By perseverance, the snail reached the ark.

(Charles H. Spurgeon, 19th century)

(immortalized by E.H. Gillis, *In de Gloria*)

Wij hebben misschien wel een klein projectje voor u.

(Tim Nawrot, 2008)

Papa *lust* altijd maar werken.

(Kasper Scheers, 2013)

VOORWOORD

"Wij hebben misschien wel een klein projectje voor u."

Het is mei 2008 en ik ben met de trein op weg naar Brussel, voor een van mijn laatste werkdagen aan het INBO. Heel toevallig kom ik Tim Nawrot tegen, per uitzondering ook naar Brussel sporend in plaats van naar Leuven. Tim is een jaar eerder mijn promotor geweest bij mijn statistiekthesis in Hasselt. Hij vraagt hoe het met mij gaat. Goed, zeg ik, maar wel op zoek naar werk. Waarop Tim dus antwoordt dat hij in het labo pneumologie misschien wel een klein projectje heeft voor mij, in de lijn van mijn thesis. Waarop ik daadwerkelijk in Leuven begin te werken, voor 4 maanden. En waarop die 4 maanden uiteindelijk 3 jaren worden als wetenschappelijk medewerker en daarna nog eens 5 jaar als doctoraats-student. Niet slecht voor een klein projectje.

De start van mijn doctoraat is er dus gekomen dankzij een onwaarschijnlijk toevallige ontmoeting in de trein. Dat dit doctoraat ook afgewerkt is geraakt, is allerminst te danken aan het toeval. Wel aan een heel aantal mensen, en tot hen richt ik nu het woord.

Ben en Tim, allereerst wil ik jullie bedanken voor jullie promotorschap. Jullie hebben mij na een fase van job- en studiehopping de kans gegeven om opnieuw wat stabiliteit in mijn professionele leven aan te brengen. Ben vertrouwde mij de uitvoering toe van zijn lang gekoesterde 'Hiltonstudie' plannen en ik hoop dat ik dat vertrouwen niet beschaamd heb met de realisatie van wat uiteindelijk de GeFiSto studie is geworden. Tim liep vlak na mijn aankomst in Leuven wel over naar Hasselt, maar bleef mij heel actief volgen en wist tussen zijn honderden e-mails per dag toch steeds de belangrijke (namelijk die van mij) eruit te pikken. Ben en Tim, jullie bleven altijd geduldig en koelbloedig, ook wanneer ik een deadline weer iets te dicht had laten naderen en er nog een abstract of manuscript moest nagelezen worden. De gevraagde feedback kwam ondanks mezelf toch steeds op tijd, was bovendien altijd van hoog niveau en droeg in grote mate bij tot het welslagen van dit doctoraat.

Dear jury members, prof. Paul Cullinan (Imperial College, London), prof. Marc Claeys (UA), prof. Cathy Matheï (KU Leuven) and prof. Tatiana Kouznetsova (KU Leuven), thank you very much for your time to read the whole manuscript (nearly 200 pages after all) and for being here today for a gripping and instructive discussion of my work. I greatly appreciate the effort.

Van de vele collega's en ex-collega's die in de loop van de jaren in het labo Pneumologie gepasseerd zijn, wil ik er een aantal even apart vermelden. Peter en Jeroen, als co-bazen naast Ben verstaan jullie de kunst om, net als Ben zelf, niet boven maar tussen de 'juniors' te staan en op gemoedelijke maar zeer wetenschappelijke wijze iedereen bij te staan met raad en daad. Anita, jij was een betrouwbaar baken in de mij vreemde wereld van de administratie (hoe zat dat ook weer met bestelbonnen en facturen voor vervoer op droog ijs?). 'Oude garde' (waarvan de meesten wel jonger dan ikzelf), bedankt om vooral in de eerste jaren van mijn verblijf de boel op te vrolijken met paintball, lunchen in de Alma, spelletjesavonden, twee lichtjes legendarische cantussen, quizzen met Glourious Basterds en Köt van Asem, looptoertjes rond de kerk van Winksele, voetbal kijken in Duitsland en nog zo veel meer. Ik wil zeker ook alle collega's bedanken die zo weinig van statistiek kennen dat ik wel een statistisch genie leek, en mij op die manier bestaansrecht gaven in het labo (en *en passant* mijn publicatielijst pimpten). Wie zich in bovenstaande beschrijvingen nog niet heeft kunnen terugvinden, weet dat jullie allemaal een groot of klein steentje hebben bijgedragen aan de – wat mij betreft – positieve sfeer in het labo, en zo aan mijn humeur en werkijver. Bedankt dus!

Lidia and Yang, you both deserve special mention, not only because of the smooth atmosphere in our little office, but certainly because of your well-appreciated share in the GeFiSto study, which simply could not have existed without you. I can especially recommend Lidia for all patient-related research at home and abroad: 100% professional, always friendly, never nervous, with a natural flair for setting study volunteers at ease. En ze spreekt zelfs Nederlands!

Beste GeFiSto-vrijwilligers, bedankt om jullie gedurende een heel jaar over te leveren aan de grillen van twee wetenschappers op zoek naar bewijsmateriaal voor de nefaste invloeden van fijn stof. Niet

alleen de 20 proefkonijnen, ook onze twee verpleegsters Mieke en Rita zorgden ervoor dat de buitenlandse GeFiSto-avonturen een sociaal en wetenschappelijk succes werden. Dankuwel allemaal, jullie waren geweldig.

Mijn ouders verdienen een speciale vermelding. De financiële bijstand tijdens mijn studies, later de vaak welgekomen opvang van de kinderen en de permanente morele steun waren hen nog niet genoeg. Ze boden zichzelf namelijk zonder verpinken aan als onderzoeksobject voor de GeFiSto-studie en zorgden bovendien met een indrukwekkend staaltje netwerken voor bijna de helft van de kandidaat-vrijwilligers (en van de uiteindelijke studiegroep). Respect! En ook: bedankt!

De laatste zes jaar zijn drie mini-mensjes een voor een mijn leven binnengewandeld en hebben het grondig overhoop gehaald. Kasper, Oskar en Leonie, een concrete bijdrage aan mijn doctoraat hebben jullie niet geleverd (of toch: bedankt voor de leuke citaten, jongens), maar dat geeft niet. Jullie impact is namelijk veel waardevoller dan dat. Een werkdag mag nog zo lang, vermoeiend, lastig, tegenvallend geweest zijn, gewoon het ’s avonds thuiskomen en het zien van jullie koddige gezichtjes en het luisteren naar jullie verhalen, doet mij al de rest snel vergeten en beseffen wat de essentie is.

Marieke, jou vermeld ik als laatste, maar weet dat dat een compliment is (je weet hoe goed ik ben in het omfloerst geven van complimentjes). Vergelijk het met een wetenschappeljke publicatie: de eerste auteur is degene die het meeste werk heeft gedaan, dan volgen anderen die hun steentje hebben bijgedragen en de baas, de belangrijkste onderzoeker, de man/vrouw die het hele project heeft mogelijk gemaakt, sluit de rij. En die projecten-mogelijk-maker, Marieke, dat ben jij. Ik zal nooit kunnen snappen, maar ook nooit vergeten, hoe jij je uit de slag hebt getrokken tijdens de GeFiSto-trip naar Milaan. Twee kinderen van 3 en 1 jaar oud, papa 10 dagen weg (Kasper: “Mama, papa komt nooit meer terug he?”), opa en omi ook mee, zelf een nieuwe voltijdse job, en dat alles in een half huis zonder keuken. Moest ik nu een hoed ophebben, ik zette hem drie keer na elkaar af. Dus Marieke, bedankt voor je opofferingen, je kranigheid, je alles. En omdat ik het volgens jou veel te weinig zeg, doe ik het nu, voor al die honderden lezers: ik zie u graag!

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LIST OF ABBREVIATIONS

ADS	Asian dust storms
AIC	Akaike's Information Criterion
ARPA Lombardia	Regional Agency for the Protection of the Environment in Lombardy
BAL	Bronchoalveolar lavage
BC	Black carbon
BOS	Bronchiolitis obliterans syndrome
BP	Blood pressure
CC	Compliance coefficient
CC	Case-control
CCO	Case-crossover
CI	Confidence interval
CIMT	Carotid intima-media thickness
CMV	Cytomegalovirus
CO	Carbon monoxide
COH	Cohort
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive protein
CS	Cross-sectional
CV	Cardiovascular
DALYs	Disability adjusted life years
DBP	Diastolic blood pressure

DC	Distensibility coefficient
EBB	Endobronchial biopsy
ECO	Ecological study
EEA	European Environment Agency
ESCAPE	European Study of Cohorts for Air Pollution Effects
EU	European Union
GBD	Global Burden of Disease
Hb	Hemoglobin
Hct	Hematocrit
HEM	Hemorrhagic stroke
HR	Hazard ratio
HRV	Heart rate variability
ICD-10	International Classification of Diseases, 10^{th} revision
IRCEL	Belgian Interregional Environment Agency
IS	Ischemic stroke
ISHLT	International Society for Heart and Lung Transplantation
LAD	Lymphocytic airway disease
LB	Lymphocytic bronchiolitis
LTx	Lung transplantation
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean volume of red blood cells
MOOSE	Meta-Analysis of Observational Studies in Epidemiology
MPV	Mean platelet volume

NA	Negative affect
NICU	Neonatal Intensive Care Unit
NO	Nitrogen oxide
NO_2	Nitrogen dioxide
NP	Non-professional
OR	Odds ratio
PA	Positive affect
PAD	Physical activity duration
PAF	Population attributable fraction
PAH	Polycyclic aromatic hydrocarbon
PANAS	Positive and Negative Affect Schedule
PM	Particulate matter
PM_x	Particulate matter with an aerodynamic diameter ≤ x μm
POD	Postoperative day
ΔP	Pulse pressure
PWV	Pulse wave velocity
RBC	Red blood cells
RHI	Reactive hyperemia index
SBP	Systolic blood pressure
SD	Standard deviation
SES	Socioeconomic status
SIDS	Sudden Infant Death Syndrome
SO_2	Sulphur dioxide
TBB	Transbronchial biopsy

TIA	Transient ischemic attack
TS	Time-series
UFP	Ultrafine particles
UNECE	United Nations Economic Commission for Europe
VOC	Volatile organic compound
WHO	World Health Organization
YEM	Young's Elastic Modulus

INTRODUCTION

AIR POLLUTION

Air pollution is the introduction of any substance in the atmosphere that can harm living beings (plants, animals or humans), or the natural or even the built environment in general. Pollutants range in composition from simple gaseous molecules to particles with a very complex composition.

