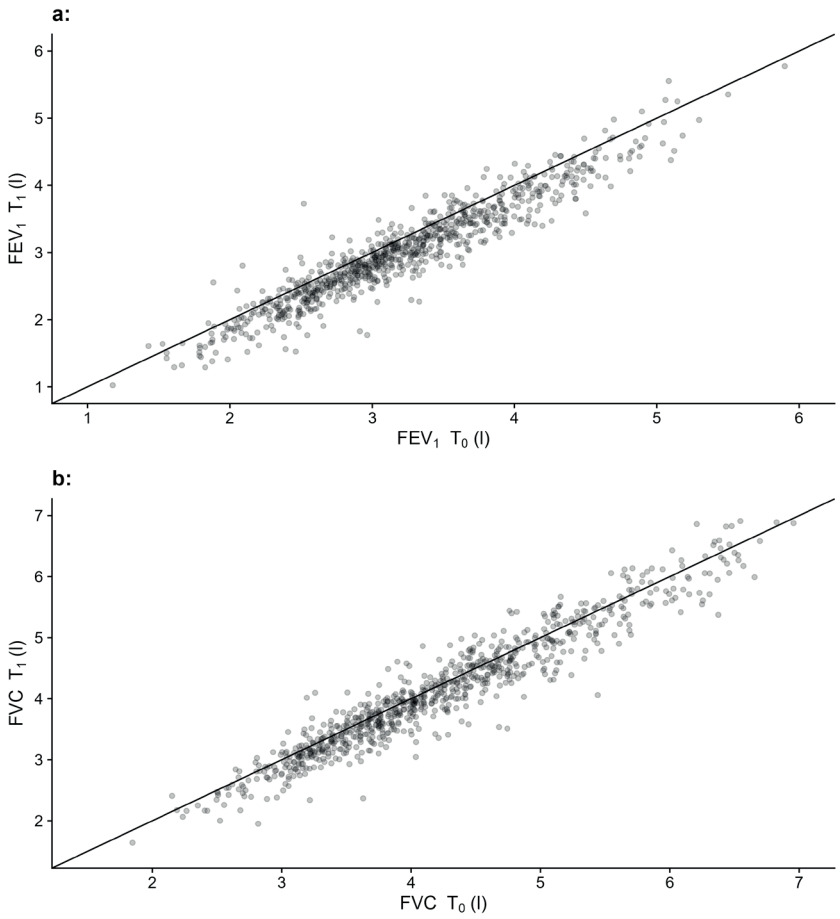


**Figure 1:** $FEV_1$ (**a**) and FVC (**b**) at baseline vs follow-up of the dataset used for analysis, the solid line depicts the line of unity

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# Supplementary Tables and Figures

**Supplementary Table S1:** lung function parameters in 2014-2015 ($T_0$) and 2021-2022 ($T_1$) measured during the VGO study

| | $T_0$ (N=847) | $T_1$ (N=847) | p-value* |
|---|---|---|---|
| **$FEV_1$ (L)** | 3.21 (0.74) | 3.01 (0.75) | <0.001 |
| **FVC (L)** | 4.22 (0.99) | 4.12 (0.98) | 0.02 |
| **$FEV_1$/FVC %** | 76.10 (6.51) | 73.7 (7.32) | <0.001 |
| **PEF (L/sec)** | 8.51 (2.04) | 8.05 (2.11) | <0.001 |
| **MMEF (L/sec)** | 2.75 (1.06) | 2.30 (1.02) | <0.001 |

Data are presented as mean (SD). * t-test.

**Supplementary Table S2:** dichotomous respiratory health outcomes of VGO 7-year follow-up study

| | Overall (N=847) |
|---|---|
| **New onset airway obstruction ($FEV_1$/FVC<0.7)** | |
| no | 609 (71.9%) |
| yes | 106 (12.5%) |
| Baseline airway obstruction | 132 (15.6%) |
| **Self-reported doctor diagnosed asthma** | 60 (7.08%) |
| **Daily cough >3m last year** | 144 (17.2%) |
| **Daily phlegm >3m last year** | 120 (14.3%) |
| **Wheezing last year** | 90 (10.8%) |
| **Self-reported COVID-19** | |
| no | 536 (63.3%) |
| yes, tested | 253 (29.9%) |
| yes, suspected | 58 (6.85%) |
| **COVID-19 <8wks of visit** | 72 (8.63%) |

Data are presented as n (%).

**Supplementary Table S3:** VGO follow-up study participant characteristics stratified by farm childhood

| Living on a farm in childhood | no (N=543) | yes (N=300) | p-value* |
|---|---|---|---|
| **Distance to nearest farm (m)** | 448 (253) | 380 (241) | <0.001 |
| **Farm visit in past year** | 242 (45.2%) | 179 (60.7%) | <0.001 |
| **Multiple reasons for farm visit** | | | |
| No, did not visit | 293 (54.8%) | 116 (39.3%) | <0.001 |
| No, single reason | 195 (36.4%) | 135 (45.8%) | |
| yes | 47 (8.79%) | 44 (14.9%) | |
| **Childhood farm job** | 200 (36.8%) | 268 (89.3%) | <0.001 |
| **Atopy** | 183 (34.0%) | 64 (22.0%) | <0.001 |

Data are presented as mean (SD) or n (%). * t-test or chi$^2$.

**Supplementary Table S4:** VGO participant baseline characteristics, comparing follow-up responders and non-respondersSupplementary Table S5: linear model results for percentage predicted baseline lung function with interaction for week average NH3 and follow-up examination

| | Non-responders (N=1525) | responders (N=969) | p-value* |
|---|---|---|---|
| **Age at baseline** | 56.5 (11.7) | 56.4 (9.99) | 0.946 |
| **female** | 875 (57.4%) | 488 (50.4%) | <0.001 |
| **Height (cm)** | 170 (0.90) | 172 (0.90) | <0.001 |
| **BMI (kg/m$^2$)** | 27.2 (4.45) | 26.9 (3.94) | 0.140 |
| **Education level** | | | |
| low | 439 (29.2%) | 168 (17.5%) | <0.001 |
| intermediate | 662 (44.0%) | 450 (46.8%) | |
| high | 402 (26.7%) | 344 (35.8%) | |
| **Early-life livestock exposure** | | | |
| no | 642 (42.7%) | 391 (40.5%) | 0.295 |
| farm job | 370 (24.6%) | 229 (23.7%) | |
| farm childhood | 492 (32.7%) | 345 (35.8%) | |
| **Atopy** | 447 (30.0%) | 280 (29.4%) | 0.779 |
| **Smoking** | | | |
| non | 629 (41.2%) | 430 (44.4%) | 0.023 |
| former | 735 (48.2%) | 467 (48.2%) | |
| current | 161 (10.6%) | 72 (7.43%) | |

**Supplementary Table S4:** Continued

| | Non-responders (N=1525) | responders (N=969) | p-value* |
|---|---|---|---|
| **Lung function** | | | |
| percent predicted$FEV_1$† | 98.3 (16.0) | 100 (14.5) | 0.001 |
| percent predicted FVC† | 102 (13.4) | 103 (12.8) | 0.040 |
| percent predicted MMEF† | 92.5 (33.5) | 96.8 (32.8) | 0.002 |
| $FEV_1$/FVC (%) | 95.4 (9.26) | 96.5 (8.11) | 0.002 |
| **Livestock exposure** | | | |
| Endotoxin (EU/m3)‡ | 0.247 (0.169) | 0.247 (0.157) | 0.898 |
| $PM_{10}$ (µg/m3)‡ | 0.302 (0.187) | 0.311 (0.176) | 0.214 |
| Distance to nearest farm (m) | 446 (273) | 427 (252) | 0.065 |

Data are presented as mean (SD) or n (%). Education levels: low = lower secondary school or less; intermediate = intermediate vocational education or upper secondary school; high = higher education or university.† percentage of predicted value calculated conform GLI 2012 reference equations.
‡ Baseline annual average concentration at the home address by dispersion modeling. * t-test or $chi^2$.

**Supplementary Table S5:** linear model results for percentage predicted baseline lung function with interaction for week average NH3 and follow-up examination

| | $FEV_1$ % predicted | | FVC % predicted | | $FEV_1$/FVC % | | MMEF % predicted | |
|---|---|---|---|---|---|---|---|---|
| | **β** | **95% CI** | **β** | **95% CI** | **β** | **95% CI** | **β** | **95% CI** |
| **Week prior avr. $NH_3$** (µg/m3) | -6.52 | -12.88, -0.16 | -0.75 | -2.44, 0.94 | -2.88 | -5.45, -0.31 | -3.8 | -6.79, -0.81 |
| **Smoking history** | | | | | | | | |
| current vs never | -22.63 | -27.48, -17.78 | -7.11 | -8.4, -5.82 | -1.77 | -3.73, 0.19 | -8.61 | -10.9, -6.33 |
| former vs never | -2.13 | -4.99, 0.73 | -1.81 | -2.57, -1.05 | 1.38 | 0.22, 2.53 | -0.34 | -1.68, 1.01 |
| **Born in study area** | 1.19 | -2.08, 4.45 | -0.6 | -1.47, 0.27 | 2.45 | 1.13, 3.77 | 1.82 | 0.29, 3.36 |
| **Farm childhood** | 6.86 | 3.93, 9.8 | 0.82 | 0.04, 1.6 | 1.88 | 0.69, 3.06 | 2.68 | 1.3, 4.06 |
| **Follow-up examination** | 0.91 | -6.03, 7.86 | 0.99 | -0.86, 2.83 | -0.39 | -3.19, 2.42 | 0.22 | -3.05, 3.49 |
| **Week average NH3 x Follow-up examination** | 3.74 | -6.2, 13.67 | -0.32 | -2.96, 2.32 | 2.51 | -1.51, 6.52 | 2.51 | -2.17, 7.19 |

All variables were mutually adjusted. % predicted values were calculated conform 2012 GLI reference equations.

**Supplementary Table S6:** Results of generalized additive model for farm childhood, additionally adjusted for distance to the nearest farm, farm visits, childhood farm jobs, COVID-19 within 8 weeks of follow-up examination and change in week prior average $NH_3$ concentration

| | ΔFEV1 (ml/y) | | ΔFVC (ml/y) | | ΔFEV1/FVC (%/y) | | ΔPEF (ml/s per year) | | ΔMMEF (ml/s per year) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | β | 95%CI | β | 95%CI | β | 95%CI | β | 95%CI | β | 95%CI |
| **Base model** | | | | | | | | | | |
| Farm childhood | -4.66 | -9.21, -0.11 | -2.75 | -8.16, 2.66 | -0.04 | -0.11, 0.03 | -5.05 | -25.46, 15.37 | -10.69 | -20.50, -0.88 |
| **Distance to farm** | | | | | | | | | | |
| Farm childhood | -4.48 | -9.08, 0.11 | -2.00 | -7.45, 3.45 | -0.05 | -0.12, 0.02 | -5.75 | -26.38, 14.88 | -10.59 | -20.5, -0.67 |
| log distance to the nearest farm (m) | 0.85 | -2.23, 3.93 | 3.57 | -0.08, 7.22 | -0.03 | -0.08, 0.01 | -3.33 | -17.15, 10.50 | 0.49 | -6.16, 7.13 |
| **Farm visits** | | | | | | | | | | |
| Farm childhood | -4.76 | -9.40, -0.13 | -2.89 | -8.40, 2.62 | -0.04 | -0.12, 0.03 | -6.98 | -27.84, 13.89 | -9.49 | -19.52, 0.53 |
| farm visits | 1.90 | -2.44, 6.24 | 1.12 | -4.04, 6.29 | 0.01 | -0.06, 0.08 | 9.59 | -9.97, 29.15 | 2.45 | -6.94, 11.85 |
| **Farm jobs during childhood** | | | | | | | | | | |
| Farm childhood | -5.11 | -10.43, 0.22 | -1.01 | -7.33, 5.30 | -0.08 | -0.17, -0.0004 | -8.04 | -31.91, 15.83 | -14.15 | -25.61, -2.68 |
| Farm job during childhood | 0.82 | -4.28, 5.91 | -3.21 | -9.27, 2.84 | 0.07 | -0.005, 0.15 | 5.53 | -17.34, 28.40 | 6.40 | -4.59, 17.38 |
| **COVID-19 prior to examination** | | | | | | | | | | |
| Farm childhood | -4.48 | -9.09, 0.14 | -2.85 | -8.34, 2.63 | -0.04 | -0.05, -0.04 | -2.26 | -22.87, 18.36 | -9.84 | -19.75, 0.06 |
| COVID-19 within 8 wks of examination | -1.42 | -9.10, 6.27 | -6.33 | -15.47, 2.81 | 0.06 | 0.07, 0.07 | 16.11 | -18.23, 50.46 | 3.87 | -12.64, 20.37 |
| **Change in week prior avr. NH3** | | | | | | | | | | |
| Farm childhood | -4.68 | -9.24, -0.13 | -2.71 | -8.12, 2.70 | -0.04 | -0.11, 0.03 | -4.69 | -25.11, 15.73 | -10.89 | -2.96, 17.25 |
| Change in week prior avr. NH3 (µg/m3) | -0.02 | -0.15, 0.11 | 0.04 | -0.12, 0.20 | -0.001 | -0.003, 0.001 | 0.36 | -0.23, 0.96 | -0.21 | -0.49, 0.08 |

All variables were mutually adjusted. Models were additionally adjusted for age, gender, height, BMI, education level, smoking history and atopy.

**Supplementary Table S7:** generalized additive model covariate results in participants with and without atopy

| | ΔFEV1 (ml/y) | | ΔFVC (ml/y) | | ΔFEV1/FVC (%/y) | | ΔPEF (ml/s per year) | | ΔMMEF (ml/s per year) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | β | 95%CI | β | 95%CI | β | 95%CI | β | 95%CI | β | 95%CI |
| **Smoking current vs never** | | | | | | | | | | |
| atopic | -39.00 | -57.91, -20.09 | -54.58 | -77.10, -32.05 | 0.04 | -0.24, 0.32 | -77.14 | -159.60, 5.33 | 2.79 | -36.28, 41.86 |
| non-atopic | -11.18 | -23.51, 1.15 | -9.72 | -24.34, 4.90 | -0.10 | -0.30, 0.09 | -89.86 | -146.11, -33.62 | -7.07 | -34.40, 20.26 |
| **Smoking former vs never** | | | | | | | | | | |
| atopic | -12.16 | -20.76, -3.56 | -16.74 | -26.99, -6.49 | -0.02 | -0.15, 0.10 | -31.13 | -68.64, 6.38 | -11.10 | -28.87, 6.67 |
| non-atopic | -4.39 | -9.66, 0.87 | -2.60 | -8.84, 3.64 | -0.06 | -0.14, 0.03 | 12.23 | -11.78, 36.25 | -13.03 | -24.70, -1.36 |
| **farm childhood** | | | | | | | | | | |
| atopic | -0.66 | -11.58, 10.25 | 2.64 | -10.36, 15.64 | 0.03 | -0.14, 0.19 | 20.57 | -27.04, 68.18 | -15.89 | -38.45, 6.66 |
| non-atopic | -6.58 | -12.61, -0.54 | -1.97 | -9.13, 5.18 | -0.13 | -0.22, -0.03 | -18.50 | -46.04, 9.03 | -14.23 | -27.61, -0.85 |
| **Childhood farm job** | | | | | | | | | | |
| atopic | 0.29 | -9.24, 9.82 | -2.75 | -14.10, 8.60 | -0.02 | -0.16, 0.12 | -27.70 | -69.26, 13.86 | 4.20 | -15.49, 23.88 |
| non-atopic | 1.19 | -4.82, 7.19 | -3.54 | -10.66, 3.58 | 0.12 | 0.03, 0.22 | 20.57 | -6.81, 47.96 | 7.29 | -6.01, 20.60 |

Models were adjusted for age, sex, height, BMI and education level. If relevant, models were additionally adjusted for smoking history, farm childhood and childhood farm job.