Based on the environment of exposure, air pollution can be classified as household, occupational, or ambient air pollution. Household air pollution, caused by the use of solid biomass fuels for cooking and heating, is a serious health issue, especially in rural areas in low- and middle-income countries.[1] Indoor exposure to second-hand smoke and to chemical (e.g. cleaning products) and microbial agents (e.g. moulds) is also considered as household air pollution exposure. Occupational exposure can be similar to ambient exposure in terms of pollutant characteristics (e.g. in traffic-related jobs), but it also comprises indoor exposure in industrial environments, e.g. in mining, heavy metal industry, or the production of nanoparticles. Moreover, duration of exposure and/or concentration of pollutants is usually higher. Both household and occupational exposures are beyond the scope of this thesis.

Although pollutants can easily penetrate into the indoor environment, ambient air pollution is generally considered, and measured, as pollution of the outdoor atmosphere, of the air that we breathe in daily life. Greenhouse gases in the atmosphere (e.g. CO_2 and methane, CH_4) absorb and emit thermal infrared radiation and are responsible for the greenhouse effect, eventually harming the environment. So, based on the definition in the first paragraph, they are ambient pollutants as well. However, because the influence of greenhouse gases on human health is indirect, by gradually changing the climate, these agents are not considered in this thesis (even though some gases are both a greenhouse gas and a 'direct' pollutant, e.g. ozone, O_3).

From this point on, when referring to 'air pollution', I mean ambient air pollution *sensu stricto*, or the pollutants in the atmosphere with direct – proven or supposed – adverse effects on human health.

Sources

Air pollutants can end up in the atmosphere through natural causes (e.g. forest fires, volcanic eruptions, wind-blown soil dust) or human activities. The main anthropogenic emissions originate from motorized transport, industry, agriculture and building heating (**Figure 1**). Primary pollutants are emitted directly into the atmosphere, where they can undergo chemical or physical changes to become secondary pollutants. For example, nitrogen oxide (NO) and nitrogen dioxide (NO_2) are both emitted by road transport engines. NO is highly unstable and will undergo photochemical reactions to form NO_2, which has a longer lifetime. In turn, on days with high UV radiation, NO_2 is responsible for the formation of another secondary pollutant, ozone. Similarly, particulate matter (PM) can be emitted directly into the atmosphere, but particles can also aggregate or react with other substances (gaseous, fluid, or solid), leading to the formation of highly complex secondary particles.[2]

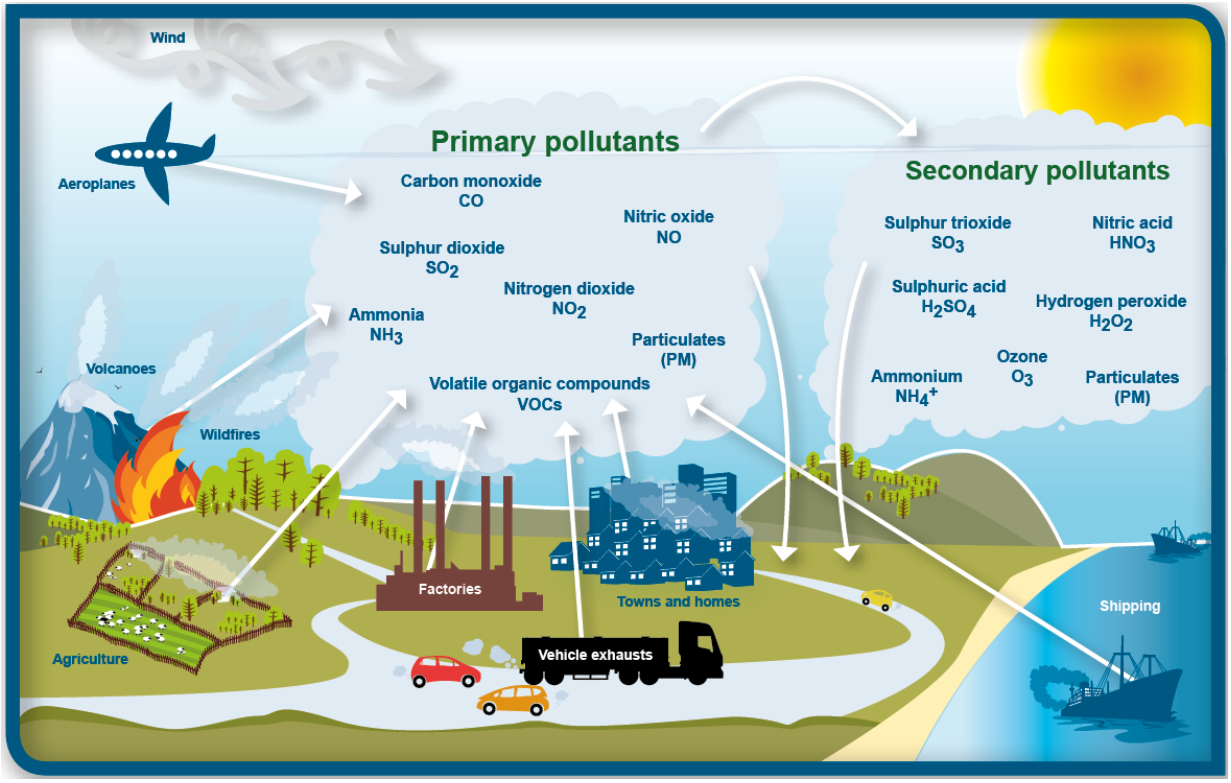

Figure 1. Sources and types of primary and secondary pollutants. Taken from Scotland's Environment .[3]

Main pollutants

Several substances that are found in the atmosphere have been identified as harmful for living beings, and for human health in particular. The main pollutants are PM and the gases NO_2, ozone, volatile organic compounds (VOCs), carbon monoxide (CO), and sulphur dioxide (SO_2).[4]

Based on the current literature, there is now a consensus that PM is the fraction of air pollution most reliably associated with human disease.[5,6] In Flanders, Belgium, PM accounts for about 75% of those disability adjusted life years (DALYs) that are due to environmental factors.[2,7] Secondary particles in particular are potentially hazardous. Formed by condensation of gases and liquids and by further deposit of other substances on their surface, they are a complex mixture of harmful molecules such as heavy metals, polycyclic aromatic hydrocarbons (PAH), dioxins, and so on.[2]

The behavior and health effects of PM do not only depend on chemical composition, but also on the aerodynamic diameter, i.e. the size, of the particles. PM is usually classified in three size classes: thus, PM_{10}, $PM_{2.5}$, and $PM_{0.1}$ include particles with aerodynamic diameters smaller than 10, 2.5, and 0.1 µm, respectively (**Figure 2**). In an atmospheric PM mixture, usually about 50 to 80% is $PM_{2.5}$;[8] the remainder is called the coarse fraction ($PM_{2.5-10}$). Ultrafine particles (UFP, $PM_{0.1}$) make up only a small portion of the total PM mass, but they are considered the most hazardous fraction, because the surface/volume ratio (and hence, reactivity) increases with decreasing volume, and they can penetrate deeper into the airways and possibly cross the alveolar-capillary barrier.[5,9] Although UFP can be measured in experimental circumstances, monitoring stations established to quantify environmental air pollution, historically have measured only PM_{10} (since 1997 in Belgium) and, more recently, $PM_{2.5}$ (since 2005 in Belgium). Therefore, epidemiological studies usually rely on data on PM_{10} or $PM_{2.5}$ to estimate the health effects of exposure to the smallest PM fractions.

In my PhD work, presented in this thesis, the emphasis was on health effects of exposure to PM. However, in the GEFISTO study (see **Chapter 4**), we also considered NO_2 and black carbon (BC) as exposure variables. Mainly emitted by vehicle exhausts, NO_2 is a good marker of traffic exposure, and it is recognized as an important contributor to the onset or development of human disease (either

directly or through its role in the formation of ozone).[10] BC is a component of $PM_{2.5}$ and has received special attention in epidemiological research of air pollution, as it is a good indicator of the combustion-derived and potentially very harmful parts of PM.[11]

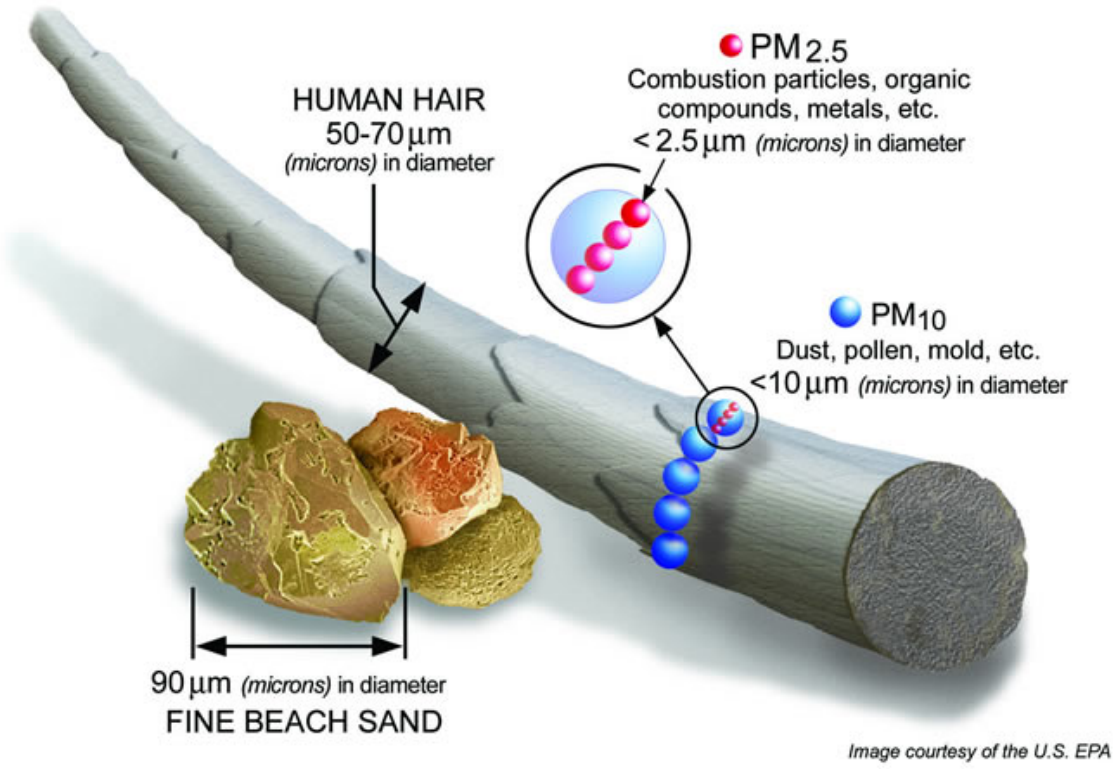

Figure 2. Size of PM_{10} and $PM_{2.5}$, relative to human hair and fine beach sand. Taken from U.S. EPA[12]

HEALTH EFFECTS OF AIR POLLUTION

Brief history

The first indications for an increased morbidity and mortality in relation to exposure to air pollution were found during short-lived episodes of extreme pollution, such as the *1930 Meuse Valley Fog*[13] and the *Great Smog of 1952* in London.[14] Health effects at low to moderate ambient PM concentrations and long-term effects have thoroughly been investigated only from the 1990s. Large-scaled prospective cohort studies, such as the Harvard Six Cities Study[15] and the American Cancer Society Study[16] found that long-term PM exposure was associated with respiratory and cardiovascular disease

and mortality. Meanwhile, a rapidly increasing number of time-series studies demonstrated that short-term (daily) variation in mortality,[17,18] respiratory symptoms,[19] and cardiovascular events[20] could, at least partly, be explained by concurrent short-term variation in air pollution levels. These cohort and time-series studies[15-20] are only a few examples; the early literature on ambient air pollution and human health has repeatedly been reviewed.[9,21,22] A more complete overview of the history of air pollution research is provided by H.R. Anderson.[23]

Current state

In the past 25 years, an enormous amount of epidemiological evidence for the detrimental health effects of exposure to ambient air pollution has been collected. To illustrate the diversity of health endpoints and target populations that have been investigated, I created a non-exhaustive summary of recent meta-analyses of studies regarding air pollution and human health (**Table 1**). This overview clearly demonstrates significant effects of both daily variation and long-term exposure on respiratory[24-29] and cardiovascular diseases,[28-34] but also on less obvious disorders, such as adverse pregnancy outcomes,[35,36] impaired neurological development and accelerated cognitive decline,[37] and type 2 diabetes.[38] For an even longer list of meta-analyses on environmental health, I refer to a systematic review that was published in 2015.[39]

Based on this still growing evidence, air pollution has now been recognized as a major threat to human health worldwide. According to the Global Burden of Disease (GBD) 2010 study,[40] worldwide, 3.7 million deaths and 3.1% of global DALYs were attributed to ambient PM pollution, placing it in the top 10 of risk factors (**Figure 3**). Similarly, a recent study conducted in Europe[41] concluded that 3 to 7% of the annual burden of disease in six participating countries was associated with nine environmental risk factors, among which ambient $PM_{2.5}$ was by far the leading risk factor.

Table 1. Overview of recent meta-analyses on human health effects of short-term and long-term exposure to air pollution.

Study reference	Health outcome	Population	Study types	Pollutant and increment	Unit and pooled estimate per increment	No. of studies
Short-term exposure						
[33] Pieters 2012	Heart rate variability (SDNN)	General (excluding occupational exposure)	panel	10 µg/m³ $PM_{2.5}$	**% -1.25 (-1.81; -0.68)**	17
[34] Mustafic 2012	Myocardial infarction	General population	TS + CCO	10 µg/m³ $PM_{2.5}$	**RR 1.025 (1.015; 1.036)**	13
				10 µg/m³ PM_{10}	**RR 1.006 (1.002; 1.009)**	13
[28] Atkinson 2014	CV hospital admissions	General population	TS	10 µg/m³ $PM_{2.5}$	**% 0.90 (0.26; 1.53)**	7
	Resp. hospital admissions				% 0.96 (-0.63; 2.58)	6
	All-cause mortality				**% 1.04 (0.52; 1.56)**	23
	CV mortality				**% 0.84 (0.41; 1.28)**	18
	Respiratory mortality				**% 1.51 (1.01; 2.01)**	16
[30] Shah 2015	Stroke (hospital admissions and mortality)	General population	TS + CCO	10 µg/m³ $PM_{2.5}$	**RR 1.011 (1.011; 1.012)**	41
				10 µg/m³ PM_{10}	**RR 1.003 (1.002; 1.004)**	78
[24] Zhang 2016	COPD hospital admissions	General (East Asia)	TS + CCO	10 µg/m³ $PM_{2.5}$	**RR 1.022 (1.013; 1.032)**	5
	Asthma hosp. admissions	Children (East Asia)			**RR 1.022 (1.019; 1.026)**	4
Long-term exposure						
[29] Faustini 2014	All-cause mortality	General population	Cohort	10 µg/m³ $PM_{2.5}$*	**RR 1.045 (1.007; 1.088)**	11
	CV mortality				**RR 1.196 (1.091; 1.310)**	17
	Resp. mortality				**RR 1.050 (1.009; 1.094)**	8
[35] Hu 2014	Hypertension disorder of pregnancy	Pregnant women	Retrospective cohort	5 µg/m³ $PM_{2.5}$	OR 1.15 (0.94; 1.40)	5
				10 µg/m³ PM_{10}	OR 1.10 (0.96; 1.26)	6

Study reference		Health outcome	Population	Study types	Pollutant and increment	Unit and pooled estimate per increment	No. of studies
[38]	Balti 2015	Type 2 diabetes	General population	Cohort	10 µg/m³ $PM_{2.5}$	**OR 1.11 (1.03; 1.20)**	5
[25]	Barone-Adesi 2015	Lung function (FEV_1)	Children (4-16y)	Cohort	10 µg/m³ NO_2	**% -0.7 (-1.1; -0.3)**	9
[26]	Chen 2015	Lung cancer incidence	General population	Cohort and CC	10 µg/m³ $PM_{2.5}$	**OR 1.11 (1.00; 1.22)**	6
		Lung cancer incidence	Professional drivers		Vs. NP drivers	**OR 1.27 (1.19; 1.36)**	14
[27]	Hamra 2015	Lung cancer incidence and mortality	General population	Cohort	10 µg/m³ NO_2	**RR 1.04 (1.01; 1.08)**	15
[36]	Lamichhane 2015	Low birth weight	Neonates, worldwide	Retrospective cohort	10 µg/m³ PM_{10}	**g -10.31 (-13.57; -7.05)**	5
		Preterm birth (adjusted for smoking)			10 µg/m³ PM_{10}	**OR 1.23 (1.04; 1.41)**	3
[32]	Provost 2015	CIMT (subclinical atherosclerosis)	General population	Cohort, CS	5 µg/m³ $PM_{2.5}$	**% 1.66 (0.86; 2.46)**	6
				Cohort, long.	5 µg/m³ $PM_{2.5}$	**µm/y 1.04 (0.01; 2.07)**	3
[31]	Scheers 2015	Stroke incidence (including mortality)	General (Europe and North America)	Pro- and retro-spective cohort	5 µg/m³ $PM_{2.5}$	**HR 1.06 (1.02-1.11)**	10
					10 µg/m³ PM_{10}	**HR 1.02 (0.98-1.07)**	9
[37]	Clifford 2016	Cognitive function	Children, elderly	Cohort and cross-sectional	No formal meta-analysis, but: • decreased brain development in children • cognitive decline in elderly		31

Significant results are highlighted in **bold**.

CV = cardiovascular; NP = non-professional; CIMT = carotid intima media thickness

Study types: CC = case-control; CCO = case-crossover; CS = cross-sectional; TS = time-series

* Similar results were found with NO_2 as the exposure variable

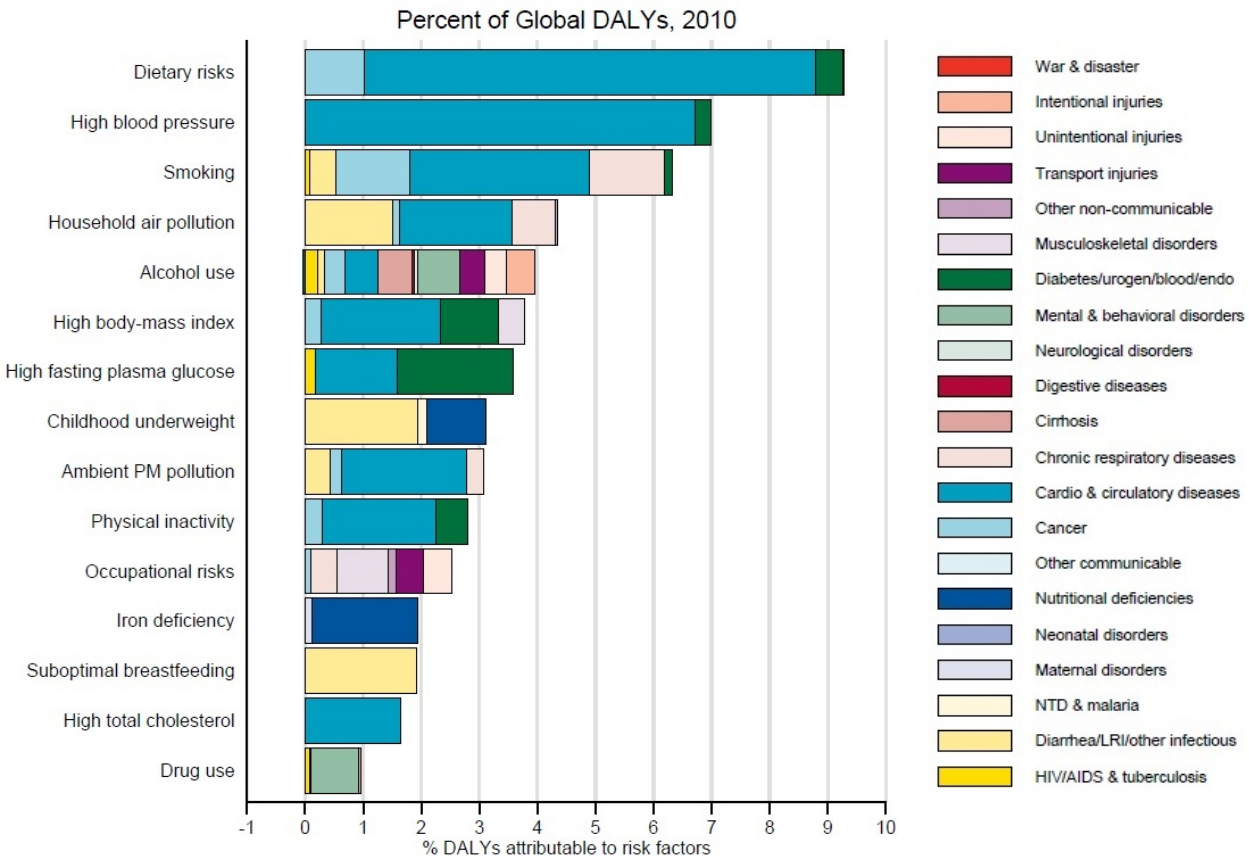

Figure 3. Burden of disease attributable to 15 leading risk factors in 2010. Adapted from GBD Study 2010[40]

Biological pathways

In addition to the epidemiological approach, numerous *in vitro* experiments, studies with animal models, and well-controlled experiments using human volunteers have been conducted to better understand and underpin the causal link between air pollution and human health, and to unravel the pathophysiological mechanisms involved in the process. Based on four comprehensive reviews,[4-6,42] published in the last seven years, and mainly focusing on cardiovascular disease, I will briefly discuss these biological pathways.