**Supplementary Table S8:** Generalized additive model associations between covariates and baseline lung function

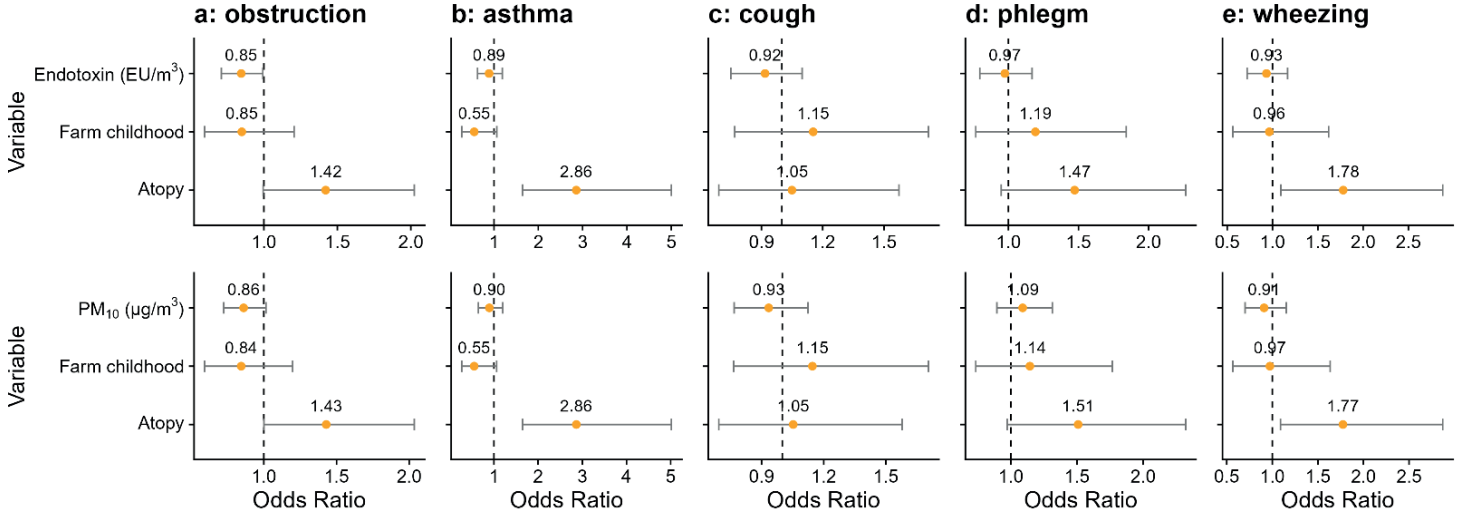
| | FEV1 (ml) | | FVC (ml) | | FEV1/FVC (%) | | PEF (ml/s) | | MMEF (ml/s) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | β | 95%CI | β | 95%CI | β | 95%CI | β | 95%CI | β | 95%CI |
| **Age (per 10y)** | -251.58 | -288.84, -214.31 | -209.29 | -251.18, -167.41 | -2.14 | -2.63, -1.65 | -322.18 | -431.56, -212.79 | -459.91 | -532.8, -387.02 |
| **Female** | -528.59 | -622.35, -434.83 | -711.31 | -816.68, -605.94 | 0.32 | -0.91, 1.55 | -2278.73 | -2553.92, -2003.54 | -427.83 | -611.2, -244.46 |
| **Height (cm)** | 35.37 | 30.25, 40.49 | 52.89 | 47.14, 58.65 | -0.10 | -0.17, -0.03 | 56.95 | 41.92, 71.97 | 19.95 | 9.94, 29.96 |
| **BMI (kg/m2)** | -9.18 | -16.81, -1.56 | -17.62 | -26.19, -9.05 | 0.07 | -0.03, 0.17 | 4.43 | -17.96, 26.82 | 4.07 | -10.85, 18.99 |
| **Education level (vs low)** | | | | | | | | | | |
| medium | 13.49 | -80.54, 107.53 | -10.26 | -115.93, 95.41 | 0.40 | -0.83, 1.63 | 116.82 | -159.16, 392.81 | 39.21 | -144.69, 223.11 |
| high | 31.96 | -66.60, 130.51 | 42.72 | -68.04, 153.48 | 0.14 | -1.15, 1.43 | 259.55 | -29.71, 548.81 | -38.77 | -231.51, 153.98 |
| **Smoking history (vs never)** | | | | | | | | | | |
| current | -190.86 | -346.06, -35.67 | -16.88 | -191.29, 157.53 | -4.13 | -6.16, -2.10 | -413.01 | -868.51, 42.49 | -574.79 | -878.31, -271.26 |
| former | -8.13 | -75.80, 59.54 | 31.49 | -44.56, 107.55 | -0.68 | -1.57, 0.21 | 117.29 | -81.33, 315.92 | -47.69 | -180.04, 84.66 |
| **Farm childhood** | 40.06 | -28.16, 108.27 | -21.22 | -97.88, 55.44 | 0.94 | 0.05, 1.83 | 23.28 | -176.92, 223.48 | 169.25 | 35.84, 302.65 |
| **Atopy** | -13.55 | -83.78, 56.68 | 53.45 | -25.48, 132.38 | -1.30 | -2.22, -0.38 | -8.23 | -214.37, 197.9 | -95.79 | -233.15, 41.57 |

All variables were mutually adjusted.

**Supplementary Table S9:** Results of generalized additive modelling on associations between covariates and lung function rate of change over seven years of follow-up

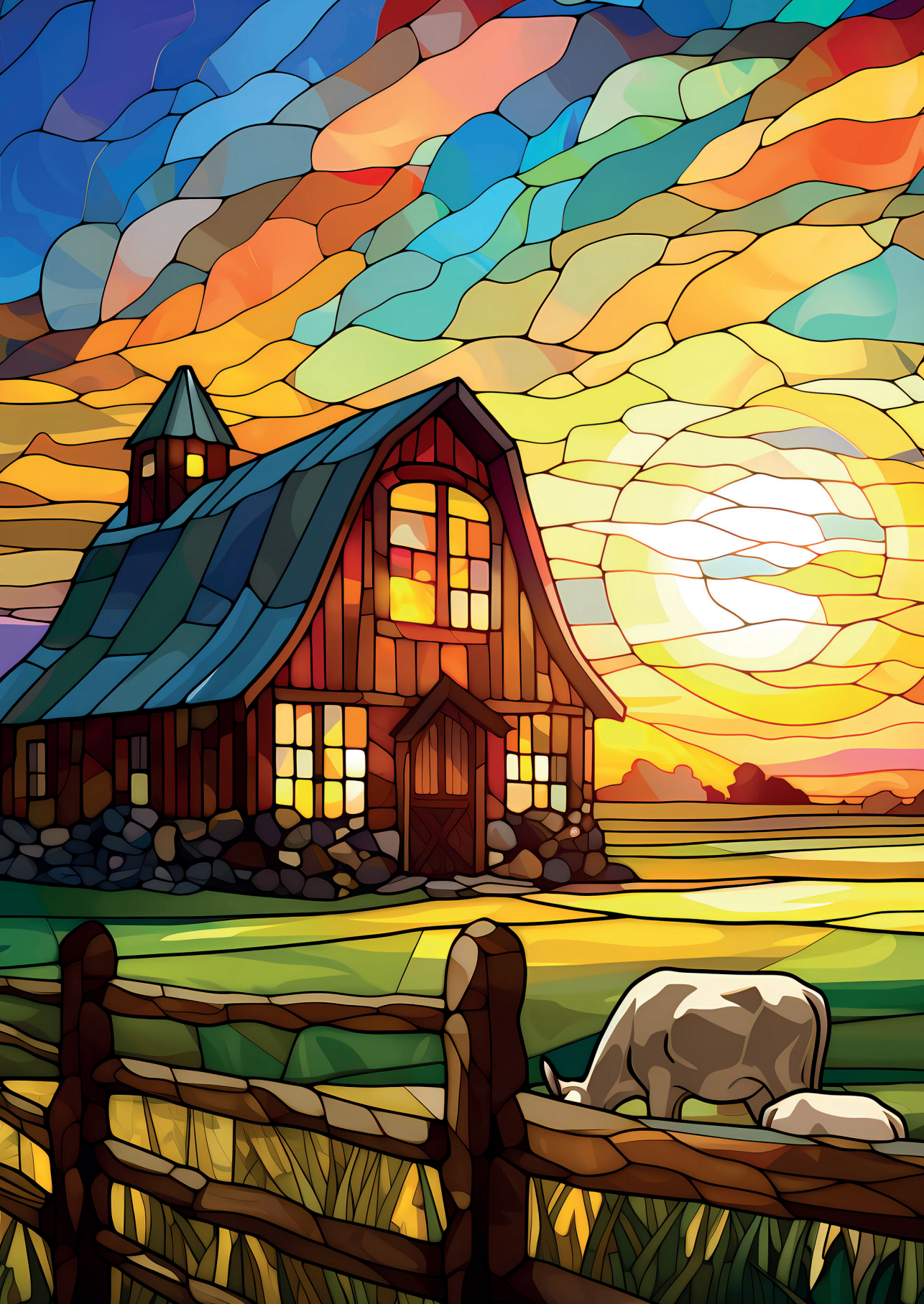
| | ΔFEV1 (ml/y) | | ΔFVC (ml/y) | | ΔFEV1/FVC (%/y) | | ΔPEF (ml/s per year) | | ΔMMEF (ml/s per year) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | β | 95% CI | β | 95% CI | β | 95% CI | β | 95% CI | β | p value |
| **Age (per 10y)** | -3.84 | -6.33, -1.35 | -4.85 | -7.80, -1.90 | -0.02 | -0.06, 0.02 | -22.60 | -33.75, -11.45 | -2.28 | -7.64, 3.08 |
| **Female** | 1.37 | -4.88, 7.63 | -5.86 | -13.29, 1.57 | 0.06 | -0.03, 0.16 | -16.95 | -45.00, 11.11 | 21.15 | 7.66, 34.63 |
| **Height (cm)** | -0.23 | -0.58, 0.11 | -0.11 | -0.51, 0.30 | 0.001 | 0.00, 0.01 | -0.98 | -2.51, 0.56 | 0.13 | -0.61, 0.86 |
| **BMI (kg/m$^2$)** | -0.53 | -1.04, -0.02 | -1.24 | -1.84, -0.64 | 0.01 | 0.00, 0.02 | -2.09 | -4.37, 0.19 | 0.36 | -0.74, 1.46 |
| **Education level** | | | | | | | | | | |
| intermediate (vs low) | 4.04 | -2.23, 10.31 | 3.55 | -3.90, 11.00 | 0.08 | -0.02, 0.17 | 24.17 | -3.97, 52.31 | 2.19 | -11.33, 15.72 |
| high (vs low) | 2.07 | -4.51, 8.64 | 4.95 | -2.86, 12.76 | 0.01 | -0.09, 0.12 | 11.85 | -17.64, 41.34 | -2.27 | -16.44, 11.91 |
| **Smoking history** | | | | | | | | | | |
| Current (vs never) | -18.84 | -29.19, -8.49 | -22.76 | -35.06, -10.46 | -0.05 | -0.21, 0.11 | -83.24 | -129.69, -36.8 | -3.34 | -25.66, 18.98 |
| Former (vs never) | -6.66 | -11.18, -2.15 | -6.84 | -12.20, -1.48 | -0.05 | -0.12, 0.03 | 1.04 | -19.21, 21.3 | -12.63 | -22.37, -2.90 |
| **Farm childhood** | -4.66 | -9.21, -0.11 | -2.75 | -8.16, 2.66 | -0.04 | -0.11, 0.03 | -5.05 | -25.46, 15.37 | -10.69 | -20.5, -0.88 |
| **Atopy** | 2.63 | -2.06, 7.31 | 1.15 | -4.41, 6.72 | 0.02 | -0.05, 0.09 | 3.09 | -17.93, 24.11 | 6.76 | -3.34, 16.87 |

All variables were mutually adjusted.




**Supplementary figure 1:** Logistic regression model associations between livestock-related air pollutants and **a**: new onset airway obstruction, defined as $T_0$FEV1/FVC > 0.7 and T1 FEV1/FVC < 0.7; **b:** self-reported doctor diagnosed asthma; **c:** chronic daily cough > 3 months last year; **d:** chronic daily phlegm >3months last year; **e:** wheezing last year

*Note:* all variables have been mutually adjusted. Models were additionally adjusted for age, sex, height, BMI, education level and smoking history. Predicted livestock-related air pollutant concentrations at residential addresses were winsorized.



Chapter 5

# Impact of COVID-19 containment measures on perceived health and health-protective behavior: a longitudinal study

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## Abstract

This longitudinal study aimed to assess the impact of COVID-19 containment measures on perceived health, health protective behavior and risk perception, and investigate whether chronic disease status and urbanicity of the residential area modify these effects.

Participants (n=5,420) were followed for up to 14 months (September 2020-October 2021) by monthly questionnaires. Chronic disease status was obtained at baseline. Urbanicity of residential areas was assessed based on postal codes or neighborhoods. Exposure to containment measures was assessed using the Containment and Health Index (CHI). Bayesian multilevel-models were used to assess effect modification of chronic disease status and urbanicity by CHI.

CHI was associated with higher odds for worse physical health in people with chronic disease (OR=1.09, 95% credibility interval (CrI)=1.01,1.17), but not in those without (OR=1.01, CrI=0.95,1.06). Similarly, the association of CHI with higher odds for worse mental health in urban dwellers (OR=1.31, CrI=1.23,1.40) was less pronounced in rural residents (OR=1.20, CrI=1.13,1.28). Associations with behavior and risk perception also differed between groups.

Individuals with chronic disease and those living in urban areas are differentially affected by government measures put in place to manage the COVID-19 pandemic. This highlights the importance of considering vulnerable subgroups in decision making regarding containment measures.


## Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), sparked an ongoing pandemic after it was first detected in Wuhan, China.[1] Apart from the direct health effects from the infection itself,[2] a range of indirect effects has emerged from the COVID-19 pandemic driven by fear of infection, stigma, anxiety and depression.[3-6] Likewise, the stringency of government measures to manage the outbreak has been shown to adversely affect health and wellbeing.[7] To date, most studies on indirect effects of the COVID-19 pandemic are cross-sectional in nature, while the stringency of containment measures has changed significantly over time.[8] Insights into the physical and mental health effects related to the stringency of containment measures issued by the government over time

are, therefore, crucial in developing a better understanding of the indirect effects of COVID-19 associated with the containment measures in vulnerable groups.

People with identified risk factors for becoming seriously ill from COVID-19, such as diabetes, cardiovascular and respiratory disease,[9] were found to be more susceptible to these indirect effects in a study investigating the early phase of the pandemic.[7] To illustrate, a study in the United States among 1,382 people with diabetes reported a substantive increase in both general and diabetes-related stress as well as social isolation, which significantly affected disease management.[10] Likewise, a survey among diabetes nurses in 27 European countries reported significant increases in physical and mental health issues in the population suffering from diabetes.[11] It has been shown that changes in the healthcare system of the Netherlands were associated with a decline in health status and an increase in psychological stress among patients with chronic cardiopulmonary disorders.[12] This suggests that indirect effects of the pandemic, mediated by containment measures, could be modified by pre-existing chronic disease. Alongside the vulnerability due to chronic conditions, an individual's living environment could play a role in the indirect health effects of the pandemic. A study in the United States showed that cancer patients in urban areas, compared to rural areas, were more likely to practice COVID-19 protective behaviors.[13] This suggests that urbanicity of the residential area could be of importance in assessing the impact of the COVID-19 pandemic.



From September 2020 until November 2021, we performed a monthly online survey among in total 5,420 participants of three Dutch cohort studies. The present study aimed to assess whether people with and without chronic disease (defined as: diabetes, cardiovascular disease, obesity, asthma or COPD) were differentially affected, in terms of perceived health and health-protective behavior, by the stringency of containment measures over time. In addition, we explored whether the impact of government stringency differed with urbanicity of the residential area.

## Materials and Methods

### Study population and design

Participants for the "IMPACT" study were recruited from three existing Dutch prospective cohort studies, the Occupational and Environmental Health Cohort Study (AMIGO),[14] the Livestock Farming and Neighboring Residents' Health study (VGO),[15] and the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study.[16] The design and sample selection for these studies have previously been

described in detail. Briefly, AMGIO was designed to be representative of the general working population of the Netherlands. The cohort consists of 14,298 adults recruited between April 2011 and July 2012 from patient registries of 99 general practices (GP) spread across the country. Similarly, the 8,772 adults participating in the VGO study were enrolled from the registries of 21 GPs in a livestock dense area in the south-east of the Netherlands in 2012. The PIAMA birth cohort (n=3,963) was established by enrolling pregnant women registered at one of 50 participating Dutch prenatal healthcare clinics between March 1996 and May 1997. A subset of 1,912 participants of the PIAMA study could be contacted by email for the IMPACT study, resulting in a total of 24,982 eligible for the present study.

## Ethical approval

The Medical Research Ethics Committee (MERC) of the University Medical Centre Utrecht (UMCU) reviewed the study protocol (nr. 20/242) and ruled that official MERC approval was not required, because no invasive procedures were performed. All participants provided written informed consent before enrolment within the declaration of Helsinki framework.

## Data collection

Participants were invited by post (VGO) or email (AMIGO and PIAMA). Each cohort had slightly different start dates and follow-up periods, from September 2020 to August 2021 for AMIGO, from December 2020 to August 2021 for PIAMA, and from December 2020 to October 2021 for VGO. Participation started with a baseline questionnaire assessing general characteristics and chronic disease status. Chronic disease was defined as having at least one of the following conditions: 1) asthma or Chronic Obstructive Pulmonary Disease (COPD), 2) cardiovascular disease, 3) diabetes mellitus, or 4) obesity (BMI>30 kg/$m^2$). Monthly follow-up questionnaires, sent at the beginning of each month, were used to collect information on perceived physical and mental health, COVID-19 related health-protective behavior and risk perception during the 4 weeks prior to completing the questionnaire date. All questionnaires were provided and completed through a (mobile) web-based application (COVapp). The questionnaires can be found in the supplementary material. To investigate differences between participants living in urban or rural areas, urbanicity of the residential area was obtained from the Dutch Central Bureau of Statistics (CBS) using the 4 digits of the postal code (AMIGO, VGO) or neighborhood (PIAMA) which were collected in 2014 (VGO), 2015 (AMIGO) and 2017-2018 (PIAMA).[17] The five CBS categories of urbanicity were dichotomized, defining urban as >1000 addresses per $km^2$.

## Outcomes: perceived health, COVID-19 related health-protective behavior, and COVID-19 risk perception

We used 5-point physical and mental health scores ranging from excellent to poor from the monthly questionnaires as outcomes. As the category 'poor' was rarely chosen (Supplementary figures S1 and S2), we merged the two lowest categories ('poor' and 'fair'), resulting in a 4-point ordinal scale for the analyses (excellent, very good, good, fair/poor). We investigated COVID-19 related protective behavior using the self-reported 1) average daily number of close contacts within the recommended social distancing of 1.5 meters (excluding household members, categorized as 0-1, 2-5, 6-10, 11-20, >20), 2) how often these contacts lasted longer than 10 minutes (<half, half or >half of close contacts) and 3) how often personal protective equipment (PPE) was used during these contacts (not at all, <half-, half-, >half of close contacts). We investigated COVID-19 related risk perception using perceived probability of (re)-acquiring COVID-19 (highly unlikely, unlikely, neutral, likely, highly likely) and perceived probability of becoming seriously ill from COVID-19 (highly unlikely, unlikely, neutral, likely, highly likely). Lastly, because healthcare availability could explain relationships between chronic disease status and perceived health, we explored two healthcare specific outcomes: healthcare avoidance in fear of acquiring COVID-19 in healthcare environments (does not describe me/my situation at all, does not describe me, neutral, describes me, describes me perfectly) and worrying about missed/postponed healthcare appointments (does not describe me/my situation at all, does not describe me, neutral, describes me, describes me perfectly).