Three important pathways, starting from the inhalation of particles into the alveoli, are distinguished (**Figure 4**). First, pulmonary oxidative stress and inflammation can cause systemic oxidative stress and inflammation by the release and subsequent lung-blood transport of inflammatory mediators (cytokines, activated WBC, platelets), eventually leading to several chronic disorders. This hypothesis has been corroborated by epidemiological and experimental studies, showing significant

associations between PM and markers of systemic inflammation, such as C-reactive protein (CRP), IL-6, and platelets,[43-47] although in other studies no overt changes were observed.[48,49] Furthermore, chronic ailments such as progression of atherosclerosis, endothelial cell dysfunction, and thrombosis have also been related to exposure to air pollution.[4,46,50-52]

A second pathway consists of an imbalance of the autonomous nervous system, caused by interactions between inhaled particles and lung receptors, and potentially leading to acute detrimental events such as arrhythmia, decreased heart rate variability, hypertension, and (again) endothelial dysfunction.[33,49,52-55]

In a third physiological pathway, intermediate and ultimate effects are similar to the first pathway, but the first step is different. In the classical pathway, inflammatory mediators cross the lung-blood barrier, whereas in this proposed alternative pathway, the pollutants themselves (ultrafine particles, soluble metals, VOCs) penetrate into the circulatory system through diffusion from the alveoli to the lung capillaries. Direct translocation of inhaled nanoparticles has been shown in animal studies[56-58] and experiments with human volunteers.[59]

Through each of the pathways mentioned above, inhaled pollutants can also affect the nervous system. Indeed, neuroinflammation and oxidative stress, generated by either direct translocation of ultrafine particles through the blood-brain barrier or by translocation of inflammatory mediators, are potentially responsible for acute events such as ischemic stroke and chronic diseases such as Parkinson's disease, Alzheimer's disease, and neurodevelopmental disorders.[60,61]

Note that these pathways mainly describe the propagation of cardiovascular and cerebro-vascular diseases. For a similar overview on respiratory disease, I refer to a recent review by Xing et al.,[62] and more information on (epi)genetic effects and carcinogenicity of long-term exposure to air pollution is provided by Loomis et al.[63] and references therein.

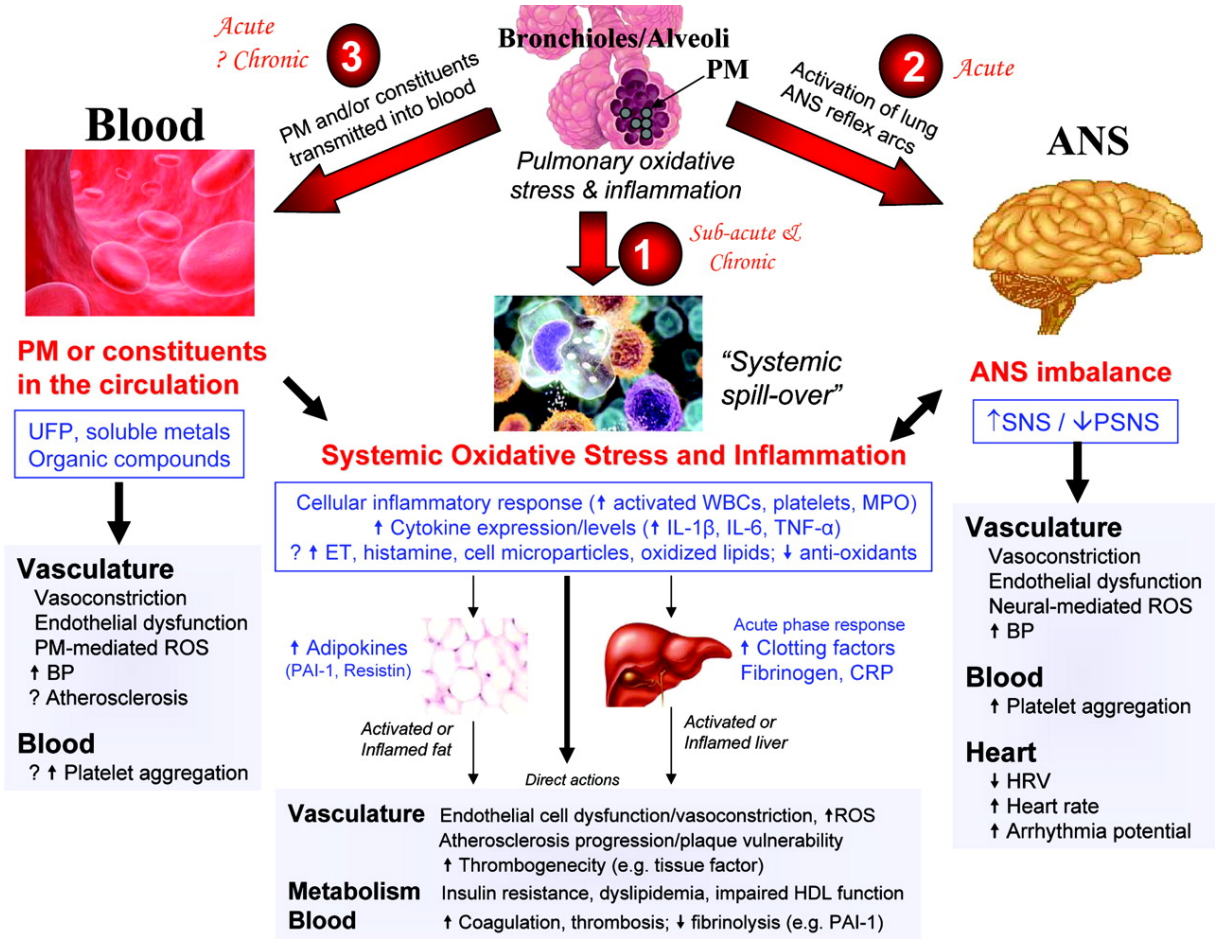

Figure 4. Biological pathways linking PM exposure with cardiovascular disease. Taken from Brook et al. 2010.[42]

Susceptible and vulnerable populations

Air pollution has been linked with many adverse health outcomes, but often these associations are stronger in certain susceptible or vulnerable subpopulations than in the general population.[22] Before discussing which subpopulations are more at risk for adverse effects of air pollution, it is necessary to clarify the exact meaning of, and the difference between, the concepts "susceptibility" and "vulnerability".

In a recent review, Sacks et al.[64] provide a list of definitions used in previous reports and reviews, concluding that susceptibility usually refers to biological or intrinsic factors (e.g. genetics, sex, age) and vulnerability to extrinsic factors (e.g. socioeconomic status (SES), lifestyle). However, they blend both concepts in an own definition for the term "susceptible" (in the context of PM exposure): "Individual and population-level characteristics that increase the risk of PM-related health effects in a

population including, but not limited to: genetic background, birth outcomes (e.g. low birth weight, birth defects), race, sex, life stage, lifestyle (e.g. smoking status, nutrition), pre-existing disease, SES (e.g. educational attainment, reduced access to health care) and characteristics that may modify exposure to PM (e.g. time spent outdoors)." Similarly, in a comprehensive meta-analysis on short-term exposure and health effects in specific subpopulations, Bell et al.[65] also merge the concepts of vulnerability and susceptibility into a common term: "effect modifiers".

Bell et al.[65] found strong and significant increased risk in elderly (> 65y or other age cut-offs), and suggestive evidence of higher risk with lower SES, measured as either education or income. The increased susceptibility of elderly can be explained by two factors: a gradual decline in physiological processes over time, and a higher prevalence of pre-existing cardiovascular and other diseases (see below). In their narrative review, Sacks et al.[64] pointed out that not only elderly, but also children are more susceptible because chronic exposure to air pollution can impair the growth of their respiratory system, leading to adverse effects, such as wheeze, cough, and asthma exacerbations. Increased susceptibility of infants and children already starts during pregnancy, resulting in preterm birth, low birth weight, increased risk of infant mortality, and adverse cardiovascular and respiratory outcomes during their later life.[66]

Bell et al.[65] also found non-significant higher risks of PM-related death and hospitalization in women, but Sacks et al.[64] concluded that evidence for effect-modification by sex was not consistent, and the large-scale ESCAPE study in Europe even found an opposite result, with higher hazard ratios (HR) for all-cause mortality related to air pollution in men than in women.[67] Genetic factors, such as polymorphisms in genes whose protein products are important in inflammatory or antioxidant processes, and obesity can also modify the relationship between exposure to air pollution and health outcomes. People with pre-existing diseases are another large group with elevated susceptibility for adverse effects of air pollution. Among these are cardiovascular diseases (e.g. hypertension, ischemic heart disease, history of myocardial infarction or ischemic stroke), respiratory diseases (asthma, COPD), and type 2 diabetes.[64]

METHODS IN AIR POLLUTION RESEARCH

Timeframe

In air pollution research, duration of the time window of exposure is an important factor, having obvious consequences for study design, statistical methodology, and interpretation of the results. Usually, exposure to air pollution is either defined as “short-term / acute” or “long-term / chronic”. Studies on short-term exposure try to link measurements of daily, or even hourly, variation in exposure to pollutants, to the subsequent onset of a particular acute health effect (including mortality). In long-term or chronic exposure studies, researchers aim to capture the average level of exposure during several years (usually in the place of residence) and to find associations with the incidence of certain diseases, which can be of acute or chronic nature.

By analogy with toxicological animal studies, we can also distinguish subacute (up to 28 days) and subchronic (up to 90 days) exposure in-between acute and chronic exposure.[68,69] In epidemiology, relatively little attention has been paid to subacute and subchronic effects, compared to the concepts of acute and chronic exposure.

Experimental studies

As mentioned before, experimental studies are important tools in air pollution research to unravel the biological mechanisms that explain the health effects found in epidemiological studies. The major benefits of *in vitro* experiments or studies using animal models or human volunteers, are a controlled and precise administration of the desired concentrations of the pollutant, and the possibility of measuring their effects directly in the target tissue. Moreover, in experimental settings, effect modifiers and confounders can easily be controlled for, thus facilitating statistical analysis and interpretation of results.

On the other hand, results from *in vitro* and animal experiments have to be translated to the human situation with care, also because experimental pollutant concentrations are usually much higher than those inhaled in real life. Obviously, for ethical and practical reasons, studies with human

study subjects are limited in exposure time (minutes to hours), and hence, they can only be used for short-term acute effects and are not suitable for the investigation of effects of air pollution that emerge only after a longer period of exposure.