## Exposure: stringency of COVID-19 containment measures

The stringency of the government measures to contain the COVID-19 outbreak was assessed using the Containment and Health Index (CHI) provided by the Oxford COVID-19 Government Response Tracker (OxCGRT).[18] The CHI is an additive index ranging from 0 to 100, describing the severity of measures put in place by a government to manage the outbreak at any given date during the pandemic. Provided as a time series, day-to-day CHI values are based on 20 indicators divided in 3 categories: 1) Containment and closure (school closing, travel restrictions), 2) Economic response (income support, dept relief) and 3) Health systems (testing and vaccination policies). Monthly questionnaires assessed outcomes over a 4-week period prior to each questionnaire. Therefore, monthly averages of daily CHI values were used to quantify exposure to government measures in the month prior to each monthly questionnaire (e.g. March average CHI was assigned to April questionnaires).

## Statistical analysis

Data cleaning was performed by first removing participants missing baseline age, sex, BMI, urbanicity and chronic disease status. (n=56; 1%), and then removing individual time points missing all outcome values (n=14,275; 25%). To address missing data in the remaining dataset for the independent variables (at most 10.4% for a single variable), models were fitted on data imputed using the MICE package (version 3.14.15). Baseline age, sex, BMI, urbanicity and chronic disease status were imputed at the participant level (method=2lonly.pmm). After 100 iterations of the imputation algorithm with default settings, five imputed datasets were generated. Outcome variables were included in the imputation procedure on record level but imputed outcome values were not used in subsequent analyses.

Statistical analysis was performed using R (version 4.2.1) and RStudio.[19] We used a Bayesian multi-level model to accommodate the ordinal outcomes and time-series structure of the data as implemented in the BRMS package (version 2.18.0).[20] Besides the default prior, number of iterations and warm-up (burn in), we used the "logit" link function and a first order autoregressive term to account for the correlation of observations within individuals over time. Chronic disease status, CHI (scaled to interquartile range, IQR) and urbanicity were included as explanatory variables. As the distribution pattern of the CHI over time showed a distinct seasonal pattern (Figure 1), we included season as a potential confounder. This was done using sine and cosine functions of the observation date to estimate the amplitude and phase of the seasonal cycle. To increase precision of the estimates, models were additionally adjusted for age, sex, BMI and recruitment cohort (AMIGO, VGO, PIAMA).

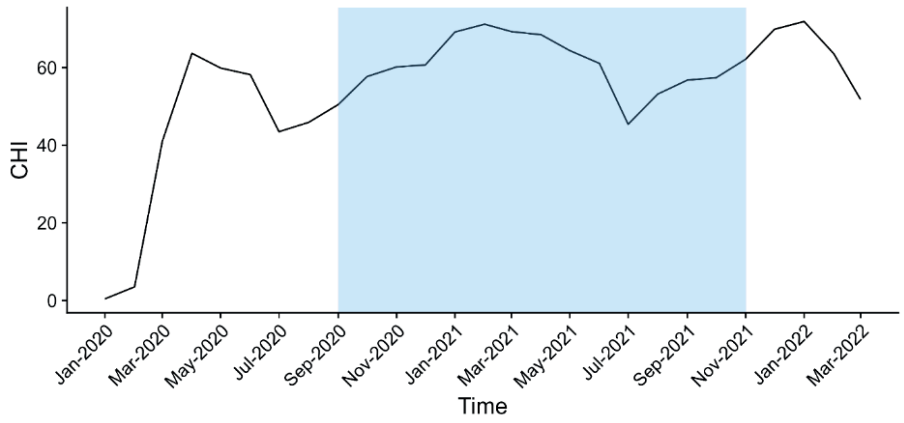


**Figure 1:** Monthly mean Containment and Health Index (CHI), depicting stringency of COVID-19 containment measures in the Netherlands over time. The study period is indicated by the shaded area

Models with chronic disease - CHI and urbanicity – CHI interaction terms were used to assess whether the relationships between CHI and perceived health, COVID-19 related behavior and risk perception differ between people with and without a chronic disease and for urban versus rural populations. As notable differences in age and recruitment procedures exist between PIAMA and the other cohorts, a sensitivity analysis was performed excluding PIAMA participants. Likewise, a complete case analysis was performed in parallel to assess the impact of the imputation procedure. Results were expressed as odds ratios (OR) with 95% credibility intervals (CrI).

## Results

### Study population

A total of 5,420 respondents were included, consisting of 3,383 (62.4%) AMIGO, 1,184 (21.8%) VGO and 853 (15.7%) PIAMA participants. The overall response rate was 22%, PIAMA had the highest response rate (44.6%) followed by AMIGO (23.7%), and VGO (13.5%). An overview of the general characteristics of the study population can be found in Table 1. Participants with (compared to without) chronic disease were older (58.7y vs 53.3y) had higher BMI (28.6 kg/m$^2$ vs 24.2 kg/m$^2$), a higher proportion of females (55.8% vs 52.8%) and a similar proportion of urban residents (53.2% vs. 52.8%).Within the chronic disease group, obesity was the most prevalent chronic condition (42.5%), followed by asthma or COPD (35.6%), cardiovascular disease (35.1%) and diabetes (14.2%). Overall, 17% of the participants reported a (suspected) SARS-CoV-2 (re-)infection before or during the study. As shown in Figure 1, CHI ranged from 71.2 (most stringent, in February 2021) to 45.4 (least stringent, in July 2021).

Differences in baseline characteristics, and distribution of missing data, between cohorts can be found in supplementary table S1. AMIGO and VGO were relatively similar in terms of mean age (61.0 vs 59.6 years), percentage female sex (52.9% vs 50.5%) and BMI (26.1 vs 25.7 kg/m2). PIAMA participants were younger (mean age 24.5 years), more often female (64.5%) and had a slightly lower average BMI (23.6 kg/m$^2$). PIAMA was the most urbanized cohort (73.0%), followed by AMIGO (58.0%) and VGO (23.4%). As PIAMA participants were considerably younger, with exception of asthma, chronic disease was more prevalent in the other cohorts. The distributions of the outcomes over time can be found in supplementary figures S1-S9. A comparison between IMPACT study responders and non-responders using previously collected data, can be found in supplementary table S2.

**Table 1:** COVID-19 IMPACT study population characteristics per chronic disease status

| | No chronic disease (N=3,383) | Chronic disease (N=1,516) | Overall[1] (N=5,420) | P-value |
|---|---|---|---|---|
| Age [years] | 53.2 [24.0, 78.0] | 58.7 [24.0, 78.1] | 55.0 [23.1, 78.1] | <0.001 |
| Sex, female | 1,889 (55.8%) | 766 (50.5%) | 2,936 (54.2%) | <0.001 |
| BMI [kg/m$^2$]* | 24.2 [15.6, 30.0] | 28.6 [16.1, 56.7] | 25.6 [15.6, 56.7] | <0.001 |
| Urbanicity of residential area | | | | 0.558 |
| <1000 addresses/km$^2$ | 1,501 (44.4%) | 693 (45.7%) | 2,446 (45.1%) | |
| >1000 addresses/km$^2$ | 1,801 (53.2%) | 800 (52.8%) | 2,861 (52.8%) | |
| Asthma or COPD | - | 540 (35.6%) | 540 (10.0%) | - |
| Diabetes | - | 215 (14.2%) | 215 (4.0%) | - |
| Cardiovascular disease | - | 532 (35.1%) | 532 (9.8%) | - |
| Obese (BMI>30 kg/m$^2$) | - | 645 (42.5%) | 645 (11.9%) | - |
| COVID-19 before or during study | 592 (17.5%) | 270 (17.8%) | 921 (17.0%) | 0.823 |
| Cohort | | | | <0.001 |
| AMIGO | 2,016 (59.6%) | 1,082 (71.4%) | 3,383 (62.4%) | |
| PIAMA | 632 (18.7%) | 140 (9.2%) | 853 (15.7%) | |
| VGO | 735 (21.7%) | 294 (19.4%) | 1,184 (21.8%) | |

Data are presented as mean [range] or n (%). P-values: Wilcoxon or chi$^2$ test. *BMI=mass(kg)/(height (m))$^2$. [1]Missing chronic disease status (N=521) not shown. Disease status was imputed if baseline age, sex, urbanicity and cohort were available (N=512).

## Associations of CHI with perceived health

We investigated whether CHI was associated with perceived mental health scores, using main effects models (Figure 2) and models with interaction terms (CHI x chronic disease and CHI x urbanicity; Table 2). In the main effects model, an IQR increase in CHI (IQR CHI = 11.5) was associated with increased odds of a worse mental health score (OR=1.27, CrI=1.20,1.34). Likewise, participants with at least one chronic disease reported a worse mental health score (OR=1.59, CrI=1.31,1.92). Urbanicity, however, was not found to be significantly associated with mental health (OR=0.93, CrI=0.79,1.11). The interaction models showed no modification of the effect of CHI by chronic disease. However, the association of CHI with mental health was found to be more pronounced in urban (OR=1.31 CrI=1.23,1.40) compared to rural areas (OR=1.20, CrI=1.13,1.28).

Models with perceived physical health score as an outcome showed no statistically significant main effect of CHI, while chronic disease was associated with worse physical health (OR=2.46, CrI=2.03,3.01). Models with interaction terms showed that the association with CHI was limited to participants with chronic disease (OR=1.09, CrI=1.01,1.17) compared those without chronic disease (OR=1.01, CrI=0.95,1.06). No association between urbanicity and physical health was observed (OR=0.89, CrI=0.75,1.05) and no interaction between CHI and urbanicity was found.

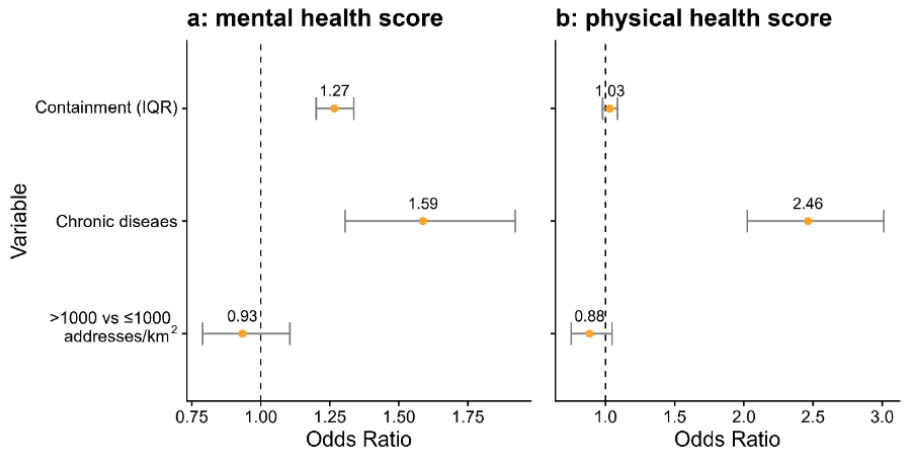


**Figure 2 a:** Bayesian multilevel main effect models for **a:** perceived mental health score main effect model, **b:** perceived physical health score main effect model. Models were adjusted for age, sex, BMI, recruitment cohort and season

Main effect model results for COVID-related behavior outcomes can be found in supplementary figure S10. As expected, more stringent containment measures were associated with a lower number of close (within 1.5m) personal contacts (OR=0.53, CrI=0.49,0.56). Participants with chronic disease reported fewer contacts (on the ordinal scale) with persons within 1.5m compared to those without chronic disease (OR=0.70, CrI=0.56,0.89). No main effect of urbanicity (OR=0.92, CrI=0.75,1.13) on the number of close contacts was observed. Interaction models showed that the association of CHI tended to be stronger in participants with chronic disease (OR=0.48, Cri=0.44,0.53) compared to those without (OR=0.55, Cri=0.51,0.59). Likewise, the association of CHI was found to be stronger in urban (OR=0.50, CrI=0.47,0.54) compared to rural areas (OR=0.57, CrI=0.52,0.61).

**Table 2:** Interaction model results for Containment and Health Index with chronic disease andurbanization, including group specific effects

| | Chronic disease | | Urbanization | |
|---|---|---|---|---|
| **Outcomes (ordinal variables)** | **No** | **Yes** | **No** | **Yes** |
| Mental health score | 1.26 (1.19,1.33) | 1.28 (1.19,1.39 | **1.20(1.13,1.28)** | **1.31(1.23,1.40)** |
| Physical health score | **1.01(0.95,1.06)** | **1.09 (1.01,1.17)** | 1.03 (0.97,1.10) | 1.03 (0.97,1.09) |
| N close contacts* | **0.55 (0.51,0.59)** | **0.48(0.44,0.53)** | **0.57(0.52,0.61)** | **0.50(0.47,0.54)** |
| Close contacts >10min† | 0.98 (0.92,1.06) | 1.01 (0.93,1.11) | 1.00 (0.92,1.08) | 0.99 (0.92,1.07) |
| PPE usage during close contacts‡ | 1.71 (1.60,1.84) | 1.82 (1.66,2.00) | **1.64 (1.51,1.77)** | **1.83 (1.70,1.98)** |
| Perceived risk of acquiring COVID-19 | 1.51 (1.41,1.62) | 1.48 (1.35,1.62) | 1.54 (1.45,1.71) | 1.4 (1.35,1.55) |
| Perceived risk of severe COVID-19 | **1.21 (1.13,1.30)** | **1.50 (1.38,1.64)** | 1.30 (1.24,1.40) | 1.30 (1.21,1.39) |

Results are presented as odds ratios with 95% credible intervals. Abbreviations: CD = chronic disease. * Number of close contacts within 1.5m as by Dutch covid legislation. † Fraction of close contacts with a duration longer than 10 minutes. ‡ Fraction of close contacts in which personal protective equipment (PPE) was used. Bold: statistically significant interaction effects. Interaction effects were analyzed in separate models, adjusted for age, sex, BMI, chronic disease status, season and cohort.

## Associations of CHI with health-protective behavior

No association between CHI and close contact duration was observed (OR=0.99, CrI=0.93,1.06). Similarly, chronic disease and urbanicity were not associated with contact duration. In addition, no interaction was found between CHI and chronic disease or urbanicity in relation to close contact duration. On the other hand, odds for PPE usage during close contacts were shown to increase with increasing CHI (OR=1.75, CrI=1.63,1.87). Chronic disease status and urbanicity were not associated with PPE usage during close contacts. However, a borderline significant interaction term suggested that the association of CHI with PPE usage was more pronounced in participants with chronic disease (Table 2). Likewise, the effect of CHI on PPE usage was found to be stronger in urban areas.

## Associations of CHI with COVID-19 risk perception

Perceived probability of acquiring COVID-19 was found to be positively associated with CHI and chronic disease, but no association with urbanicity was identified (Supplementary figure S11A,B). A borderline significant interaction term suggested that the association with CHI was more pronounced in rural (OR=1.54, CrI=1.45,1.71) compared to urban areas (OR=1.44 CrI=1.35,1.55). No interaction between chronic disease and CHI was found. Models with outcome 'perceived probability of becoming seriously ill from COVID-19' showed positive associations with a higher CHI (OR=1.30,

CrI=1.22,1.38). Likewise, chronic disease status (OR=5.81, CrI=4.86,6.98) was strongly associated with perceived risk of severe COVID-19. A significant interaction term (OR=1.24, CrI=1.13,1.36) showed that the association with CHI was stronger in people with chronic disease. No association between urbanicity of the residential area and perceived probability of severe illness was found.

### Associations of CHI with healthcare avoidance

Models investigating healthcare avoidance (due to fear of acquiring COVID-19 in healthcare environments, Supplementary figure S11C,D) suggested that a higher CHI was associated with lower odds for healthcare avoidance (OR=0.94, CrI=0.88,1.01) but this association was not statistically significant. Chronic disease was associated with higher odds for healthcare avoidance (OR=1.26 CrI=1.12,1.53). The model with outcome 'worrying about missed or postponed healthcare appointments' showed that chronic disease (OR=1.60, CrI=1.40,1.82) but not CHI was associated with higher odds for worrying.

### Sensitivity analyses

As the PIAMA cohort differs substantially from both AMIGO and VGO in terms of age and recruitment procedure, we performed a sensitivity analysis to investigate whether this influenced our results. To this end, the interaction models (between CHI and chronic disease as well as CHI and urbanicity) were re-analyzed using data from AMIGO and VGO participants only, thus excluding PIAMA participants. Results of these models (supplementary table S3) show that cohort differences did not significantly affect our results. A comparison of the results with a complete case analysis can be found in supplementary tables S4 and S5. Besides the narrower credibility intervals, indicating that the multiple imputation analysis was more efficient, no apparent differences were found.

## Discussion

In this study we investigated whether the intensity of national COVID-19 containment measures, expressed by CHI, differentially affected people with or without a chronic disease and residents of urban versus rural areas. We found that associations of CHI with perceived health, health protective behavior, and risk perception were more pronounced in participants with a chronic disease and residents of urban areas. Mental health decreased with increasing CHI. While this association was not dependent on chronic disease status, it was shown to be more pronounced in residents of urban areas. This decrease in mental health, could be (partly) explained by our finding that CHI was associated with increased risk perception for COVID-19 infection and severity. The relationship between CHI and perceived probability of a severe COVID-19 infection was found to be stronger among those suffering from a chronic disease. Additionally, participants with a chronic disease reported worsening physical health with increasing CHI. This was not seen in participants without a chronic disease, indicating that chronic disease confers a predisposition to worsening physical health during the peaks of the COVID-19 pandemic.

These findings are in line with cross-sectional reports of worse health in chronically diseased individuals during the pandemic.[11,21,22] Our longitudinal analyses showed that this association is related to fluctuations in the stringency of containment measures over time. It has been suggested that the decline in health is related to unavailable or inaccessible healthcare.[12,21,23] In this study, we show that healthcare avoidance (due to fear of acquiring COVID-19 at a healthcare facility) decreased with increasing CHI. This suggests that containment measures provide a sense of security in relation to the use of healthcare services aiding the continuation of regular healthcare. However, having a chronic disease was associated with healthcare avoidance. Thus, ensuring and propagating patient safety in healthcare environments is crucial during pandemics.

Individuals with a chronic disease reported fewer close contacts (within 1.5m) during which they used PPE more often. This increase in health protective behavior may be explained by increased COVID-19 risk perception which is supported by our finding that chronic disease was strongly associated with perceiving an increased probability of infection with SARS-CoV-2 and severe COVID-19. Other factors related to chronic disease (e.g. decreased mobility) could also play a role. Similarly, living in an urban area (compared to rural) was associated with fewer contacts during which PPEs were used more often. Urbanicity, however, was not associated with perceiving an increased probability of infection or severe disease. This suggests that the inclination

of urban residents towards health protective behavior is driven by other factors like social pressure, which is in line with the Dutch public debate during the pandemic, stressing a (presumed) elevated infection risk in cities [24]. These findings could also be a reflection of the more profound change in day-to-day life in cities (empty streets, closed shops) during lockdown. We showed that the associations of CHI (which incorporates group size restrictions and PPE policies) on the number of contacts and PPE usage were more pronounced in individuals with chronic disease and urban residents respectively, indicating an increased inclination to adhere to containment measures in both groups.

Evidence on the role of urbanicity in mental health is inconclusive. There are reports of beneficial effects of living in a rural area,[25] while other studies find no associations between mental health and living in urban areas.[26] A recent study assessing the role of housing environment on mental health during the pandemic reported no associations with urbanicity and mental health indicators.[27] However, they did report that lacking access to an outdoor space (e.g. garden or balcony) was associated with worse mental health outcomes during lockdown. Likewise, an Italian study reported an association between living in apartments smaller than 60m$^2$ and increased risk of depressive symptoms in students.[28] This may potentially explain that we found a stronger association with CHI in urban areas, where typically homes are smaller and without private outdoor spaces. Also of importance are differences in available services and amenities between urban and rural areas, resulting in more pronounced changes in day-to-day life in urban areas during lockdown. We showed that VGO participants, mainly living in rural municipalities, reported better mental health compared to AMIGO participants, who are more evenly distributed along the urban-rural gradient. As our models are corrected for urbanicity, this indicates that other regional factors could play a role. The fact that VGO participants reported a higher perceived probability of acquiring COVID-19 is potentially explained by the fact that most VGO participants live in the province of Noord-Brabant, which was the epicenter of the initial start of the epidemic in the Netherlands.[29] Our finding that older individuals reported better mental health than younger participants, is in line with reports of pandemic-related mental health issues in young people,[30] which can be explained by differences in coping strategies and support structures between children, adolescents and adults.

Limitations of this study include the response rate of 22%, conferring potential influence of non-response bias. However, comparing study sample characteristics with the source population revealed relatively minor differences in age, sex and smoking habits. Another limitation are the substantial differences between cohorts.

These differences, however, mainly involve differences in recruitment and younger age of PIAMA participants (a birth cohort) compared to AMIGO and VGO which were recruited amongst adults. A sensitivity analysis without PIAMA showed that these differences did not significantly influence our results. Another limitation is the correlation between CHI and time. By taking the multi-level structure of our data into account, we were able to analyze individual outcome trajectories. However, as a result of adjustment for correlation over time between observations, our multi-level model underestimates true associations with CHI. Lastly, the absence of a baseline health score measurement before the onset of the pandemic prevents comparison to a situation without any containment measures.

The unique challenges faced (e.g. questionnaire app development, data protection clearance) in setting up this study efficiently during the initial days of the pandemic emphasize the need for `ready to go` research frameworks that are easy to deploy in future public health crises. Main strengths of this study include the longitudinal design combined with the use of CHI as a standardized assessment of exposure to containment measures, enabling estimation of the effect of CHI while controlling for individual confounders that are stable over time. Another strength is our use of multiple imputation to address missing data, resulting in more precise effect estimates than more basic approaches like complete case analysis or mean imputation.

In conclusion, our study shows that the stringency of government measures, put in place to manage the outbreak, differentially affect people with chronic disease and residents of urban areas, emphasizing the importance of considering vulnerable subgroups in decision making about containment measures in public health crises.

## Data sharing statement

The data underlying this article will be shared with researchers who provide a methodologically sound proposal on request to the corresponding author.

## Funding

The IMPACT project was supported by internal funds from the Institute for Risk Assessment Sciences (IRAS) at Utrecht University. The Prevention and Incidence of Asthma and Mite Allergy Study (PIAMA) has been funded by grants from the Netherlands Organization for Health Research and Development; the Netherlands Organization for Scientific Research; the Lung Foundation of the Netherlands; the Netherlands Ministry of Planning, Housing, and the Environment; the Netherlands

Ministry of Health, Welfare, and Sport; and the National Institute for Public Health and the Environment.

The Farming and Neighbouring Residents' Health (VGO) study was funded by the Ministry of Health, Welfare and Sports and the Ministry of Economic Affairs of the Netherlands and supported by a grant from the Lung Foundation Netherlands (3.2.11.022).

The Occupational and Environmental Health Cohort Study (AMIGO) was supported by The Netherlands Organisation for Health Research (ZonMw) within the programme Electromagnetic Fields and Health Research under grant numbers 85200001 and 85200002.

## Acknowledgements

The authors are grateful to Stichting Informatie Voorziening Zorg (SIVZ, Houten; trusted third party) for assistance with recruiting participants from the VGO cohort and ensuring VGO participants' privacy.

### Contributors statement

**Warner van Kersen:** Conceptualization, Data curation, Formal Analysis, Methodology, Project administration, Software, Visualization, Writing – original draft **Myrna M. T. De Rooij:** Conceptualization, Methodology, Project administration, Writing – review & editing **Lützen Portengen** Methodology, Writing – review & editing **Nekane Sandoval Diez**: Data curation, Writing – review & editing **Inka Pieterson**: Data curation **Marjan Tewis**: Data curation **Jolanda Boer**: Resources, Writing – review & editing **Gerard Koppelman**: Resources **Judith M. Vonk**: Resources, Writing – review & editing **Roel Vermeulen:** Conceptualization, Funding acquisition **Ulrike Gehring:** Conceptualization, Resources, Writing – review & editing **Anke Huss:** Conceptualization, Project administration, Resources, Writing – review & editing **Lidwien A.M. Smit:** Conceptualization, Supervision, Resources, Writing – review & editing.

## Conflict of interest

The authors declare no competing interests.

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Chapter 5s

# Supplementary Materials

## Questionnaire Q1. Baseline Questionnaire

I. What is your date of birth?
II. What is your sex?
    0. Male
    1. Female
III. What is your current weight? (kg)
IIV. What is your height? (cm)
V. For each of the conditions below, please indicate whether you have (had) them.
    a. Heart attack (0=no,1=yes)
    b. Narrowed arteries legs (0=no,1=yes)
    c. Stroke or TIA (0=no,1=yes)
    d. Other heart condition (0=no,1=yes)
    e. Lung disease asthma, COPD, chronic bronchitis (0=no,1=yes)
    f. Diabetes Mellitus (0=no,1=yes)

## Questionnaire Q2. Monthly Questionnaires

I. How do you rate your physical health? (in relation to the past 4 weeks)
    1. Poor
    2. Fair
    3. Good
    4. Very good
    5. Excellent
II. How do you rate your mental health? (in relation to the past 4 weeks)
    1. Poor
    2. Fair
    3. Good
    4. Very good
    5. Excellent
III. How many different people came within 1.5m of you? On average per day, excluding household members.
    0-1
    2-5
    6-10
    11-20
    >20

IV. In how many cases did these contacts last longer than 10 minutes?
1. In less than half of the cases
2. In approximately half the cases
3. In more than half of the cases

V. Did you use any personal protective equipment during these contacts? e.g. facemask, gloves or a screen?
1. No
2. In less than half the cases
3. In approximately half the cases
4. in more than half of the cases

VI. How likely do you believe it to be that you will get Coronavirus/COVID-19 (again)?
Very unlikely
Somewhat unlikely
Neutral
Somewhat likely
Very likely

VII. If you were to get Coronavirus/COVID-19, how likely do you believe it to be that you will become seriously ill?
Very unlikely
Somewhat unlikely
Neutral
Somewhat likely
Very likely

IIX. Please indicate how well the following statement describes your behaviour: I avoid healthcare because I am afraid of getting Coronavirus/COVID-19 that way.
Doesn't describe me at all
Does not describe me
Neutral
Describes me
Describes me exactly

IIX. Please indicate how well the following statement describes your behaviour: I worry about the impact of my missed/postponed healthcare appointments.
Doesn't describe me at all
Does not describe me
Neutral
Describes me
Describes me exactly

# Supplementary Tables and Figures

**Supplementary Table S1:** COVID-19 IMPACT study population characteristics per cohort, including missing data

| | AMIGO (N=3,383) | PIAMA (N=853) | VGO (N=1,184) | P-value |
|---|---|---|---|---|
| **Age (y)** | 61.0 [39.0, 76.8] | 24.5 [23.1, 25.3] | 59.6 [26.2, 78.1] | <0.001 |
| Missing | 0 (0%) | 2 (0.2%) | 0 (0%) | |
| **Sex, female** | 1,788 (52.9%) | 550 (64.5%) | 598 (50.5%) | <0.001 |
| Missing | 0 (0%) | 2 (0.2%) | 0 (0%) | |
| **BMI (kg/m2)*** | 26.1 [16.1, 56.7] | 23.6 [15.6, 48.5] | 25.7 [16.8, 42.9] | <0.001 |
| Missing | 320 (9.5%) | 84 (9.8%) | 171 (14.4%) | |
| **Urbanicity** | | | | <0.001 |
| <1000 addresses/km$^2$ | 1,422 (42.0%) | 165 (19.3%) | 859 (72.6%) | |
| >1000 addresses/km$^2$ | 1,961 (58.0%) | 623 (73.0%) | 277 (23.4%) | |
| Missing | 0 (0%) | 65 (7.6%) | 48 (4.1%) | |
| **Chronic disease** | 1,082 (32.0%) | 140 (16.4%) | 294 (24.8%) | <0.001 |
| Asthma or COPD | 347 (10.3%) | 102 (12.0%) | 91 (7.7%) | 0.0152 |
| Diabetes | 174 (5.1%) | 3 (0.4%) | 38 (3.2%) | <0.001 |
| Cardiovascular disease | 408 (12.1%) | 4 (0.5%) | 120 (10.1%) | <0.001 |
| Obese (BMI>30) | 493 (14.6%) | 43 (5.0%) | 109 (9.2%) | <0.001 |
| Missing | 285 (8.4%) | 81 (9.5%) | 155 (13.1%) | |
| **COVID-19 before or during study** | 491 (14.5%) | 212 (24.9%) | 218 (18.4%) | |
| Missing | 0 (0.00%) | 0 (0.00%) | 0 (0.00%) | |

Data are presented as mean [range] or n (%). P-value: Kruskal-Wallis or chi$^2$ test. *BMI=mass(kg)/(height (m))$^2$.

**Supplementary Table S2:** COVID-19 IMPACT characteristics of responders and non-responders

| | IMPACT Sample (N=5,420) | Non-responders (N=19,216) | Source Population (N=24,636) | P value* |
|---|---|---|---|---|
| **Cohort** | | | | |
| PIAMA | 853 (15.7%) | 1,059 (5.5%) | 1,912 (7.8%) | <0.001 |
| AMIGO | 3,383 (62.4%) | 10,915 (56.8%) | 14,298 (58.0%) | |
| VGO | 1,184 (21.8%) | 7,242 (37.7%) | 8,426 (34.2%) | |
| **Age** | | | | |
| Mean (SD) | 55.1 (15.8) | 56.7 (13.3) | 56.4 (13.9) | <0.001 |
| Median (Q1,Q3) | 59.0 (48.5,67.3) | 58.0 (49.0,67.0) | 58.2 (48.9, 67.0) | |
| Min-Max | 24.0 – 79.2 | 24.3 – 79.4 | 24.0 – 79.4 | |

**Supplementary Table S2:** Continued

| | IMPACT Sample (N=5,420) | Non-responders (N=19,216) | Source Population (N=24,636) | P value* |
|---|---|---|---|---|
| Missing | 527 | 0 | 527 | |
| **Sex** | | | | |
| Male | 2,240 (45.8%) | 8,687 (45.2%) | 10,927 (45.3%) | 0.432 |
| Female | 2,647 (54.2%) | 10,528 (54.8%) | 13,175 (54.7%) | |
| Missing | 533 | 1 | 534 | |
| **Smoking Status** | | | | |
| Never | 2,055 (45.2%) | 5,693 (42.8%) | 7,748 (43.4%) | <0.001 |
| Current smoker | 504 (11.1%) | 2,091 (15.7%) | 2,595 (14.5%) | |
| Former smoker | 1,992 (43.8%) | 5,509 (41.4%) | 7,501 (42.0%) | |
| Missing | 869 | 5,923 | 6,792 | |

*Linear model ANOVA or Pearson's chi-squared test.

**Supplementary Table S3:** Interaction effects of Containment and Health Index with chronic disease and urbanization, all three IMPACT cohorts combined and sensitivity analysis excluding PIAMA participants

| | Interaction CHI x CD | | Interaction CHI x Urban | |
|---|---|---|---|---|
| **Outcomes (ordinal variables)** | **IMPACT study (Pooled)** | **Excluding PIAMA** | **IMPACT study (Pooled)** | **Excluding PIAMA** |
| Mental health score | 1.02 (0.94,1.10) | 1.05 (0.98,1.14) | **1.09 (1.02,1.27)** | 1.05 (0.98,1.13) |
| Physical health score | **1.08 (1.00,1.17)** | 1.07 (0.98-1.15) | 1.00 (0.93,1.07) | 1.01 (0.94,1.09) |
| N close contacts* | **0.89 (0.81,0.97)** | **0.87 (0.79,0.96)** | **0.88 (0.81,0.96)** | **0.88 (0.81,0.96)** |
| Close contacts >10min† | 1.03 (0.94,1.13) | 1.04 (0.94,1.15) | 1.00 (0.92,1.09) | 0.98 (0.89,1.08) |
| PPE usage during close contacts‡ | 1.06 (0.98,1.17) | 1.02 (0.94,1.12 | **1.12 (1.03,1.22)** | **1.20 (1.10,1.31)** |
| Probability of acquiring COVID-19 | 0.98 (0.89,1.08) | 0.98 (0.88,1.08) | 0.92 (0.84,1.00) | 0.93 (0.85,1.02) |
| Probability of severe COVID-19 | **1.24 (1.13,1.36)** | **1.22 (1.11,1.35)** | 1.00 (0.92,1.08) | 1.08 (0.99,1.17) |

Results are presented as odds ratios with credible intervals. Abbreviations: CD = chronic disease. * Number of close contacts within 1.5m as by Dutch covid legislation. † Fraction of close contacts with a duration longer than 10 minutes. ‡ Fraction of close contacts in which personal protective equipment (PPE) was used. Bold: statistically significant interaction effects. Models were adjusted for age, sex, BMI, chronic disease status, season and cohort.

**Supplementary Table S4:** Interaction effects of Containment and Health Index with chronic disease and urbanization, complete case analysis and multiple imputation results

| | Interaction CHI x CD | | Interaction CHI x Urban | |
|---|---|---|---|---|
| **Outcomes (ordinal variables)** | **Complete case** | **Multiple imputation** | **Complete case** | **Multiple imputation** |
| Mental health score | 1.03 (0.95,1.12 | 1.02 (0.94,1.10) | **1.09 (1.01,1.18)** | **1.09 (1.02,1.27)** |
| Physical health score | **1.07 (1.00,1.17** | **1.08 (1.00,1.17)** | 1.01 (0.94,1.08) | 1.00 (0.93,1.07) |
| N close contacts* | **0.89 (0.81,0.98)** | **0.89 (0.81,0.97)** | 0.96 (0.88,1.04) | **0.88 (0.81,0.96)** |
| Close contacts >10min† | 1.03 (0.94,1.14) | 1.03 (0.94,1.13) | 1.01 (0.94,1.09) | 1.00 (0.92,1.09) |
| PPE usage during close contacts‡ | **1.12 (1.00,1.23** | 1.06 (0.98,1.17) | 0.93 (0.86,1.00) | **1.12 (1.03,1.22)** |
| Probability of acquiring COVID-19 | 0.98 (0.88,1.09) | 0.98 (0.89,1.08) | 0.90 (0.81,1.00) | 0.92 (0.84,1.00) |
| Probability of severe COVID-19 | **1.33 (1.20,1.48)** | **1.24 (1.13,1.36)** | 0.99 (0.90,1.10) | 1.00 (0.92,1.08) |

Results are presented as odds ratios with credible intervals. Abbreviations: CD = chronic disease. * Number of close contacts within 1.5m as by Dutch covid legislation. † Fraction of close contacts with a duration longer than 10 minutes. ‡ Fraction of close contacts in which personal protective equipment (PPE) was used. Bold: statistically significant interaction effects. Models were adjusted for age, sex, BMI, chronic disease status, season and cohort.