Epidemiological studies

Numerous epidemiological studies have identified exposure to ambient air pollution as an important cause of respiratory and cardiovascular morbidity and mortality. Beyond any doubt, these epidemiological studies have contributed most to an increased understanding of the detrimental impact of air pollution on human health in real-world settings at ambient levels. Studies on health effects of short-term and long-term exposure differ fundamentally in terms of study design, analytical methodology and public health implications.

Short-term exposure to air pollution. Short-term studies investigate whether daily variation in ambient air pollution concentrations can be associated with a similar and concurrent variation in acute adverse health effects in the target population, such as mortality (general and cause-specific) or hospital admissions for cardiovascular (e.g. MI), cerebrovascular (e.g. ischemic stroke) or respiratory disease (e.g. asthma exacerbations). Results emerging from this type of studies have to be interpreted as a warning for potential excess mortality and morbidity during (or shortly after) brief periods of elevated air pollution. The announcement of smog alerts can help minimize the impact of such a pollution peak on public health by imposing temporary speed and traffic limits on the one hand, and warning the population to refrain from certain activities (e.g. physical exercise) on the other hand. An example of an elaborate real-life smog alert protocol can be found on the website of the city of Brussels.[70]

Most early publications on short-term effects of air pollution have used a time-series (TS) approach, whereas the case-crossover (CCO) design represents a relatively newer approach to study acute health effects. Both methods link large register-based datasets on the target outcome with daily measures of air pollution, but they use clearly different statistical modelling techniques. The TS design uses Poisson regression models to link daily counts of the outcome (e.g. mortality) with daily air

pollution data. Increasingly sophisticated models have been used to control for secular, seasonal and weekly time trends and to avoid confounding by other factors such as temperature and varying individual characteristics. Many of these covariates are non-linear, which complicates modelling even more, and different results can be obtained with different modelling assumptions and covariates included.[71]

In contrast, the CCO approach does not require complicated model building and is less dependent on model assumptions. It was developed in the early 1990s to study effects of brief and transient exposures on the change in risk of acute and discrete events.[72] Thus, the CCO approach is suitable, and has been increasingly used, for short-term air pollution research.[73] Its major power, compared to the classical TS design, is the ability to avoid confounding by individual characteristics. All the study subjects have experienced the event. The hazard period is defined as the average time period that is relevant for the acute event, and this period is compared with control times. Thus, subjects serve as their own controls at an individual level. The choice of the control period is crucial to avoid or limit selection bias and to safely assume the absence of time trends. The time-stratified design for the selection of control days has been shown to be the best selection method to avoid statistical bias (see **Figure 1** in **Chapter 1** for a representation of the bidirectional time-stratified design).[74,75]

Long-term exposure to air pollution. For short-term variation in ambient PM levels, the research question is *when* acute adverse events are most likely to occur (temporal variation in exposure), whereas for long-term exposure, the question is rather *where* people are most at risk (spatial variation in exposure). Additionally, studies on short-term effects of air pollution are inadequate to explain the prevalence and development of chronic air pollution-related diseases, such as lung cancer. These can only be interpreted in association with elevated pollutant concentrations over a much longer time span. Long-term exposure studies are able to detect an effect of even small differences in exposure on the development of physiological processes underlying acute events and chronic diseases alike. Translated to public health policy, this means that hot spots of air pollution (e.g.

industrial areas, busy roads, densely built city centers) deserve special attention and that the long-term concentrations of ambient air pollutants need to be kept as low as possible.

Long-term air pollution studies are usually cohort studies. The retrospective cohort approach, linking register-based health outcomes (mortality or hospital admissions) to estimated long-term air pollution exposure, is certainly the quickest and cheapest method. However, because of their essentially ecological nature, these studies lack information on important confounding risk factors at the individual level, such as smoking status or SES. Since the 1980s, large-scale prospective cohort studies have been set up to assess the health effects of long-term exposure air pollution. By design, results are only obtained after several years of intensive, time-consuming, and expensive collection of data through repeated interviews with participants and sometimes own measurements of air pollution. However, because of the incorporation of detailed data on individual covariates, the prospective cohort design is superior to the retrospective approach. Two famous examples are the Harvard Six Cities study in the United States[76] and the ESCAPE project in Europe.[77] Both cohort studies have found effects of long-term exposure to air pollution on multiple health endpoints, including overall and cause-specific mortality, respiratory and cardiovascular diseases.

PUBLIC HEALTH ASPECTS

As mentioned before, both indoor and ambient air pollution are now recognized as serious threats for public health worldwide,[40,41] an observation that is reflected in statements and measures made by official instances, such as the World Health Organization (WHO).

Air quality standards and guidelines

As the first scientific evidence of detrimental effects of ambient air pollution on public health was published, international health organizations started formulating guidelines to tackle the problem of air pollution. The 1979 Geneva Convention on Long-range Transboundary Air Pollution, drafted by the United Nations Economic Commission for Europe (UNECE), was the first international legally binding instrument to take on the issue of air pollution on a broad regional basis.[78] This convention formulated

rather vague agreements for the "contracting parties" (European countries) on air quality management, research and development and exchange of information.

In contrast, the WHO formulated more concrete, although not binding, air quality guidelines in 1987 (last updated in 2005).[79] The WHO proposes maximum values for both daily and averaged yearly ambient concentrations of four pollutants, PM, NO_2, ozone, and SO_2, based on scientific health based research.

The European Union (EU) and the United States both imposed binding air quality limit values for their member countries and states of the federation, respectively.[80,81] However, these limit values are substantially more relaxed than the evidence-based guidelines by the WHO. EU and US air quality standards and WHO guidelines for PM are summarized in **Table 2**. As mentioned above, apart from these air quality standards, local authorities have implemented *ad hoc* regulations to cope with brief periods of increased air pollution, such as the smog alert protocol in Brussels.

Recent trends in ambient PM concentrations

Belgium. According to the most recent annual report (2014)[82] by the Belgian Interregional Environment Agency (IRCEL), air quality has improved in the last 17 years. **Figure 5** shows the time trends for ambient PM_{10} and $PM_{2.5}$ concentrations. Annual PM_{10} means (panel A) slowly decreased from 1997 to 2008, when the worldwide economic crisis and concurrent decline in industrial activity (including heavy road traffic) marked an additional drop in ambient concentrations. This was followed by a further decrease and a remarkably good last year for which data are available (2014). Similar drops in 2008 and 2014 are present for the number of days above the daily limit for PM_{10} (panel B) and for annual $PM_{2.5}$ means (panel C).

In 2014, no area in Belgium had annual mean PM_{10} or $PM_{2.5}$ concentrations above the European limit values or more than 35 daily means above 50 µg/m³ PM_{10}. However, when taking the more stringent WHO guidelines as the benchmark, still 21% of the population was exposed to annual mean PM_{10} levels above the target value. For annual mean $PM_{2.5}$ levels and number of days above the daily limit for PM_{10}, these figures are even 78% and 97%, respectively.

Table 2. Air quality references values for PM, as stated by the WHO, EU and USA environmental agencies.

Pollutant	Time window	WHO guideline (2005)		EU limit (2008)		USA standard (2012)	
		Conc. (μg/m³)	Excee-dances*	Conc. (μg/m³)	Excee-dances*	Conc. (μg/m³)	Excee-dances*
PM_{10}	24 hours	50	3	50	35	150	1
	1 year	20	n/a	40	n/a	Not implemented	
$PM_{2.5}$	24 hours	25	3	Not implemented		35	7
	1 year	10	n/a	25	n/a	15	n/a

* Maximal number of exceedances permitted per year.

Europe. For current ambient concentrations of PM and other pollutants in the EU, I refer to the most recent report by the European Environment Agency (EEA).[83] Even though overall air quality has slightly improved over the past few years in a similar way as in Belgium, the situation is still problematic in many densely populated areas within the EU. No data are presented for the annual mean levels, but from Maps 3.1 and 3.2 in the report, we learn that in, e.g., the Po valley in Italy, the southern (industrial) half of Poland, the whole of Bulgaria, and also for some measurement stations in Belgium, the Netherlands and the Ruhr area, PM_{10} and $PM_{2.5}$ daily limits were exceeded on more than 35 days in 2013. The majority of measurement stations in the EU would not comply with the much more stringent WHO guidelines for daily PM values.

World. Mainly thanks to emission reductions in industry, energy supply, and road traffic, air pollution levels in most high-income countries (e.g. Belgium, Western Europe, the United States) have been slowly decreasing over the past decade, although they are still too high in particular areas with a high industrial activity or traffic density. This positive evolution is in sharp contrast to the sometimes enormously high and still increasing levels of air pollution to which people are exposed in other parts of the world.[84]

In low-income countries, mainly in Asia and sub-Saharan Africa, indoor air pollution by cooking and heating with biomass fuels is a major health threat. In 2012, 4.3 million deaths were attributable to household air pollution worldwide.[1]

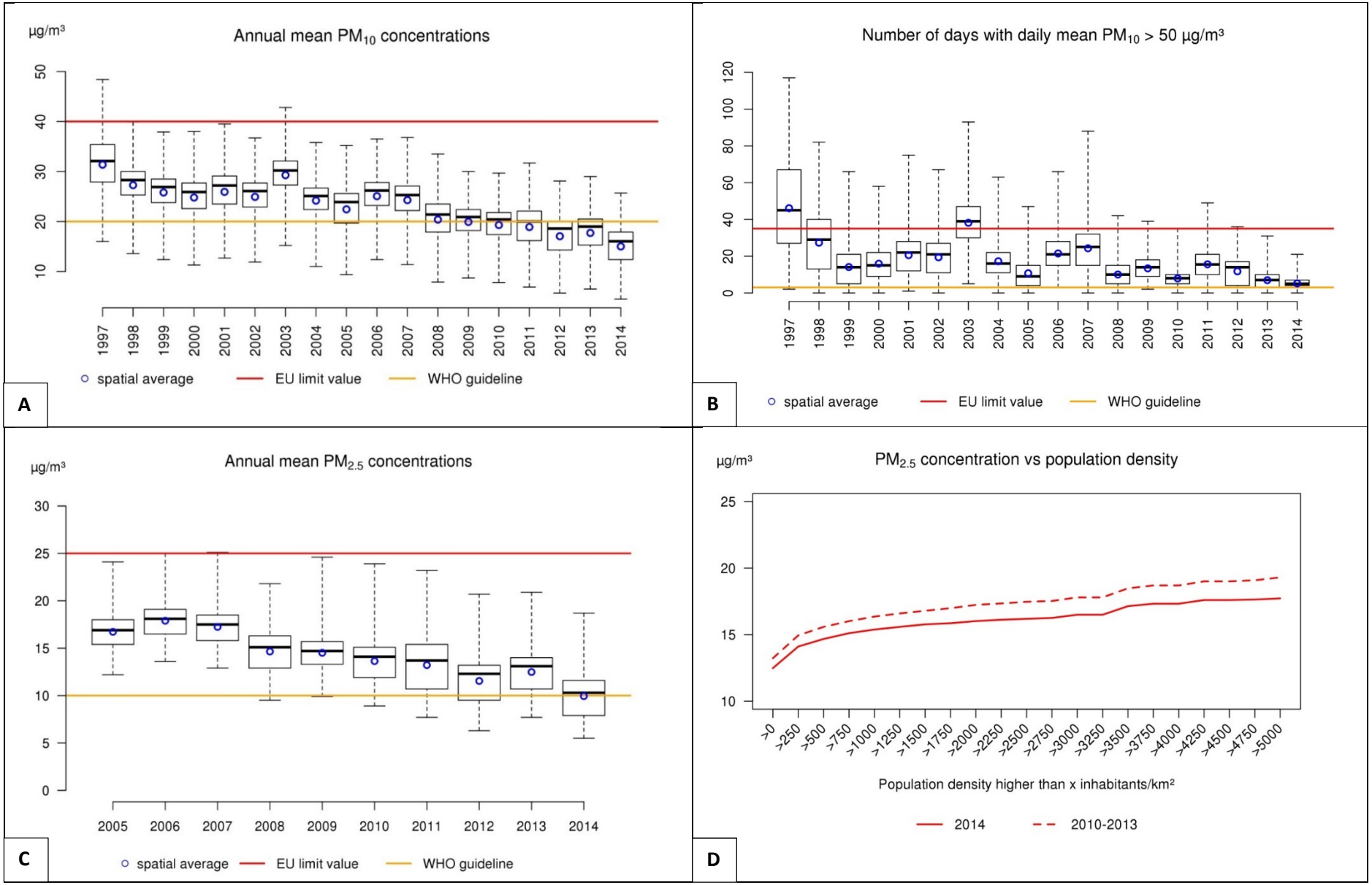
µg/m³
Annual mean PM10 concentrations
50
40
30
20
10
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
spatial average
EU limit value
WHO guideline
A
Number of days with daily mean PM10 > 50 µg/m³
120
100
80
60
40
20
0
spatial average
EU limit value
WHO guideline
B
µg/m³
Annual mean PM2.5 concentrations
30
25
20
15
10
5
0
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
spatial average
EU limit value
WHO guideline
C
µg/m³
PM2.5 concentration vs population density
25
20
15
10
>0
>250
>500
>750
>1000
>1250
>1500
>1750
>2000
>2250
>2500
>2750
>3000
>3250
>3500
>3750
>4000
>4250
>4500
>4750
>5000
Population density higher than x inhabitants/km²
2014
2010-2013
D

Figure 5 (previous page). Panels A-C: Time trends in air quality, as measured by PM monitoring stations in Belgium. Panel D: Exposure to $PM_{2.5}$ related to population density in Belgium. Taken from the IRCEL annual report on air quality in Belgium.[82]

Additionally, in fast-growing and populous economies such as China, India, and Brazil, ambient air quality in many urban areas is alarmingly poor. This is due to a rapid increase in industrial activity (usually powered by coal), heavy road traffic, personal car and motorbike traffic, combined with a lack or complete absence of regulations controlling the quality and quantity of emissions. For example, in the mega-cities (> 14 million inhabitants) Delhi, Kolkata (both in India), Dhaka (Bangladesh), and Beijing (China), annual PM_{10} levels in 2015 were about 230, 140, 160, and 110 $\mu g/m^3$, respectively.[84]

As a result, when weighed by population, an increasing trend in global ambient PM levels is detected, despite the decrease in high-income countries mentioned above. Consequently, the vast majority of the 3.7 million deaths attributable to ambient air pollution worldwide, namely 88%, occur in low- and middle-income countries.[1]

Current state and future perspectives

In spite of the favorable evolution in ambient PM concentrations in Belgium and several other high-income countries, there is no reason to relax or even maintain current limit values for exposure. In contrast, sustained efforts and new measures are needed to reduce the burden of disease from air pollution worldwide. In 2012, Brunekreef et al.[85] published "Ten principles for clean air" to provide guidance for future public health policy. I will briefly discuss some of the principles stated in the paper.

Principle 3: There is an urgent need to reduce concentrations [of PM] significantly. Indeed, studies that modeled the shape of the exposure-response relationship, indicate that the association between PM concentration and health endpoints such as mortality, is linear, with no evidence for a threshold or a 'safe level'[86-88]. Moreover, the exposure-response curve can even be steepest at the lowest concentrations.[89] Extended follow-up of the Harvard Six Cities cohort study showed that a reduction in ambient PM concentration has already led to a reduction in mortality risk and that a further reduction in exposure is likely to have continuing beneficial public health effects.[90,91]

Principle 4: UFP and BC need to be considered in future research. As mentioned before, UFP and BC are currently not routinely measured by most official monitoring stations. Adverse health effects of living near busy roads cannot be explained by PM_{10} or $PM_{2.5}$, because of their relatively homogenous distribution on a small scale. In contrast, UFP and BC are not only the most hazardous fractions of PM (see above), they also show much steeper gradients close to roadways. Hence, they are more likely to be the real cause of the observed effects, and it is recommended to monitor these fractions more regularly and to strengthen policies to reduce their emissions from motor vehicles.

Principle 6: Real-world emissions of NO_2 from modern diesel engines are much higher than anticipated. Climate change caused by increasing emissions of greenhouse gases such as CO_2, is a serious problem and initiatives to reduces these emissions should be encouraged. However, the "Eco Premium" that the Belgian federal government implemented in 2010 (and wisely abandoned in 2012) was a failure and bears a lot of irony in its name. A price reduction of 15% was awarded to the purchase of cars that emitted less than 105 g/km CO_2. Sales of small diesel cars peaked, because these were the only ones with such a low theoretical CO_2 emission. However, real-life emissions of CO_2 are much higher than in lab tests under optimal circumstances, and moreover, NO_x and PM emissions by diesel vehicles exceed those by cars driven by other fuels.[92] This received public attention and indignation when the Volkswagen emissions scandal ("dieselgate") erupted in September 2015.[93] These examples plainly show that adverse health effects caused by emissions of diesel vehicles have long been (and are still being) underestimated or even neglected by constructors and policy makers alike.

Principle 8: Combustion of biomass fuel produces toxic pollutants. Similar to the financial promotion of diesel cars in Belgium from 2010 to 2012, the use of biomass burning (e.g. wood) is currently being promoted in Europe to reduce greenhouse gas emissions. However, combustion products from ordinary wood stoves and hearths are as toxic as those from fossil fuels.[85] So again, policy makers, producers and consumers bear a shared responsibility in the pursuit of a cleaner atmosphere.

Principle 9: Compliance with current limit values for major air pollutants in Europe confers no protection for public health. An increasing number of countries (including Belgium) comply with the EU limit values, but this is certainly no reason to weaken or relax air pollution control policies. The EU air quality standards can be seen as a compromise between economic interests and health concerns, and are far above recommendations by the WHO. Studies of the ESCAPE project have shown adverse health effects well below the EU limit values WHO standards.[67,94] Combined with the linear dose-response relationship mentioned above, these observations should encourage European and national policy makers to further decrease ambient air pollution down to levels below the WHO guidelines values.

Principle 10: The benefits of EU policies to reduce air pollution outweigh the costs by a large amount. Economic arguments against more stringent measures to further reduce emission of pollutants (e.g. filters are expensive, production and consumption will decrease) are counteracted by the medical and economic costs of pollution-related morbidity.[95,96] In fact, monetized benefits from air pollution regulation (stemming from reduced loss of productivity and reduced illness burdens) outweigh the costs by far, as shown by cost-benefit analyses .[97,98]

These state-of-the-art principles were written for the situation in Europe. As mentioned above, in developing countries, tackling the problem of indoor air pollution by cooking and heating with biomass fuels is an additional challenge. In mega-cities in countries with a fast growing economy, such as China and India, the environmental situation is precarious, and urgent and vigorous measures are needed to decrease air pollution exposure to levels now found in European and North American cities, as a first step.

AIMS

The general objective of this PhD project was to gain more insight in the relationship between ambient air pollution and human health, with a special interest in the exposure range below or around the EU air quality standards, but above the WHO guidelines, as we currently have in Belgium. We selected three subpopulations, which are assumed to be more susceptible to PM air pollution: infants, elderly, and patients who underwent a lung transplantation (LTx), to evaluate health effects of low to medium-high levels of ambient air pollution.

- In a CCO analysis of a time-series database, I investigated whether short-term (daily) variation in environmental PM_{10} concentrations was associated with risk of infant mortality (<1y of age) in Flanders. I explicitly put forward the question whether the EU air quality standard for short-term exposure is sufficiently stringent, in the light of our study results.
- In a collaboration with the people of the Lung Transplantation Unit, we hypothesized that graft rejection after LTx can be linked to PM air pollution. We also investigated which underlying physiological mechanisms are involved, and we evaluated the potentially beneficial role of the antibiotic azithromycin.
- In a panel study of 20 healthy elderly volunteers, we aimed to quantify subacute effects of air pollution. We explored whether a decrease or increase of PM exposure (compared to Flanders) during several days was associated with biomarkers of respiratory, cardiovascular and general health, and whether there was any evidence for a threshold value at the lower end of the exposure-response curve. We aimed to measure exposure to PM at an individual level.
- By performing a systematic review and meta-analysis on stroke incidence in relation to long-term PM exposure, I contributed to the growing database of valuable reviews and meta-analyses on health effects of air pollution.

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CHAPTER 1

DOES AIR POLLUTION TRIGGER INFANT MORTALITY IN WESTERN EUROPE?

A CASE-CROSSOVER STUDY

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ABSTRACT

Background: Numerous studies show associations between fine particulate air pollutants [particulate matter with an aerodynamic diameter ≤ 10μm (PM_{10})] and mortality in adults.

Objectives: We investigated short-term effects of elevated PM_{10} levels on infant mortality in Flanders, Belgium, and studied whether the European Union (EU) limit value protects infants from the air pollution trigger.

Methods: In a case-crossover analysis, we estimated the risk of dying from non-traumatic causes before 1 year of age in relation to outdoor PM_{10} concentrations on the day of death. We matched control days on temperature to exclude confounding by variations in daily temperature.

Results: During the study period (1998-2006), PM_{10} concentration averaged 31.9 ± 13.8 μg/m³. In the entire study population (n=2,382), the risk of death increased by 4% [95% confidence interval (CI), 0–8%; p=0.045] for a 10 μg/m³ increase in daily mean PM_{10}. However, this association was significant only for late neonates (2–4 weeks of age; n=372), in whom the risk of death increased by 11% (95% CI, 1–22%; p=0.028) per 10 μg/m³ increase in PM_{10}. In this age class, infants were 1.74 (95% CI, 1.18–2.58; p=0.006) times more likely to die on days with a mean PM_{10} above the EU limit value of 50 μg/m³ than on days below this cut-off.