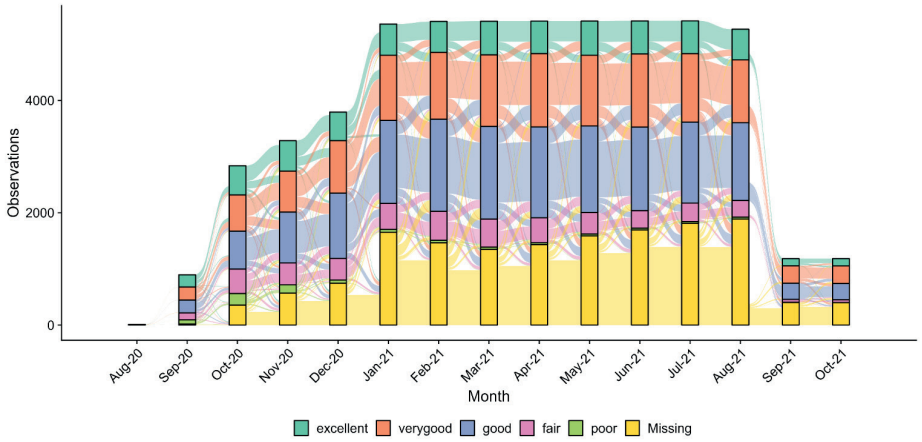
**Supplementary Table S5:** Main effect models results for all IMPACT study outcomes, complete case analysis and multiple imputation results

| | Complete case | Multiple imputation |
|---|---|---|
| **Outcomes (ordinal) and predictors** | **OR + (95%CrI)** | **OR + (95%CrI)** |
| **Mental health score** | | |
| Chronic disease | 1.68 (1.34,2.10) | 1.59 (1.31,1.92) |
| Containment | 1.29 (1.22,1.36) | 1.27 (1.20,1.34) |
| >1000 addresses/km2 | 0.93 (0.77,1.12) | 0.93 (0.79,1.11) |
| **Physical health score** | | |
| Chronic disease | 2.51 (2.01,3.11) | 2.46 (2.03,3.01) |
| Containment | 1.02 (0.97,1.08) | 1.03 (0.98,1.09) |
| >1000 addresses/km2 | 0.89 (0.74,1.06) | 0.88 (0.75,1.05) |
| **N close contacts*** | | |
| Chronic disease | 0.66 (0.51,0.85) | 0.70 (0.56,0.89) |
| Containment | 0.51 (0.48,0.55) | 0.53 (0.49,0.56) |
| >1000 addresses/km2 | 0.89 (0.71,1.12) | 0.92 (0.75,1.13) |

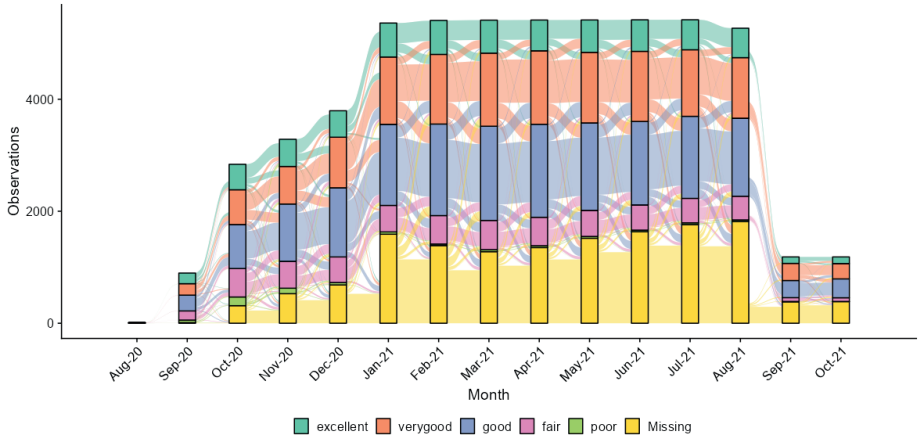
**Supplementary Table S5:** Continued

| Outcomes (ordinal) and predictors | Complete case OR + (95%CrI) | Multiple imputation OR + (95%CrI) |
|---|---|---|
| **Close contacts >10 min†** | | |
| Chronic disease | 0.91 (0.76,1.10) | 0.96 (0.81,1.15) |
| Containment | 1.00 (0.93,1.08) | 0.99 (0.93,1.06) |
| >1000 addresses/km2 | 0.93 (0.79,1.09) | 0.96 (0.83,1.12) |
| **PPE usage during close contacts‡** | | |
| Chronic disease | 1.05 (0.86,1.28) | 1.03 (0.87,1.22) |
| Containment | 2.00 (1.85,2.16) | 1.75 (1.63,1.87) |
| >1000 addresses/km2 | 0.94 (0.80,1.12) | 0.94 (0.82,1.08) |
| **Probability of aquiring COVID-19** | | |
| Chronic disease | 1.30 (1.06,1.60) | 1.35 (1.15,1.58) |
| Containmentw | 1.55 (1.44,1.67) | 1.50 (1.41,1.60) |
| >1000 addresses/km2 | 0.94 (0.78,1.12) | 0.97 (0.85,1.12) |
| **Probability of severe COVID-19** | | |
| Chronic disease | 8.87 (6.89,11.41) | 5.81 (4.86,6.98) |
| Containment | 1.36 (1.27,1.46) | 1.30 (1.22,1.38) |
| >1000 addresses/km2 | 1.04 (0.84,1.29) | 1.04 (0.90,1.21) |
| **Healthcare-avoidance** | | |
| Chronic disease | 1.55 (1.23,1.96) | 1.26 (1.12,1.42) |
| Containment | 0.89 (0.73,1.09) | 0.94 (0.88,1.01) |
| >1000 addresses/km2 | 0.78 (0.67,0.91) | 0.88 (0.80,0.97) |
| **Worries about missed healthcare** | | |
| Chronic disease | 1.80 (1.46,2.22) | 1.60 (1.40,1.82) |
| Containment | 0.84 (0.72,0.98) | 0.94 (0.88,1.01) |
| >1000 addresses/km2 | 0.97 (0.81,1.15) | 0.96 (0.87,1.06) |

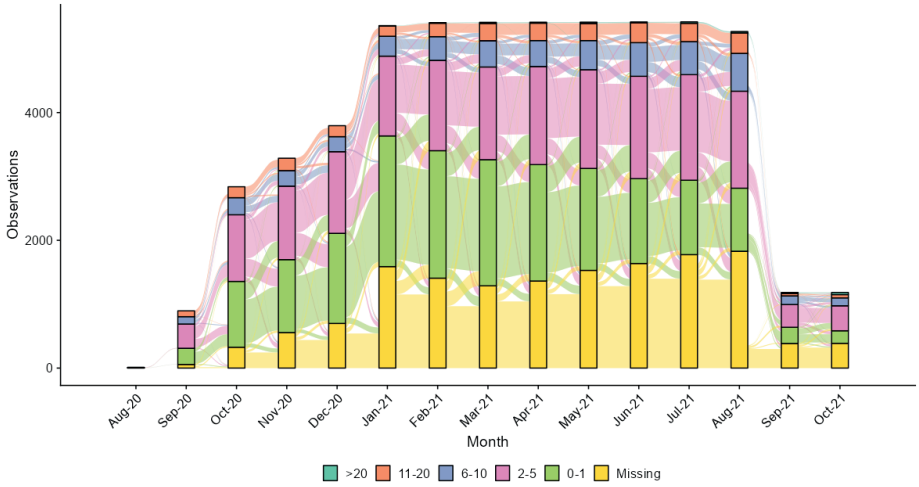
Results are presented as odds ratios with credible intervals. * Number of close contacts within 1.5m as by Dutch covid legislation. † Fraction of close contacts with a duration longer than 10 minutes. ‡ Fraction of close contacts in which personal protective equipment (PPE) was used. Variables were mutually adjusted for. Models were adjusted for age, sex, BMI, chronic disease status, season and cohort.



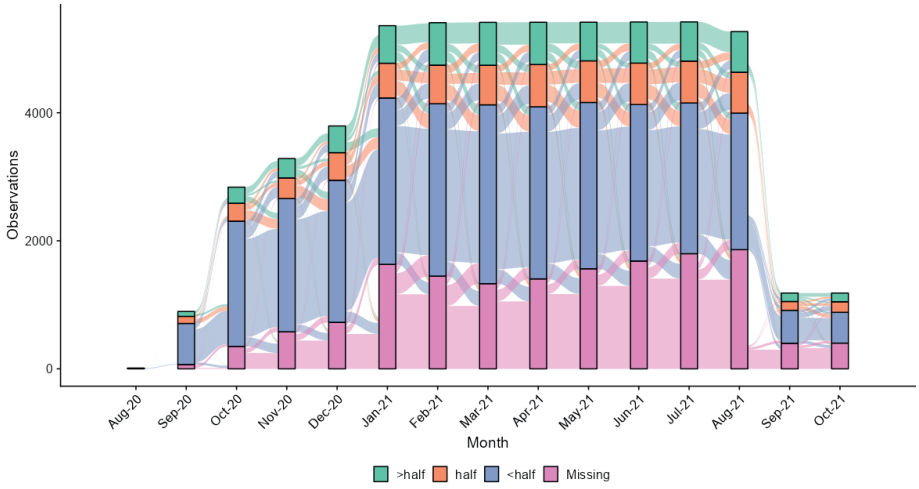

**Supplementary figure S1:** Alluvial plot of mental health scores (5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next



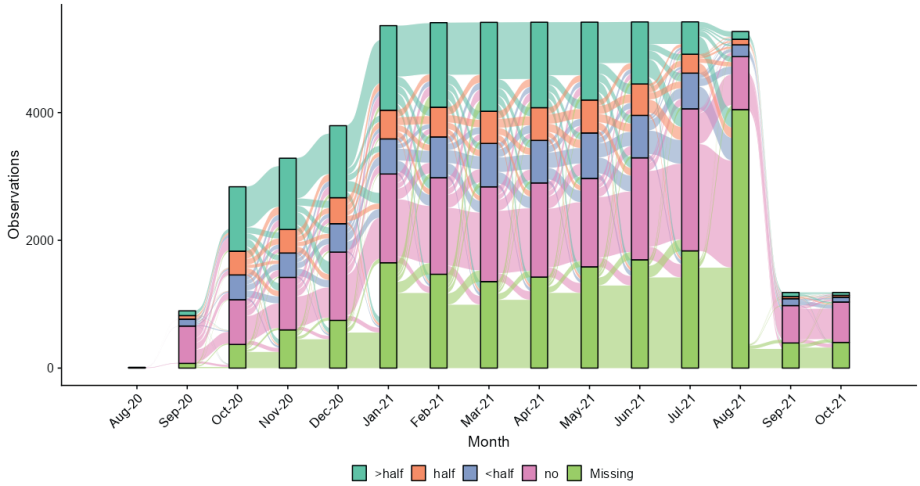

**Supplementary figure S2:** Alluvial plot of physical health scores (5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next



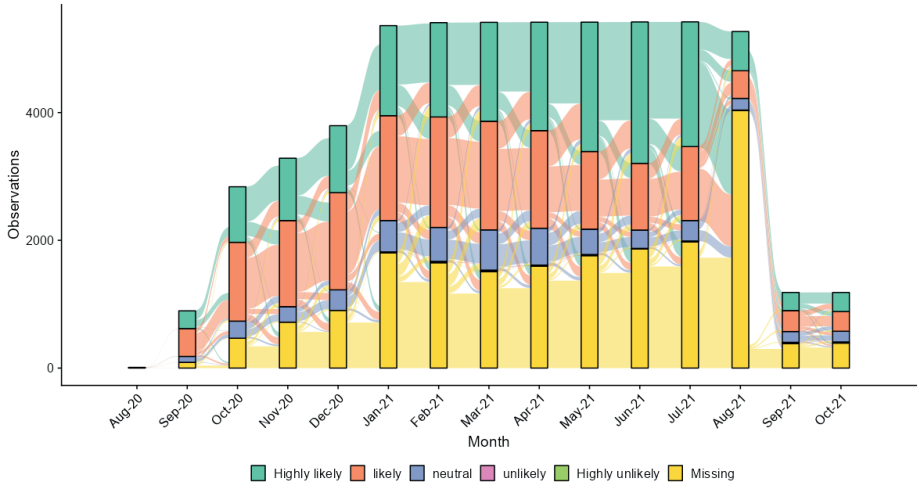

**Supplementary figure S3:** Alluvial plot of `n close interactions <1.5m`(5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next



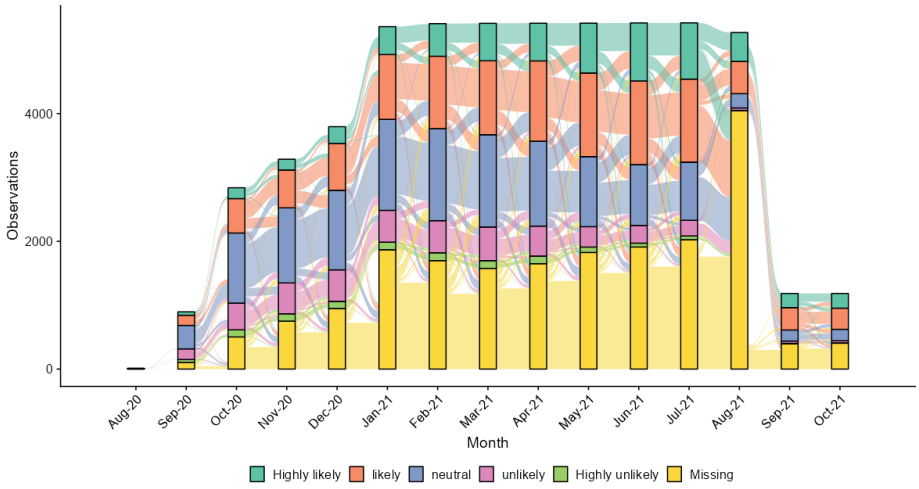

**Supplementary figure S4:** Alluvial plot of'close (<1.5m) interactions lasting >10m`(3-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next



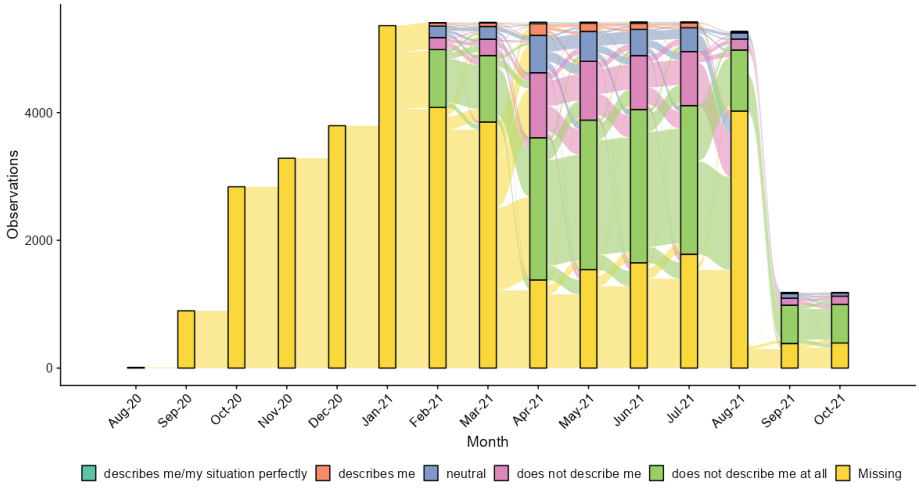

**Supplementary figure S5:** Alluvial plot of 'personal protective equipment usage during close (<1.5m) contacts`(4-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next




**Supplementary figure S6:** Alluvial plot of 'Perceived probability of acquiring COVID-19`(5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next

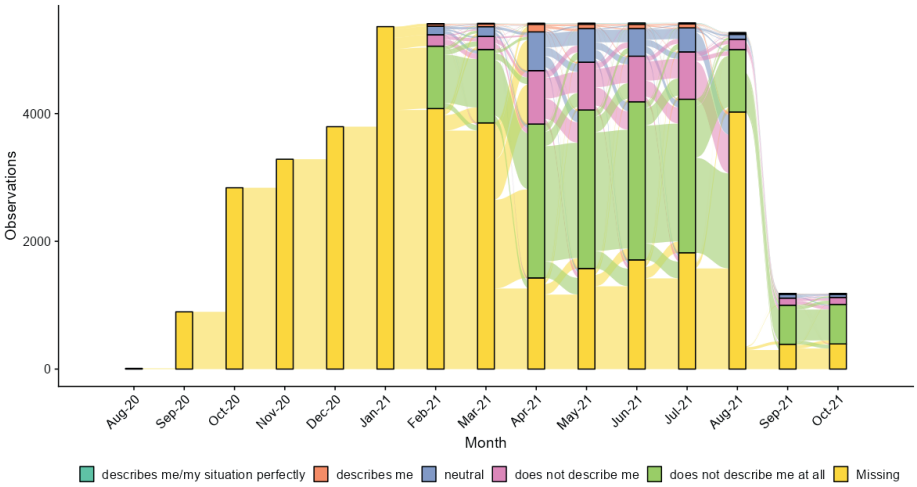



**Supplementary figure S7:** 'perceived probability of becoming seriously ill from COVID-19' (5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next



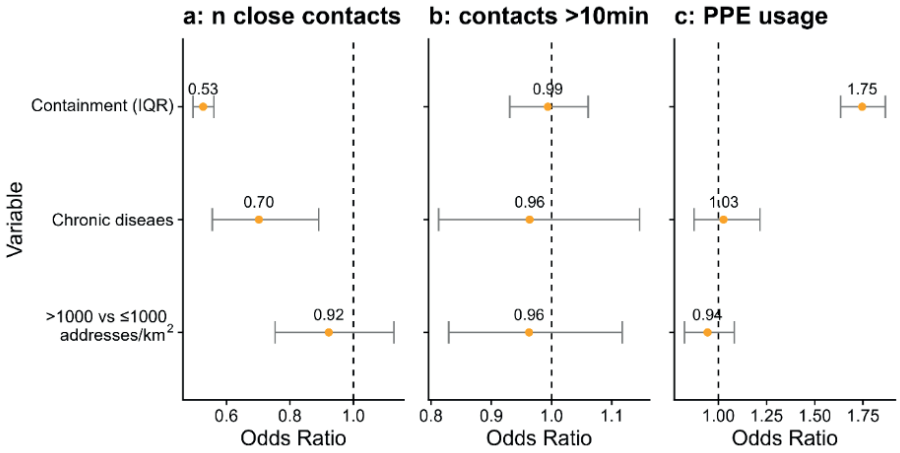

**Supplementary figure S8:** 'I avoid healthcare in fear of acquiring COVID-19 in healthcare environments`(5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next

*Note:* this question was added in February 2021 (VGO) and April (AMIGO, PIAMA)