Conclusions: Even in an affluent region in Western Europe, where infant mortality is low, days with higher PM air pollution are associated with an increased risk of infant mortality. Assuming causality, the current EU limit value for PM_{10}, which may be exceeded on 35 days/year, does not prevent PM_{10} from triggering mortality in late neonates.

INTRODUCTION

In the past few decades, numerous studies have demonstrated that short-term exposure to elevated levels of air pollution has detrimental effects on human health. Most of these studies detected positive associations between particulate air pollution [particulate matter with an aerodynamic diameter ≤ 10 or ≤ 2.5 μm (PM_{10} or $PM_{2.5}$)] and general mortality, or the triggering of acute cardiovascular events, especially in the elderly and people with pre-existing cardiovascular and respiratory conditions (Alfaro-Moreno et al. 2007; Pope 2000; Zanobetti and Schwartz 2005).

In 1952, infant mortality doubled during the London smog (De Angelo and Black 2008; U.K.Ministry of Health 1954), but only recently has there been renewed concern about a possible link between exposure to air pollution and children's health. Children are considered particularly susceptible to air pollution, because their lungs and immune system are immature during the first few years of life. Prenatal exposure to elevated levels of air pollution has been associated with early fetal loss, preterm delivery, and lower birth weight (Bell et al. 2007; Schwartz 2004). Several studies have investigated the association in infants (< 1 year of age) between PM air pollution and all-cause mortality, respiratory diseases or Sudden Infant Death Syndrome (SIDS), yielding mixed results (Hajat et al. 2007; Kaiser et al. 2004; Lin et al. 2004; Romieu et al. 2004; Tsai et al. 2006; Woodruff et al. 2008; for review, see Glinianaia et al. 2004). Most of these studies focused on urban areas in the United States or countries in transition, such as Brazil, Mexico and Taiwan, whereas the number of studies conducted in Western Europe is very limited.

The European Union (EU) set two limit values for PM_{10} concentrations: annual mean levels of PM_{10} must not exceed 40 μg/m³, and daily averages must not exceed 50 μg/m³ on more than 35 days/year. In contrast, the World Health Organization (WHO) argues that annual averages of PM_{10} levels should not exceed 20 μg/m³ and that daily averages should not exceed 50 μg/m³ on more than 3 days/year (WHO 2006).

Using a case-crossover analysis, we investigated whether there is an association between short-term elevations of PM_{10} levels and infant mortality over a recent 9-year period (1998-2006) in

the region of Flanders, Belgium, and we evaluated the effectiveness of the current EU limit values by exploring the possibility of a threshold value in the exposure-response curve. The densely populated Flemish region (> 6 million inhabitants in an area of 13500 km², i.e. a population density of about 450 inhabitants/km²) has very low rates of infant mortality by international standards (United Nations 2007), but also among the highest concentrations of PM_{10} in Europe, as well as frequent exceedings of the prevailing EU limit values for PM_{10} (Beelen et al. 2009; Nawrot et al. 2007). Main sources of PM_{10} emission are traffic, industry and agriculture.

In our analyses, we took into account the effect of socioeconomic status (SES), because SES has been shown to be a possible modifier of the association between air pollution and health (Carbajal-Arroyo et al. 2010).

MATERIALS AND METHODS

Collection of data

Mortality data. We obtained data of daily infant mortality in Flanders during the period 1998-2006 from the Flemish Agency for Care and Health (Brussels, Belgium). These data were anonymous, but the following information was provided: date of death; postal code of municipality of residence; official cause of death, according to the International Classification of Diseases, 10th revision (ICD-10;WHO 1993); maturity at birth (a binary variable: mature or premature, i.e. < 37 weeks of gestation); and age at death, categorized (according to the WHO classification) as early neonatal (≤ 7 days of age), late neonatal (8 - 28 days of age), or postneonatal (29 - 365 days of age).

Air pollution data. In Belgium, PM_{10} and several other indicators of ambient air quality are continuously measured by a dense network of automatic monitoring sites (http:// www.irceline.be). Nineteen of these measurement stations have been in use in the region of Flanders from 1998 on, and they are situated 25 km apart from each other on average. Using a land use regression model (Janssen et al. 2008), we calculated the daily exposure level of PM_{10} at the municipality level for each mortality case. This model provides interpolated PM_{10} values from the Belgian telemetric air quality network in 4 x 4

km grids. The interpolation is based on a detrended kriging interpolation model that uses land cover data obtained from satellite images (Corine land cover data set) (Janssen et al. 2008).

Temperature data. Temperature is a known confounder of the association between air pollution and mortality (Hajat et al. 2002; Huynen et al. 2001; Katsouyanni et al. 1997; Nawrot et al. 2007). We obtained daily average temperatures from the Belgian Royal Meteorological Institute (Uccle, Belgium). The Region of Flanders is very uniform for temperature, because both altitudinal and latitudinal gradients are extremely small: elevations range from 0 to 200 m above sea level, and the distance between the northernmost and southernmost part is only 100 km. The region is not larger than the State of Connecticut (USA). Therefore, we used temperature data from the central and representative station in Uccle (Brussels, Belgium).

Socioeconomic status. We created three classes of SES at the municipality level, based on salary level, economic activity, degree of unemployment, and housing grade equipment (Dexia Bank NV 2007).

Analytical strategy

Case-crossover design. We investigated the association between air pollution and infant mortality using a case-crossover design, a technique developed by Maclure (1991) that combines features of the crossover design and the matched case-control design. Similar to a crossover study, each subject serves as his or her own control and, as in matched case-control studies, the inference is based on a comparison of exposure distribution rather than the risk of disease (Jaakkola 2003). The case-crossover design is now widely used for analyzing short-term health effects of air pollution (Carracedo-Martinez et al. 2010).

Selection of hazard period and control days. We defined the hazard period, which is the brief time period when a subject is at risk, as the day of death (event day). We selected control days based on three criteria (**Figure 1**). First, we took control days from the same calendar month and year as the event days, both before and after the event. We chose this bidirectional time-stratified design above other selection strategies to avoid issues of bias, as explained by Janes et al. (2005) and Mittleman (2005). Second, control days and event days had to be at least 3 days apart from each other to avoid

short-term autocorrelation (Levy et al. 2001). This implies a 5-day exclusion period around the event day. Third, because temperature is a known confounder of the association between air pollution and health (Hajat et al. 2002; Huynen et al. 2001; Katsouyanni et al. 1997; Nawrot et al. 2007), we selected only control days having a daily average temperature within 2°C of that on the event day. Based on this strategy, the number of control days per event ranged from zero to a maximum of 25, depending on the temperature criterion. On average, each case had 8.6 control days. Seventy-six cases (3.2%) had no control days and were, by consequence, not included in the analyses.

Shape of the association. To investigate whether there might be a threshold level in the exposure - response relationship or a plateau at higher concentrations, we studied the shape of the association between PM_{10} and risk of death by using fractional polynomials. Although linear and quadratic polynomials are commonly used, they are often inadequate to describe the shape of the association. Fractional polynomials are an alternative to classical polynomials but still fall within the realm of (generalized) linear methods. They extend the classical linear and quadratic models by allowing any power from a predefined set of values typically chosen from the set (-2, -1, -1/5, 0, 1/5, 1, 2, 3) (Royston and Altman 1994). From this family of models, the best functional form is chosen using Akaike's Information Criterion (AIC). A particular feature of the fractional polynomials is that they provide a wide class of functional forms, with only a small number of terms. Moreover, the conventional linear and quadratic polynomials are included as a subset of this extended family. Based on the best fitting model, we calculated odds ratios (ORs) for mortality in association with a 10 $\mu g/m^3$ increase in PM_{10}.

Additional analyses. To detect a possible short-term delay in the effects of exposure to PM_{10}, we performed five additional case-crossover analyses with different lag structures. In these analyses, we defined the hazard period as 1, 2 or 3 days before the day of death (lag days 1, 2, and 3, respectively) or as the moving-average exposure on 2 (event day and lag day 1) or 3 (event day and lag days 1 and 2) consecutive days. We also performed a sensitivity analysis using an alternative selection strategy, with control days being matched on day of the week instead of daily temperature, thus also including the 76 cases that had no temperature-matched control day.

We conducted stratified analyses by age class, by maturity, by SES, and by cause of death, categorized as cardiorespiratory diseases (ICD-10 codes I00-J99), SIDS (ICD-10 code R95), perinatal circumstances (ICD-10 codes P00-P96), congenital and chromosomal abnormalities (ICD-10 codes Q00 – Q99) or other. Infants who died from external causes (e.g. accidents, ICD-10 V00-Y98, n=73) were excluded from all analyses.

Finally, we transformed the exposure value into a binary variable (i.e. below or above the EU limit value of 50 µg/m³) and calculated the ORs for dying on days > 50 µg/m³ compared with days with PM_{10} levels below that value.

Statistical analyses. Database management and statistical analyses were performed with SAS software (version 9.1; (SAS Institute Inc., Cary, NC, USA). We used conditional logistic regression to evaluate the case-crossover data and to estimate the odds of all-cause and cause-specific infant mortality by exposure to PM_{10}. Results are presented as ORs with 95% confidence intervals (CIs) per 10 µg/m³ increment in PM_{10} concentration or as the OR for days above the EU limit value of 50 µg/m³ against days below that value. We calculated the population attributable fraction (PAF) as in Steenland and Armstrong (2006). All tests were two-sided with $\alpha = 0.05$.

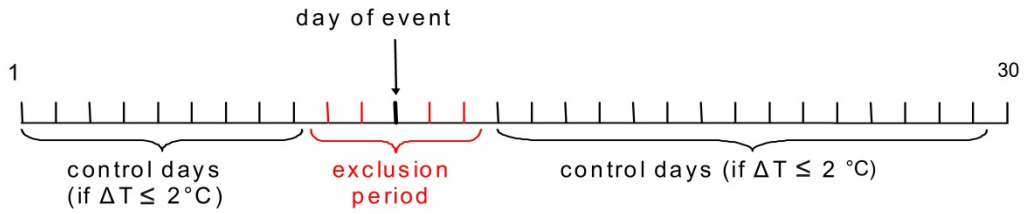

Figure 1. Bidirectional time-stratified case-crossover design. The timeline represents one calendar month. Only control days that were temperature-matched within 2°C with the day of event, were selected.