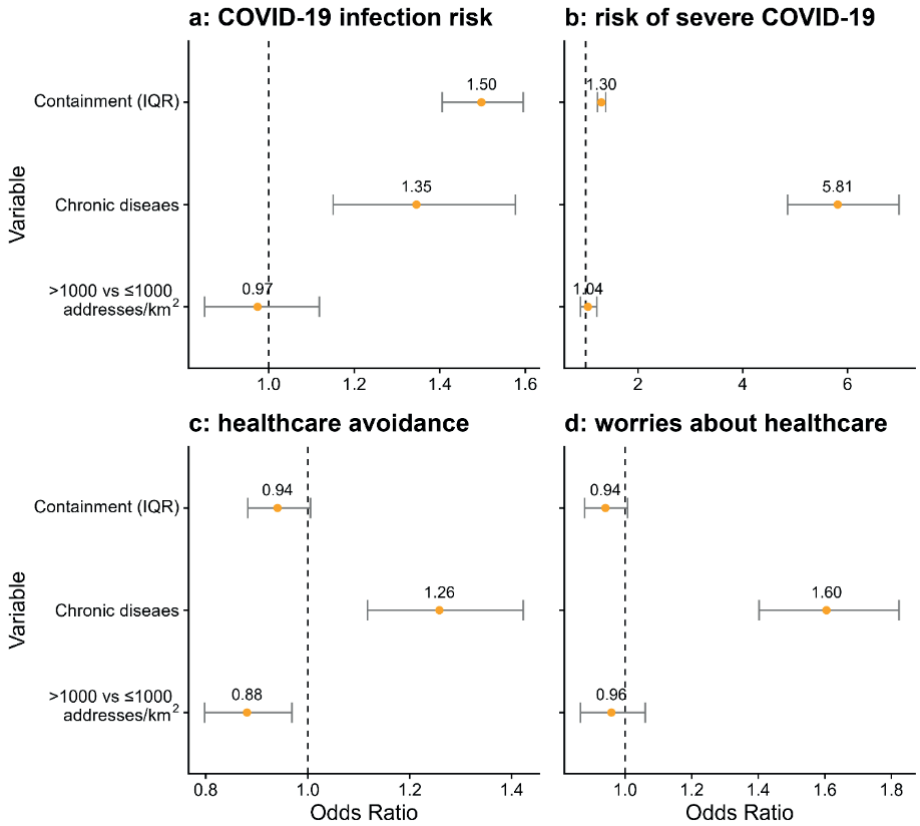



**Supplementary figure S9:** 'I worry about my missed or postponed healthcare appointments`(5-category ordinal) over the COVID-19 IMPACT study period. Bands between bars indicate the change in answer category from one month to the next

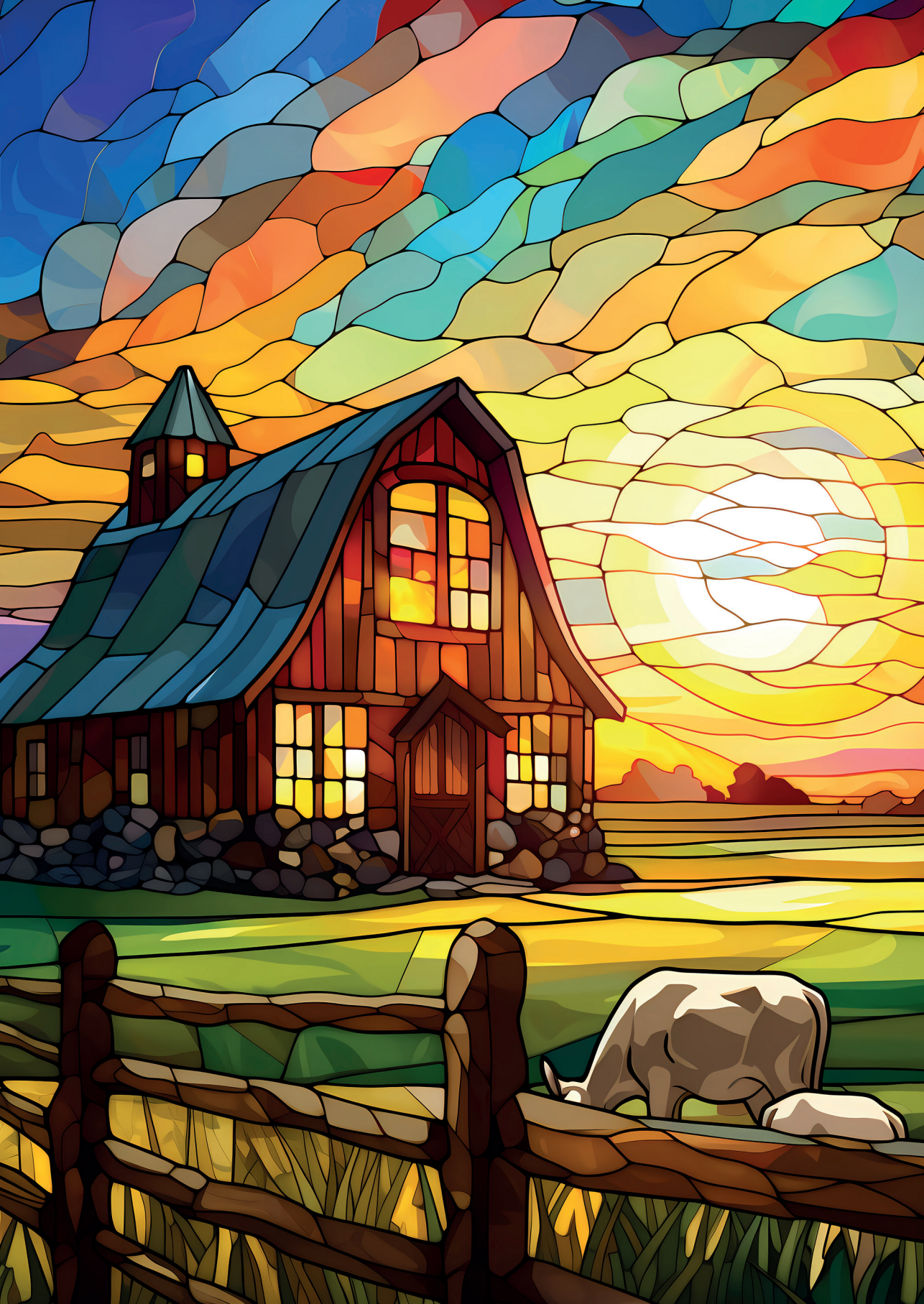
*Note:* this question was added in February 2021 (VGO) and April (AMIGO, PIAMA)




**Supplementary figure S10:** Bayesian multilevel main effect models for health protective behavior outcomes **a:** Number of close contacts (<1.5m), **b:** Use of personal protective equipment (PPE) during close contacts, **c:** Close contact duration >10 minutes. Models were adjusted for age, sex, BMI, recruitment cohort and season.




**Supplementary figure S11:** Bayesian multilevel main effect models for COVID-19 risk perception outcomes **a:** Perceived risk of COVID-19 infection, **b:** Perceived risk of severe COVID-19, **c:** Healthcare avoidance in fear of acquiring COVID-19 in healthcare environments, **d:** Worry about missed or postponed healthcare appointments. Models were adjusted for age, sex, BMI, recruitm



Chapter 6

# General Discussion

Livestock farming, after decades of intensification, has been in the spotlight for multiple reasons over the recent years. With one of the most intensive livestock sectors in a densely populated country,[1] the Netherlands is at the forefront of the societal debate on how to reconcile economically viable food production with animal wellbeing, public health challenges (e.g. air pollution, zoonoses, and antimicrobial resistance) and environmental impacts of livestock farm emissions like biodiversity loss and climate change. In response, the Dutch government has stated that an ambitious transition towards circular agriculture is needed.[2–4] From the perspective of livestock farming, circular agriculture revolves around decreasing dependency on imported animal feed, optimizing the utilization of waste products and reducing emissions (e.g. feeding animals locally grown crops from soil fertilized with their own manure). The reasoning behind the transition emphasizes local environmental impacts (e.g. nitrogen deposition) and climate change. Attention for the potential co-benefits to human health, that will likely result from reductions in farm emissions, could further stimulate circular agriculture.[5]

This dissertation describes novel insights into the impacts of air pollution from livestock farms on respiratory health in nearby residents. Associations with both acute (chapter 2) and long term (chapter 4) respiratory health effects were elucidated. Early life exposure to livestock farms was shown to be associated with accelerated lung function decline later in life (chapter 4). Influences on the microbiota of the upper respiratory tract, a potential mechanism for respiratory health effects, were explored in chapter 3. Additionally, valuable knowledge was gained regarding potential secondary health effects of COVID-19 containment measures in the Netherlands (chapter 5). This thesis furthers the scientific basis for a multidisciplinary approach in elucidating the impact of livestock farm emissions in people living in livestock dense areas.

## Acute and long-term respiratory health effects of livestock emissions in nearby residents

Chapter 2 shows that ambient $NH_3$ concentrations measured in the VGO area, two-days prior to spirometry, are associated with acute lung function decrements in a panel of COPD patients. In the United States, panel studies in children with asthma and non-diseased adults reported similar negative associations between lung function and prior day exposure to ammonia ($NH_3$) or fine particulate matter with a diameter up to 2.5 µm ($PM_{2.5}$).[6–8] Overall, these studies show that day-to-day variation in proxies for the complex mixture of pollutants emitted by livestock farms, is related

to acute changes in lung function. Cross-sectional studies report similar associations of temporal and spatial livestock exposure proxies with lung function decrements, increased risk of respiratory symptoms, pneumonia, disease exacerbations and mortality from respiratory diseases.[9–18] Studies performed in non-diseased adults and adolescents show that the associations between livestock exposure and respiratory health are not limited to people with a pre-existing respiratory disease.[9, 13–15] Moreover, recent cross-sectional studies, directly assessing exposure by means of modelled livestock attributed pollutant concentrations (e.g. endotoxin and $PM_{10}$), also report associations with decreased lung function and respiratory symptoms.[19, 20] Livestock-related exposure in these studies was expressed as annual average concentration at the home address. Additionally, an average occupancy time of 12 years was reported, making the associations with lung function suggestive of a long term effect.[20] However, studies investigating the relationship between livestock farm emissions and changes in lung function over time are scarce and have thus far been limited to occupational studies in farmers.[21] It would be premature to interpret the lack of evidence for an association between livestock farm exposures and lung function decline over a 7-year period among neighboring residents (chapter 4) as evidence against such a long-term effect. Longitudinal studies (compared to cross-sectional) have the potential to provide stronger evidence as they prospectively investigate changes in lung function in relation to exposure. However, studies with more observations per individual are needed to overcome the challenges inherent to longitudinal designs. This includes the substantial sample sizes needed to enable detection of relatively small differences in rate of change over time. The effect of exposure on lung function decline over time is further obscured by within-person variation introduced by time-varying confounders (e.g. seasonality) and covariates like equipment and personnel changes.[22] Additionally, a multi-cohort approach should be considered as the effect of livestock emissions on lung function could vary in different stages of life.



## The duality of early life farm exposure

This thesis provides novel insight into the potential impact of early life exposure to livestock farming on adult lung function. Chapter 4 shows that lung function of individuals who grew up on a livestock farm, compared to those who did not, declined faster later in life. The large number of participants who worked and lived on a farm during childhood indicates that early life exposure often reached occupational levels. At the same time, farming has been associated with longitudinal lung function decline in adolescents.[23] Similarly, childhood farm exposure has been

linked to late onset asthma in adults.[24] This suggests that the well-known protective effect of the farm environment for allergic asthma and atopy, may come at a cost later in life.[25,26] It has been hypothesized that the protective effect of the farming environment is explained by differences in microbial exposures in early life.[27] Later, it was suggested that maturation of the gut microbiota (mediated by metabolites) underlies the protective effect for asthma in farm children.[28] This is in line with the 'old-friends hypothesis', suggesting that atypical maturation of the human microbiota plays a role in the pathogenesis of inflammatory disease.[29] A study comparing Amish and Hutterite children, two rural populations that differ in terms of asthma prevalence, indicated that differences in farm scale (traditional vs industrial) could be relevant.[30] Recent studies investigating protective associations between asthma and exposures similar to the farm environment (e.g. pets, more older siblings), also suggest maturation of microbial communities to play a role.[31] The bidirectionality in the health effects associated with early life microbial exposures, for example the protective effect of endotoxin exposure on atopic conditions,[32,33] and increased risk of non-atopic asthma,[34] has also been described as 'two sides of the same coin'.[35] In conclusion, the current body of evidence suggests that the impact of growing-up on a livestock farm is too complex to be described as simply healthy or beneficial. Life-course studies assessing respiratory health trajectories, in farmers and rural residents, are needed to reveal to what extent maturation of the microbiota and 'the two sides of endotoxin exposure' shape lung function and other respiratory health outcomes.

## Livestock emissions and the microbiota of the airways

The potential mechanisms behind the effect of livestock farm-emitted air pollution on respiratory health remain to be elucidated. To that end, this dissertation explored the role of the upper respiratory tract microbiota in mediating the effect of livestock farm emissions. Valuable insights were gained into the relationship between livestock farm emissions and the upper respiratory tract microbiota of adults with COPD. Chapter 3 shows that the species richness of the oropharyngeal microbiota of COPD patients increased with endotoxin exposure. While still understudied compared to the gut microbiota, research into the role of the respiratory microbiota in health and disease is developing. Besides significant differences in community composition related to disease status and severity,[36,37] alterations related to air pollution have been clearly established.[38] However, the directionality of these associations remains elusive. Future studies aimed at establishing whether changes in microbiota drive (or follow) the respiratory health impact of air pollution are essential. Investigating

individual trajectories (e.g. reversibility) in both respiratory health and microbiota composition, in relation to air pollution exposure, would be of interest. Most studies investigating air pollution and the airway microbiota focus on bacteria, leaving open the role of viral and fungal communities (including inter-kingdom interactions) as key targets for investigation.[39] Important to mention in the context of human health and microbiota is the so-called gut-lung axis. This system of inter-organ crosstalk modulates the innate immune system, mediated by commensal micro-organisms, their fragments and metabolites.[39,40] An example of the gut-lung axis in chronic airway disease was found in exacerbating COPD patients, who showed increased gastrointestinal permeability,[41] adding to the evidence suggesting the gut microbiota to be involved alongside that of the lung.[42] The relationship between the gut and airway microbiota is also suggested to be important with regards to bacterial pneumonia, although most evidence so far stems from mouse models.[39] Nevertheless, analyzing lung and gut microbiota and their metabolites in parallel offers a promising window into the mechanism behind the increased pneumonia incidence observed near Dutch goat farms and the exact role and components of the human microbiome involved.[43] Studies including comparative microbiota analysis of samples taken from goat farms (e.g. farmers, animals, manure), pneumonia patients, control subjects and ambient air are currently underway.

## Livestock farm emissions and health in broader perspective

The complex network of livestock farming and health, with the microbiome as a possible mediating factor in between, is a captivating example of One Health. From this perspective, the classical concepts of human, animal and environmental health are inseparable. Early in the 21th century, One Health emerged as a conceptual framework for research and action on the intersection of human, animal and environmental health, when the need for interdisciplinary collaboration in the field of emerging zoonotic diseases and antimicrobial resistance became evident.[44,45] Over the recent decades, it became clear that the relationship between livestock farming and health is broader,[46] including beneficial effects of microbial exposures and the impact of chemical emissions like $NH_3$. besides health effects in farmers and nearby residents, farm emissions affect the health of farm animals and ecosystems as well. Technical end-of-pipe measures like air pollution abatement systems, have been increasingly employed to reduce emissions and protect the health of animals and farmers.[47] In densely populated areas with intensive livestock industries, vulnerable individuals (e.g. children, elderly, people with respiratory disease) could

additionally benefit from these mitigation strategies.[48] However, the efficiency of abatement systems is limited by a broad range of practical difficulties.[47] Systems like air scrubbers and biofilters, for instance, are temperature sensitive. As a result, these options are less effective during colder seasons.[47] Additionally, technical measures generally aim to reduce specific pollutants associated with odor annoyance, human health, and local environmental pollution (e.g. $NH_3$ and PM). This leaves the broader environmental impact of other livestock-related pollutants like carbon dioxide ($CO_2$) relatively unaddressed. Distance based interventions like the minimal distance (500m) between residential areas and goat farms advised by the Dutch government, do not protect the environment at all.[49] In that regard, the impact of farm emissions on climate change,[50,51] and subsequent indirect human health consequences (e.g. heat related mortality and hospitalizations) should not be overlooked.[52-55] This interplay between the global environment and human health is the focus of Planetary Health. This strongly related field complements the One Health perspective to the consequences that disturbances of Earth's natural systems have for human health.[56,57] From this global perspective, the need for structural change (e.g. circular agriculture) supported by technical interventions becomes clear.

## Livestock farm emissions and climate change

Livestock farms emit a range of pollutants that impact the environment. On the long-term, the environmental impact of livestock emissions as a driver of climate change,[50,51] could lead to large scale and broader human health effects. Greenhouse gas emissions of livestock farms are thus highly relevant for future research. Methane ($CH_4$) and nitrous oxide ($N_2O$) are the most important greenhouse gasses from livestock farms in terms of warming potential (compared to $CO_2$).[50] However, the importance of reducing the relatively small amount of livestock emitted $CO_2$ (9% of the total anthropogenic emission) should not be underestimated.[58,59] $CO_2$ accumulates in the atmosphere over centuries due to its long half-life. This means that net $CO_2$ emissions are to be reduced to zero for temperature to stabilize and that additional warming will occur until this is achieved.[60,61] This important for countries with a large livestock sector like the Netherlands, whose contribution to global $CO_2$ emissions is relatively high. On the other hand, atmospheric $CH_4$ does not accumulate but has a much stronger warming potential (28-84 times, depending on the time scale) compared to $CO_2$.[62-65] Therefore, it has been argued that $CH_4$ emission reduction should not be overlooked as a relatively small reduction would stabilize $CH_4$ related warming.[58] The majority (90%) of livestock emitted $CH_4$ results from enteric fermentation by ruminants (e.g. cows, sheep), with the remaining 10% attributed

to manure management.[51] Estimations based on the radiative forcing (change in atmospheric energy) due to anthropogenic $CH_4$, attribute roughly 12% of global warming to date to livestock emitted $CH_4$,[61] detailed livestock-climate simulations arrive at similar magnitudes (14%).[66] Livestock related $N_2O$ (mainly originating from manure) also has a significant effect on global warming, as it has a lifespan of roughly 120 years and a 265 times higher radiative potential compared to $CO_2$.[67] Evidence regarding the human health impact of climate change is developing but strongly suggestive of adverse effects. Examples include respiratory, cardiovascular or neurological outcomes (e.g. stroke) as well as changes in the spatial distribution of pathogens and their vectors.[68] Given the large livestock sector, the potential contribution of the Netherlands in reducing livestock emitted greenhouse gasses is relevant in mitigating climate change and its long term health consequences.

## Epilogue

Emissions from livestock farms affect our health and the environment in a plethora of ways. Multiple more or less effective methods to limit livestock-related emissions have been proposed. Examples include manure storage, selective breeding, low emission diets and vaccination against microorganisms responsible for methane synthesis.[50,51,58] However, taking into account the role of animal products in food security, it has been argued that measures to reduce dependency on livestock (e.g. dietary change, reducing food waste) are maybe even more essential.[58,69] Replacing protein from animal products with plant sources has been associated with reduced mortality,[70,71] adding to the body of evidence showing that such a dietary shift would benefit environmental, human and animal health.[72] This is further supported by the direct associations between human health and livestock emissions underlined in this thesis. The societal impact of structural changes like circular agriculture warrants its own line of research. Currently, the social sciences are strikingly underrepresented in One Health research.[73] A recent study, showing what makes for constructive communication in public meetings regarding the health effects of livestock farming,[73] clearly shows the added value to the One Health framework in which collaboration between stakeholders is essential. Another recently coined term that is closely related to One Health and advocates for the incorporation of the social sciences is pandemic preparedness. Methodologies from social epidemiology, like those employed in chapter 5 to assess the impact of COVID-19 policies, could provide insights into the (mental) health impact of policies aimed to transform the livestock sector. In addition, air pollution has been related with the incidence, hospitalization and mortality rate of infections in the recent COVID-19 pandemic,[75] providing an additional argument to decrease farm emissions from the view of pandemic preparedness.

Despite the substantial body of evidence in favor of structural changes, transition towards sustainable farming remains a controversial topic. There is a role for scientists in enabling the societal debate regarding controversial issues, like health effects of livestock farming, in the form of Open Science: the effort of making scientific evidence transparent and accessible for anyone. Examples of this include publishing in open access journals and the movement towards making the growing amount of computer code used for analysis (e.g. statistical modelling) accessible through platforms like GitHub or GitLab.[76] Where possible, the research presented in this dissertation has been made publicly available. Chapters 2 and 3, both published at the time of printing, are available in Open Access journals. The raw 16s sequence data (not personally identifiable), used to characterize the oropharyngeal microbiota of COPD patients (chapter 3), was shared with the NCBI Sequence Read Archive (accession number PRJNA810336). Especially regarding the ongoing societal debate on the transition to circular agriculture in the Netherlands, Open Science is indispensable to engage and educate the general public, policymakers and the livestock sector. Nevertheless, Open Science is still largely a bottom-up effort, depending on the individual researcher having the resources (e.g. funding for Open Access publishing) and opportunity (e.g. knowing how to use git) to make their work transparent and accessible. By incorporating Open Science related skills (e.g. git for data science) in scientific education, Open Science can be promoted from the top-down. Citizen science, including non-researchers (e.g. farmers) in the scientific process, should also be considered. While relatively new in environmental epidemiology,[77] citizen science supports the collaborative process essential for One Health studies and can facilitate translation of research into practice.

## Conclusion

This dissertation presents research on the respiratory health effects of livestock emissions in people living in livestock dense areas, built on cutting-edge methodologies from environmental and molecular epidemiology. The evidence provided draws attention to the public health relevance of livestock emissions. At the same time, enticing insights regarding the complex interplay between protective and adverse health effects of livestock exposure provide ample opportunities for future research. Given the equivocal state of evidence regarding the long term impact of (early-life) exposure to livestock emissions, longitudinal studies assessing the impact of livestock related air pollution on life-course trajectories of respiratory health, including lung function measurements and microbiota sampling, in farmers and nearby residents are needed. Studying the microbiota of farmers before and

after retirement (or changing careers) could provide insights into the role of the microbiome. Modeling of livestock related AMR genes in the ambient air is currently being explored as a more direct measure of exposure to livestock emissions. Other logical next steps in exposure assessment include using cluster analysis or regularization techniques (e.g. lasso regression) to combine individual emission concentrations (e.g. endotoxin and AMR) into composite livestock exposure scores. While causality and mechanisms remain to be elucidated, this should not stand in the way of the discussion regarding mitigation strategies aimed at protecting human, animal and environmental health.

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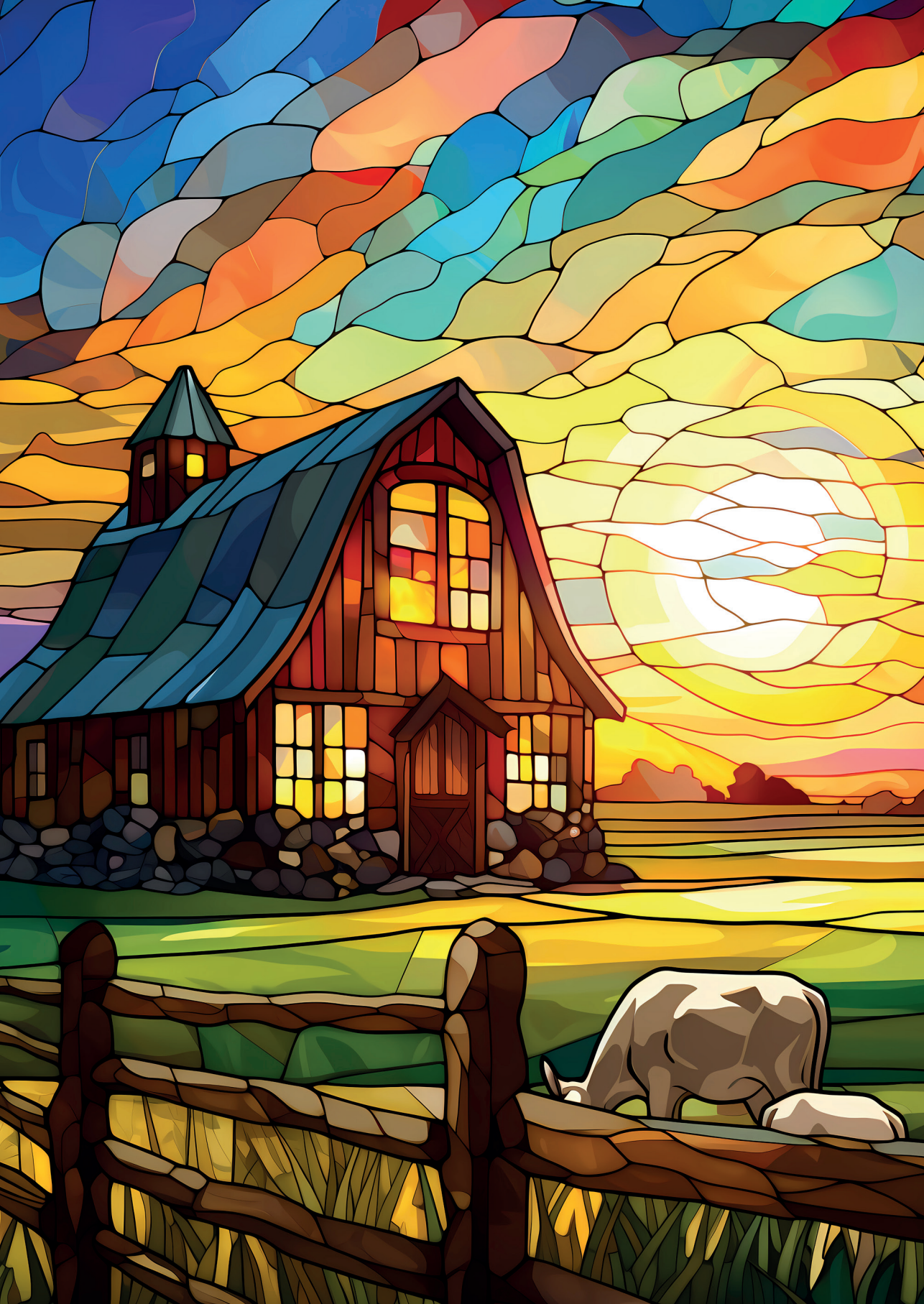

Appendices

**Summary**
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## Summary

In the Netherlands, a country with a large livestock sector in a densely populated area, increasing concerns among general practitioners and local organizations led to the first exploratory studies on health effects of neighboring residents of livestock farms. The VGO project (Dutch acronym of Livestock Farming and Neighboring Residents' Health) aimed to gain more insights into the health effects of livestock farm emissions in people who were not farmers themselves. The study area encompasses the Southeast of the Netherlands that is known for its large and intensive livestock industry. After an initial questionnaire conducted among 14,882 adults in 2012, a subset of 2,494 adults was medically examined in 2014 and 2015. Besides collection of a blood sample, nasal swab, and a fecal sample this examination included lung function testing (spirometry). Additionally, land use regression and dispersion models were developed to assess exposure to livestock-related air pollutants more precisely at the individual level. These models, developed and validated using air samples taken near farms, predict annual average livestock-emitted particulate matter and endotoxin concentrations at the home address. Endotoxin is a cell wall component of Gram-negative bacteria that induces pro-inflammatory immune responses and is ubiquitous in livestock emitted particulate matter. **Chapter 1** describes the current knowledge in the field of air pollution related to livestock farming and health impacts in people living close to farms. Residential exposure to livestock farm-emitted air pollutants has been associated with airway obstruction, lung function deficits, and respiratory symptoms. This thesis presents research performed in sub-studies of the subsequent VGO-2 and VGO-3 projects that were performed in a selection of the original VGO participants, aiming to further explore the connection between respiratory health and exposure to air pollution from livestock farms.

Exposure to livestock-related air pollution, expressed as annual or week-average air pollutant concentrations, has previously been linked to a lower lung function in VGO and other study populations. In **chapter 2**, we investigated whether day-to-day changes in livestock-related air pollution concentrations could also affect lung function. To this end, 82 VGO study participants with Chronic Obstructive Pulmonary Disease (COPD) measured their own lung function twice daily (morning and evening) during a 3-month period. Participants also kept a diary in which they recorded respiratory symptoms and whether they experienced livestock-related odor annoyance. The health data collected by the participants was compared with the daily average ammonia and particulate matter concentrations measured at two sites in the study area. We observed that a higher daily average ammonia concentration, two days before the lung function measurement, increased the risk

of a morning lung function more than 20% lower than an individual's expected (median) value. Additionally, we observed lower evening lung function on days on which participants reported livestock related odor annoyance. This shows that short-term exposure to livestock-related air pollution can affect the lung function of vulnerable individuals like COPD patients, warranting further investigation in the pathways behind these associations.

The biological mechanisms through which livestock related air pollutants affect the airways are still poorly understood. **Chapter 3** delves into the potential role of airway bacterial communities (microbiota), as a focal point for understanding how air pollutants from livestock farms could affect respiratory health. A series of three throat swabs over 12-weeks, with 6 weeks in between samples, were obtained from 99 participants with COPD and 184 control participants. The bacterial community structure was determined utilizing a bacterial 'barcoding' gene (16S rRNA) which enables identification of the species present in a sample and provides (semi-quantitative) information on their abundance. For all participants the initial sample was analysed. For twenty randomly selected participants, we also analysed the repeated samples taken 6 and 12 weeks after the initial sample. Analysis of the repeated samples revealed that the bacterial community was relatively stable over a 12-week period, indicating that a single throat swab provides a reliable image of the microbiota in an individual's upper respiratory tract. Next, we compared these snapshots of the airway microbiota with endotoxin and particulate matter concentrations at the participants home address. This revealed that higher farm-emitted pollutant concentrations were related to a higher number of bacterial species in individuals with COPD but not in control participants, suggesting that the microbiota of the upper respiratory tract of COPD patients is more susceptible to alterations induced by exposure to air pollutants than those of people without COPD.

Given the growing body of evidence supporting that people exposed to higher concentrations of livestock-emitted air pollutants have lower lung function, we expected livestock exposure to accelerate the natural decline in adult lung function that occurs around the age of 25 and onwards. This was investigated in **chapter 4** where changes in lung function between 2014-2015 and 2021-2022 were studied in association with modelled endotoxin and particulate matter concentrations at the home address for 847 adults. We did not find evidence of a relationship between livestock emissions and accelerated lung function decline. However, our results showed that participants who lived on a livestock farm during childhood experienced accelerated lung function decline later in life. This finding was surprising as it is well known that livestock farm exposures during childhood are associated with a

decreased risk for allergic disease like asthma. This protective effect for allergies is thought to be related to the maturation of the immune system, allowing it to better modulate its responses and prevent overreaction. Likewise, in the VGO population it was previously observed that participants with a farm childhood have a lower prevalence of atopy and allergic disease. At the same time, our results indicate that there might be a detrimental side to childhood livestock exposure that emerges later in life. As participants who grew up on a farm had a better lung function compared to those who did not during the initial 2014-2015 measurement, future studies with more observations over the life course are needed to establish whether the observed accelerated decline continues after the initial advantage has been nullified.

The COVID-19 pandemic coincided with the fieldwork of VGO-3, offering a major challenge, but also the opportunity to investigate the impact of an unprecedented global health crisis. The potential role of livestock farms in the outbreak in the Netherlands, was shown when minks at multiple fur farms were found to be infected with the SARS-CoV-2 virus followed by mink-to-human infections. Furthermore, chronic air pollution exposure has been linked to more severe COVID-19 outcomes. The IMPACT initiative (**chapter 5**) a successful collaboration effort to employ existing study populations to investigate the secondary impacts of the pandemic, allowed us to investigate to what extent the measures put in place to contain the outbreak affected health and wellbeing in the Netherlands. Additionally, we explored if the impact differed between participants with and without chronic disease and between urban and rural areas. For this investigation 5,420 participants were followed for up to 14 months (September 2020-October 2021) by monthly questionnaires. Stringency of containment measures during each month was measured using a national containment score ranging from 0 to 100 based on 20 indicators. Examples of these indicators include school closures, travel restrictions, testing and vaccination policies. The results showed that in months with stricter containment measures, participants that suffered from chronic disease reported worse physical health. This was not observed in participants without chronic disease. Additionally, participants in urban areas reported worse mental health during months with stricter containment. While this was also observed in rural areas, there the impact was less pronounced. This showed the importance of considering vulnerable individuals or areas in decision making regarding containment measures. On a higher level, this study showed the importance of having ready-to-go research frameworks to study the impact of health crises.

**Chapter 6** discusses the findings of this thesis in the broader context of One Health. The complex relationship between animal farming, human health and

the environment makes that farm emissions remain a pressing topic for research. Given the equivocal state of evidence regarding the long-term impact of (early-life) exposure to livestock emissions, longitudinal studies assessing the impact of livestock related air pollution on life-course trajectories of respiratory health, including lung function measurements and microbiota sampling, in farmers and nearby residents are needed. Studying the microbiota, including viruses and fungi, of farmers before and after retirement (or changing careers) could provide further insights into the reversibility of changes in these microbial communities induced by air pollutants. Potential mitigation strategies should consider structural changes to livestock production like the transition to circular farming initiated by the Dutch government. While causality and mechanisms remain to be elucidated, this should not stand in the way of the discussion regarding mitigation strategies aimed at protecting human, animal, and environmental health.

# Samenvatting

Toenemende zorgen onder huisartsen en lokale organisaties in Nederland hebben geleid tot de eerste verkennende studies naar de gezondheidseffecten van omwonenden van veehouderijen. Het VGO-project (Veehouderij en Gezondheid Omwonenden) is erop gericht om meer inzicht te krijgen in de gezondheidseffecten van emissies van veehouderijen op mensen die zelf niet op een boerderij wonen of werken. Het studiegebied beslaat het zuidoosten van Nederland dat bekend staat om zijn grootschalige en intensieve veehouderij. Na een initiële vragenlijst onder 14.882 volwassenen in 2012, werden 2.494 van deze deelnemers tussen 2014 en 2015 medisch onderzocht. Hierbij werd naast bloed, een neusuitstrijkje en een ontlastingsmonster ook een longfunctietest (spirometrie) afgenomen. Daarnaast zijn er modellen ontwikkeld om de blootstelling van iedere deelnemer aan luchtverontreiniging, afkomstig van veehouderijen, te kunnen berekenen. Deze modellen, ontwikkeld en getest met behulp van luchtmonsters uit het onderzoeksgebied, voorspellen de jaargemiddelde concentraties van door veehouderijen uitgestoten fijnstof en endotoxine op het woonadres van de deelnemers. Endotoxine is een bestanddeel van de celwand van een grote groep bacteriën (Gram-negatieven) dat ontstekingsreacties opwekt en alomtegenwoordig is in door veehouderijen uitgestoten fijnstof. **Hoofdstuk 1** beschrijft de huidige kennis op het gebied van veehouderijgerelateerde luchtverontreiniging en gezondheidseffecten bij mensen die dicht bij boerderijen wonen. Blootstelling van omwonenden aan veehouderijemissies is eerder verband gebracht met verminderde longfunctie, vernauwing van de luchtwegen (obstructie) en andere luchtwegklachten. Het onderzoek beschreven in dit proefschrift vond plaats in het kader van de VGO-2 en VGO-3 vervolgstudies. Beide zijn uitgevoerd in een selectie van de oorspronkelijke VGO-deelnemers, met als doel het verband tussen luchtverontreiniging van veehouderijen en de gezondheid van de luchtwegen bij omwonenden verder in kaart te brengen.

Blootstelling aan veehouderijgerelateerde luchtverontreiniging, uitgedrukt als jaar- of weekgemiddelde concentraties van luchtverontreinigende stoffen, is eerder in verband gebracht met een lagere longfunctie in VGO en andere onderzoekspopulaties. In **hoofdstuk 2** onderzochten we of dagelijkse veranderingen in de concentraties van veehouderijgerelateerde luchtverontreiniging ook de longfunctie kunnen beïnvloeden. Hiertoe hebben 82 deelnemers met Chronische Obstructieve Longziekte (COPD) tweemaaldaags ('s ochtends en 's avonds) gedurende een periode van 3 maanden hun eigen longfunctie gemeten. Deelnemers hielden daarnaast in een dagboek bij of er 1) sprake was van

luchtweg klachten, en 2) veehouderij gerelateerde geurhinder werd ervaren. De verzamelde gezondheidsgegevens van de deelnemers werden vergeleken met de daggemiddelde ammoniak- en fijnstofconcentraties gemeten op twee locaties in het onderzoeksgebied. Daarbij viel op dat een toename in de ammoniakconcentratie het risico vergroot op een longfunctiedaling van meer dan 20% ten opzichte van de verwachte waarde (individuele mediaan). Daarnaast zagen we lagere longfuncties op dagen waarop deelnemers geurhinder rapporteerden. Dit toont aan dat ook kortdurende blootstelling aan veehouderijgerelateerde luchtverontreiniging de longfunctie van kwetsbare mensen zoals COPD-patiënten kan beïnvloeden. De nog openstaande vraag is welke mechanismen achter deze verbanden zitten.

De biologische mechanismen waardoor veehouderijgerelateerde luchtverontreinigende stoffen de luchtwegen beïnvloeden zijn nog onduidelijk. **Hoofdstuk 3** focust op de potentiële rol hierin van de bacteriën (het zogenaamde microbioom of microbiota) die in de bovenste luchtwegen voorkomen. Om een beeld te krijgen van de microbiota in de bovenste luchtwegen, is bij 99 deelnemers met COPD en 184 controledeelnemers een serie van drie keelmonsters afgenomen over een periode van 12 weken, met tussenpozen van 6 weken. De microbiota werd in kaart gebracht met behulp van een bacterieel 'barcode' gen (16S rRNA) dat het mogelijk maakt om per monster te bepalen welke bacteriën in welke hoeveelheid aanwezig zijn. Van alle deelnemers is het eerste monster uit de serie geanalyseerd en van twintig willekeurige deelnemers zijn ook de herhaalde monsters in de analyse meegenomen. Dit laatste liet zien dat de microbiota van de bovenste luchtwegen relatief stabiel was over een periode van 12 weken, wat aangeeft dat één keelmonster een betrouwbare momentopname van de microbiota geeft. Vervolgens hebben we deze microbiota momentopnames vergeleken met endotoxine- en fijnstofconcentraties op het thuisadres van de deelnemers. Hieruit bleek dat deze door boerderijen uitgestoten verontreinigende stoffen gerelateerd waren aan een hoger aantal verschillende bacteriële soorten bij deelnemers met COPD, maar niet bij controledeelnemers, wat suggereert dat de microbiota van de bovenste luchtwegen van mensen met COPD vatbaarder is voor veranderingen door blootstelling aan luchtverontreiniging dan die van mensen zonder COPD.



Het bewijs dat blootstelling aan hogere concentraties veehouderijgerelateerde luchtverontreiniging van invloed is op de longfunctie neemt toe. Daarom verwachtten we dat blootstelling aan veehouderijemissies de natuurlijke afname van de longfunctie door veroudering bij volwassenen versnelt. Dit is onderzocht in **hoofdstuk 4**, waar veranderingen in longfunctie tussen 2014-2015 en 2021-2022 werden bestudeerd in verband met gemodelleerde endotoxine- en fijnstofconcentraties op het huisadres van 847 volwassenen. We hebben geen bewijs

gevonden voor een verband tussen veehouderijemissies en versnelde afname van de longfunctie. Echter, onze resultaten toonden aan dat deelnemers die in hun jeugd op een veehouderij woonden, later in het leven versneld in longfunctie achteruit gaat. Deze bevinding was verrassend, omdat het bekend is dat blootstelling aan veehouderijen in de kindertijd wordt geassocieerd met een verminderd risico op allergische aandoeningen zoals astma. Dit beschermende effect wordt toegeschreven aan positieve invloeden op de ontwikkeling van het immuunsysteem, waardoor het beter in staat is om immunologische reacties te reguleren en overreactie te voorkomen. Dit zien we ook in de VGO-populatie, waar deelnemers die als kind op een boerderij woonden minder vaak allergische aandoeningen hebben. Tegelijkertijd geven onze resultaten aan dat er mogelijk een nadelige kant is aan blootstelling aan veehouderijen in de kindertijd die later in het leven naar voren komt. Aangezien deelnemers die op een boerderij opgroeiden een betere longfunctie hadden tijdens de eerste meting in 2014-2015, zijn studies nodig met meer metingen gedurende de levensloop om vast te stellen of deze versnelde afname doorgaat nadat deze initiële voorsprong teniet is gedaan.

De COVID-19 pandemie viel samen met het veldwerk van VGO-3, wat een grote uitdaging bood, maar ook de kans om de impact van een ongekende wereldwijde gezondheidscrisis te onderzoeken. Een mogelijke rol van veehouderijen in de dynamiek van de COVID-19 uitbraak in Nederland kwam aan het licht toen bij nertsen op meerdere pelsdierfokkerijen werd vastgesteld dat ze besmet waren met het SARS-CoV-2-virus, gevolgd door infecties van nerts naar mens. Bovendien is chronische en kortdurende blootstelling aan luchtvervuiling in verband gebracht met ernstiger ziekteverloop en moeizamer herstel van COVID-19. Het IMPACT-project (**hoofdstuk 5**), een succesvol samenwerkingsverband met als doel in bestaande studiepopulaties de indirecte gezondheidseffecten van de pandemie te onderzoeken, stelde ons in staat om te onderzoeken in hoeverre de door de regering getroffen maatregelen de gezondheid en het welzijn in Nederland beïnvloedden. Daarnaast hebben we onderzocht of de impact verschilde tussen deelnemers met en zonder chronische aandoening en tussen stedelijke en landelijke gebieden. Voor dit onderzoek werden 5.420 deelnemers maximaal 14 maanden (september 2020-oktober 2021) gevolgd door middel van maandelijkse vragenlijsten. Hoe streng de beperkende maatregelen waren werd maandelijks vastgesteld aan de hand van een nationale 'containment-score' variërend van 0 tot 100, die gebaseerd was op 20 indicatoren. Voorbeelden van deze indicatoren zijn schoolsluitingen, reisbeperkingen maar ook test- en vaccinatiebeleid. Deelnemers met een chronische aandoening bleken zichzelf slechter te scoren op fysieke gezondheid in maanden met strengere beperkende maatregelen. Dit zagen we niet bij deelnemers zonder chronische aandoening.

Daarnaast scoorden deelnemers in stedelijke gebieden zichzelf slechter op mentale gezondheid tijdens maanden met strengere beperkingen. Hoewel dit ook het geval was in landelijke gebieden, was de impact daar minder groot. Dit laat zien dat het belangrijk is om rekening te houden met kwetsbare groepen of verschillen tussen gebieden in beleid en besluitvorming omtrent beperkende maatregelen. Overkoepelend laat deze studie zien dat het essentieel is om de infrastructuur, die nodig is voor gedegen onderzoek, klaar te hebben staan zodat de impact van gezondheidscrises bestudeerd kan worden zodra deze zich voordoen.

**Hoofdstuk 6** plaats de bevindingen van dit proefschrift in bredere context. De complexe relatie tussen veehouderij, gezondheid en het milieu maakt dat onderzoek naar de impact van veehouderijemissies nodig blijft. Gezien er nog veel onduidelijk is over de langetermijnimpact van blootstelling (op jonge leeftijd) aan veehouderijgerelateerde luchtverontreiniging, zijn langlopende studies nodig die de impact van uitstoot van veehouderijen op de luchtwegen over de gehele levensloop in kaart kunnen brengen. Longfunctiemetingen en bemonstering van de microbiota, bij boeren en omwonenden zijn daarbij essentieel. Het bestuderen van de microbiota, inclusief virussen en schimmels, van boeren voor en na pensionering (of na carrièreswitch) zou verder inzicht kunnen bieden in de omkeerbaarheid van veranderingen in de microbiota van de luchtwegen door luchtverontreiniging. Plannen om de impact van veehouderijemissies te verminderen moeten gericht zijn op structurele veranderingen in de veehouderij, zoals de transitie naar kringlooplandbouw die inmiddels door de Nederlandse regering is geïnitieerd. Hoewel de exacte mechanismen en oorzakelijke verbanden achter de impact van veehouderijemissies op de gezondheid nog moeten worden opgehelderd, mag dit het beschermen van de gezondheid van mens, dier en milieu niet in de weg staan.

# Dankwoord

Aan de slaapkamerdeur van mijn ouders bungelde ooit een kralenketting. Een rood stukje koord met daaraan geregen, houten kralen in kleuren geel, groen en blauw. Hoewel de ketting bijna 30 jaar geleden is gemaakt voor een specifieke Vader-, of Moederdag - doet er eigenlijk ook niet toe - is het kralen rijgen nooit echt gestopt. Inmiddels zijn we op het punt in mijn ketting beland waar ik een aantal kralen wat extra glans mag geven omdat dit proefschrift naar de drukker gaat. Als ik mezelf hardop afvraag bij welke kraal ik moet beginnen, komt er een herinnering boven drijven. Ter voorbereiding op sollicitatiegesprekken voor een aanstelling als PhD kandidaat, iets wat ik mezelf met mijn stotter niet zag doen, heb ik een tijdje gesolliciteerd naar posities die ik nog veel minder bij mensen die stotteren vond passen. In het voorjaar van 2018, op de fiets terug naar huis na sollicitatiegesprek bij een callcenter, rollen de tranen over mijn wangen. Ik was het niet geworden, maar tegen mijn verwachtingen in had ik een fijn gesprek gehad. Omdat dit hele boekje stoelt op de belofte die ik mezelf toen heb gedaan, om mezelf en mijn overtuigingen ten alle tijden uit te blijven dagen, lijkt het me passend dit dankwoord er ook mee te funderen.

Als eerste wil ik graag de leescommissie, bestaande uit de professoren Michel Dückers, Pieter Hiemstra, Merel Langelaar, Arjan Stegeman en Janneke van de Wijgert bedanken voor de tijd en moeite die zij hebben genomen om mijn werk kritisch te lezen. Ook alle co-auteurs, wiens commentaren een aanzienlijke bijdrage hebben geleverd aan het aanscherpen van de hoofdstukken, wil ik bedanken voor het delen van hun ervaring en expertise. Het blijft bijzonder dat ik deel heb mogen uitmaken maken van zo'n groot en interdisciplinair samenwerkingsverband als het VGO-consortium. Lidwien, bedankt voor alle feedback, ruimte en steun die je mij hebt gegeven om mezelf persoonlijk en academisch te ontwikkelen. Steevast kon ik erop rekenen dat je de tijd nam om naar me te luisteren, was je er om te voorkomen dat ik verdwaalde in geitenpaadjes, of hotels die bij aankomst veel te ver van de congres locatie bleken te liggen. In het bijzonder wil ik je bedanken voor je snelle reacties. Hoe druk je het zelf ook had, ik heb nooit ergens lang op moeten wachten. Dick en Debby, ook jullie support en constructieve feedback is van onschatbare waarde geweest. Met name in de schrijffases van de verschillende hoofdstukken van dit proefschrift heb ik veel steun gehad aan jullie commentaren en inzichten. Debby, jou wil ik daarnaast nog bedanken voor de uitnodiging om op het RIVM aan het microbioom hoofdstuk te werken. De ondersteuning die ik daar van Wouter heb gekregen heeft het oplossen van die complexe puzzel flink versoepeld, en het resultaat naar een hoger niveau getild.

Alex, zonder jou was er niet eens een microbioom hoofdstuk geweest. Als geen ander kun jij ingewikkelde materie rustig en duidelijk uitleggen. Je strak gecodeerde scripts vol met slimme automatisering, vormen nog steeds de basis van hoe ik nu zelf mijn code opzet en inricht. In het kader van 'paying it forward' heb ik recent een Git tutorial opgezet voor mijn collega's van Oulu University. Myrna, jouw manier van sturing geven aan het VGO-veldwerk team is al net zo'n blijvende inspiratie. Bedankt voor de ruimte en het vertrouwen dat je mij hebt gegeven om de IT voor het veldwerk, en de datastroom die daaruit voortkwam, onder mijn hoede te nemen. Ook ben ik je bijzonder dankbaar voor hoe je mij de kneepjes van het begeleiden van studenten hebt bijgebracht. De kroon op onze samenwerking blijft de bijzondere band die we ook buiten werktijd, vaak met lekker eten, goeie muziek en op bootjes, hebben opgebouwd. Daarover later meer, Myrna houdt werk en privé graag gescheiden dus dat doen we dan ook maar. Maartje en Aniek, ook jullie onvermoeibare inzet voor het VGO-team, de gezellige lunch wandelingen en goede gesprekken zijn links of rechtsom in dit boekje (en dus met mij) verweven. Lützen, bedankt voor de hulp bij het duiden van de statistiek en het opzetten van de analysemethode van het impact hoofdstuk. Het lijkt erop dat ik die Bayesiaanse modellen nog frequent ga gebruiken.

Ook zonder de harde werkers in het lab of in het veld had dit werk niet tot stand kunnen komen. Marieke, Sigrid, Duco, Kaitlin, Teus, Tom, Claire en Nathalie, wat hebben jullie onnoemelijk veel kilometers gereden, weer en verkeer trotserend, om alle deelnemers te bezoeken en de data te verzamelen. Bernadette en Kaitlin, bedankt voor jullie inzet bij het controleren van de grote hoeveelheid longfunctie gegevens. Ik waardeer het enorm dat ik altijd bij jullie aan kon kloppen om de details van de spirometrie te bespreken. Peter, bedankt voor de sessies die we samen hebben doorgebracht om de IMPACT database te beheren. Geheel in lijn met onze vaste afsluiter is het inderdaad nog eens wat geworden. Eef, dankzij de door jou uiterst zorgvuldig opgezette online vragenlijst was het opschonen van de verzamelde gegevens een peulenschil. Wat een verademing was dat, een voorbeeld dat ik de rest van mijn carrière meeneem. Graag wil ik ook VGO-deelnemers hartelijk bedanken, ik heb zelf mogen ondervinden hoe gastvrij onze veldwerkers door jullie werden ontvangen. Zonder jullie had dit onderzoek nooit plaats kunnen vinden.

Broer en (werk) zus, ik kan niet beschrijven wat een eer het is dat jullie als paranimfen aan mijn zijde staan. Melvin, zonder jou in mijn leven weet ik niet of ik de moed had gehad om aan dit werk te beginnen. Meubels maken, bedienen in de horeca en zelfs een EHBO-diploma, wat ben ik ongelofelijk trots op wat je allemaal doet. Myrna, jouw bijdrage aan dit proefschrift overstijgt het collegiale op alle mogelijke manieren. Bedankt dat je er altijd bent om me weer rechtop te zetten als ik even wankel.

De extra credits die je me toewenst heb ik inmiddels gevonden. Kijk even in de spiegel, dan zie je ze ook. Fleur, wat was het heerlijk om zo'n bijzonder warm en benaderbaar persoon als kamergenoot te hebben. Mariana, Yujia and my other roomies from 320c, thanks for the fantastic company and all the halloumi we devoured together. Yujia, those rice dumplings were amazing! Natuurlijk ook alle andere Aio's van het IRAS, bedankt voor de collegialiteit, support en de vele gezellige momenten. Ilse in het bijzonder, het was een feestje om met jou de Epi master te volgen. Wanneer het kan, snel weer een keer dansen!

Lieve vrienden, bedankt voor de broodnodige ontspanning naast de inspanning. Angelique, Menno, Thom, David, Arjan, Inse, Mart, Chloe, Max, Chantal en Peter. Ook al wonen we al een tijdje niet meer bij elkaar in de buurt, jullie voelen altijd dichtbij. We moeten snel maar eens een Valentijnsweekend in Finland organiseren. Olaf, Natasja, Wilco, Dominque en Kevin, ook jullie liefdevolle vriendschap is de afgelopen jaren onmisbaar geweest. Olaf, jouw interesse in mijn onderzoek (vaak op het balkon) heb ik altijd zeer gewaardeerd. Wel ook de rest de van het boekje lezen, verdorie zes jaar aan gewerkt... Hans en Stefan, ook jullie vertrouwen in mijn lab skills destijds en de vele game sessies hebben veel voor mij betekend. Misschien moet er toch maar weer een playstation komen. Dan mijn McGuire familie, jullie zijn met te veel om allemaal op te noemen. Bedankt voor de talloze cursussen, workshops en supportmeetings die we met elkaar hebben verzorgd om sterker te worden dan ons stotteren. Denis en Denise, wat ben ik dankbaar dat ik met zulke sterke en lieve mensen de opleidingen tot coach en instructeur heb mogen volgen.

Pa en ma, bedankt dat jullie mij – allebei op geheel eigen wijze – stimuleren om het beste uit het leven te halen. Mam, jouw onvoorwaardelijke liefde is als wind in mijn zeilen. Al had je denk ik niet helemaal voor ogen dat ik daarmee in Finland zou belanden. Pa, jouw praktische kijk op de wereld en je gave om dingen te maken heb ik altijd bewonderd. Het doet me goed om te zien dat je nu ook aan jezelf werkt. Tot slot wil ik jou, Veerle, vanaf de bodem van mijn hart bedanken voor je liefde en je rotsvaste vertrouwen. Jouw sprankelende lichtje maakt van elke dag een feest.

# Curriculum Vitae

Warner van Kersen was born in Gorinchem, The Netherlands, on January 10th, 1991. After obtaining his Higher General Secondary Education (HAVO) diploma at Angelus Merula College in 2008, continued his studies at the Rotterdam University of Applied Sciences where he obtained his Bachelor of Applied Science in biomedical research (GPA 3.5) in 2012. After this, Warner studied at Leiden University from which he obtained his Master of Science in biology (GPA 3.5) in 2015 with a thesis titled: "Disease in introduced ring-necked parakeets (Psittacula krameri) in the Netherlands, a non-invasive molecular approach". Between his master's and doctoral studies, he focussed on data analysis working in the pharmaceutical and packaging industry. He started his PhD at IRAS in 2018, on the project described in this thesis. Alongside his PhD, Warner followed the postgraduate Master Epidemiology at Utrecht University for which he received his Master of Science degree (GPA 3.8) in 2021. Starting In 2023, Warner has been working as a post-doctoral researcher at Oulu University, Finland, focusing on developing novel methods to assess climate change exposure and its impact on cardiorespiratory health. Additionally, he volunteers as a coach and instructor on the McGuire Programme, where he helps himself and others to overcome their stutter.


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