RESULTS

Descriptive Data

Of the 2,455 infants who died in Flanders during the study period (1998-2006; yielding a mortality of 4.67/1,000 live births), 2,382 died from nontraumatic causes, and 1,284 infants (54%) had been born before 37 weeks of gestation. **Table 1** lists ages at death and causes of death. During the study period, PM_{10} concentration averaged 31.9 ± 13.8 µg/m³ (**Figure 2**) and 321 days (an average of 35.7 days/year) had a mean daily concentration > 50 µg/m³ (population-weighted daily average for the whole region). For cases (n=2,382), the average exposure was 32.6 µg/m³ (95% CI, 15.1-59.9) and on the selected control days (n=20448) PM_{10} averaged 30.7 µg/m³ (95% CI, 14.8-56.5). Interpolated daily average PM_{10} concentrations were strongly correlated among the 308 municipalities in the study area. Correlations ranged from 0.87 to 1.00 and the strongest correlations were among neighbouring municipalities.

Case-crossover analysis

For the whole group, we found a 4% increase (95% CI, 0-8 %, p = 0.045) in the risk of death for each 10 µg/m³ increase in the concentration of PM_{10} on the event day (lag day 0) (**Table 2**). In the sensitivity analyses with up to 3 lag days or moving-average concentrations, mortality tended to be positively associated with PM_{10} as well, but these associations were not significant (data not shown). Estimates from analyses with control days matched on day of the week were comparable to those with control days matched on temperature (data not shown). Therefore, here we report only results for exposure to PM_{10} on the event day compared with temperature-matched control days.

Table 1. Non-traumatic causes of death in neonates in Flanders 1998-2006, by age class.

Cause of death (ICD-10 code)	Early neonatal (≤ 7 days of age)	Late neonatal (8 to 28 days of age)	Postneonatal (29 to 365 days of age)	Total
Cardiorespiratory diseases (I00 – J99)	3	3	44	50
Perinatal circumstances (P00 – P96)	771	197	126	1094
Congenital and chromosomal abnormalities (Q00 – Q99)	398	140	205	743
SIDS (R95)	0	0	285	285
Others	24	22	164	210
Total	1196	372	814	2382

Stratification by age class revealed stronger associations with deaths between 2 and 4 weeks of age (late neonates) than with deaths during other time periods. Specifically, a 10 μg/m³ increase in mean daily PM_{10} on the event day was associated with an 11% increase (95% CI, 1- 22%; p = 0.028) in the risk of late neonatal death. In contrast we found no evidence of effects of PM_{10} on early neonatal or postneonatal mortality. Stratified analyses revealed no significant differences in associations between PM_{10} and daily mortality among preterm versus term births (p-values for interaction ≥ 0.09, **Table 2**), although ORs were always higher for the latter group.

We further analyzed the relation between air pollution and mortality according to cause of death (**Table 3**). In the total group, we found no significant associations between PM_{10} and mortality from cardiorespiratory diseases or SIDS but significant associations in cases where the cause of death was perinatal circumstances. For late neonatal deaths, the associations were driven mainly by the group with congenital and chromosomal abnormalities (**Table 3**).

Analyses that took the EU limit value of 50 µg/m³ as a cutoff point revealed a non-significant OR for the whole group but a highly significant result for late neonatal mortality, with an OR for dying on days with PM_{10} > 50 µg/m³ of 1.74 (95% CI, 1.18-2.58; p = 0.006), compared with days below the cutoff value (**Table 2**). The corresponding AF was 43% (15-61%). When we stratified the analysis with the EU limit value by cause of death, the highest OR (for congenital and chromosomal abnormalities) just missed significance. For other causes of death, results were also not significant. For late neonatal deaths, however, stratification by cause of death revealed a highly significant result for congenital and chromosomal abnormalities (p=0.009) (**Table 3**).

In all settings, subanalyses of congenital malformations of the circulatory or respiratory system (ICD-10 codes Q20-Q28 and Q30-Q34, respectively) revealed similar ORs as in the whole group of congenital and chromosomal abnormalities (Q00-Q99), but because of a smaller sample size, these results did not reach statistical significance.

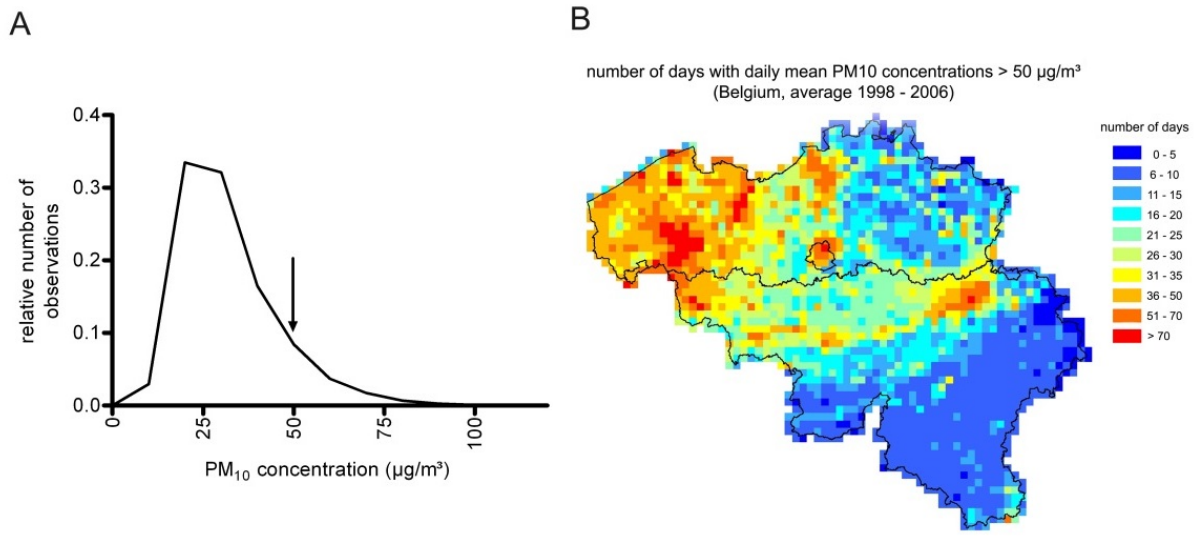

Figure 2. A) Frequency distribution of population-weighted daily mean PM_{10}-concentrations in Flanders (Belgium) during the study period (1998-2006). The arrow indicates the EU limit that may be exceeded up to 35 days per year. B) Spatial distribution of population-weighted daily mean PM_{10}-concentration, expressed as number of days with concentration > 50 µg/m³ (map of Belgium; the Flemish Region comprises the area north of the black line excluding the capital region of Brussels in the centre of the country).

Table 2. Risk of infant death associated with a 10 µg/m³ increase in PM_{10} on the event day and with ambient PM_{10} concentrations above 50 µg/m³, stratified by age category.

Age category	All (N = 2382)	Preterm (N = 1284)	Term (N = 1086)	P interaction[a]
OR for 10 µg/m³ increase in PM_{10} on event day				
All	1.04 (1.00 to 1.08)*	1.03 (0.98 to 1.08)	1.05 (0.99 to 1.11)	0.62
Early neonatal	1.04 (0.99 to 1.10)	1.03 (0.96 to 1.10)	1.07 (0.97 to 1.19)	0.49
Late neonatal	1.11 (1.01 to 1.22)*	1.10 (0.97 to 1.24)	1.13 (0.98 to 1.31)	0.77
Post neonatal	1.01 (0.95 to 1.07)	0.99 (0.88 to 1.10)	1.02 (0.94 to 1.10)	0.67
OR for days above 50 µg/m³ vs. days below 50 µg/m³ [b]				
All	1.10 (0.94 to 1.29)	0.96 (0.76 to 1.20)	1.27 (1.01 to 1.61)*	0.09
Early neonatal	0.99 (0.78 to 1.24)	0.92 (0.69 to 1.22)	1.14 (0.75 to 1.74)	0.40
Late neonatal	1.74 (1.18 to 2.58)**	1.47 (0.87 to 2.48)	2.09 (1.15 to 3.79)*	0.38
Post neonatal	1.04 (0.79 to 1.37)	0.74 (0.43 to 1.27)	1.18 (0.86 to 1.63)	0.14

Data are ORs with 95% CI. * P≤0.05 and **P≤0.01.

[a] Interaction between exposure and maturity at birth, with preterm birth defined as born before 37 weeks of gestation

[b] Based on EU limit value.

Table 3. Risk of infant death associated with a 10 μg/m³ increase in PM_{10} on the event day and with ambient PM_{10} concentrations above 50 μg/m³, stratified by cause of death.

Cause of death (ICD-10)	All (N=2382)	Early neonatal (N=1196)	Late neonatal (N=372)	Post-neonatal (N=814)
	OR for 10 μg/m³ increase in PM_{10} on event day			
Total	1.04 (1.00 to 1.08)*	1.04 (0.99 to 1.10)	1.11 (1.01 to 1.22)*	1.01 (0.95 to 1.07)
Cardiorespiratory diseases	0.98 (0.78 to 1.25)	na	na	0.98 (0.76 to 1.26)
Perinatal circumstances	1.06 (1.00 to 1.12)*	1.06 (1.00 to 1.14)*	1.09 (0.95 to 1.25)	1.01 (0.86 to 1.19)
Congenital and chromosomal abnormalities	1.04 (0.97 to 1.12)	1.00 (0.91 to 1.11)	1.16 (1.00 to 1.35)*	1.04 (0.90 to 1.20)
SIDS	0.99 (0.89 to 1.09)	na	na	0.99 (0.89 to 1.09)
	OR for days above 50 μg/m³ vs. days below 50 μg/m³ [a]			
Total	1.10 (0.94 to 1.29)	0.99 (0.78 to 1.24)	1.74 (1.18 to 2.58)**	1.04 (0.79 to 1.37)
Cardiorespiratory diseases	0.80 (0.28 to 2.25)	na	na	0.93 (0.32 to 2.71)
Perinatal circumstances	1.00 (0.78 to 1.28)	0.96 (0.72 to 1.29)	1.36 (0.77 to 2.38)	0.77 (0.34 to 1.72)
Congenital and chromosomal abnormalities	1.30 (0.98 to 1.74)	1.03 (0.69 to 1.54)	2.32 (1.24 to 4.34) **	1.38 (0.78 to 2.43)
SIDS	0.94 (0.60 to 1.48)	na	na	0.88 (0.55 to 1.41)

Data are ORs with 95% CI. * P≤0.05 and **P≤0.01.

na: not applicable due to low numbers in the specified age class.

[a] Based on EU limit value.

Analyses stratified by SES (low-, medium-, or high-SES municipality) were consistent with those estimated for the population as a whole, although ORs within SES categories were nonsignificant. ORs did not differ substantially among SES categories, as indicated by the nonsignificant interaction terms in all analyses (data not shown).

In the group of late neonates, fractional polynomial analysis revealed that a linear model adequately describes the association between infant mortality and air pollution, with no evidence for a threshold or plateau (Likelihood Ratio test for a linear model vs. a null model, p = 0.030) (**Figure 3**). More complex fractional polynomials did not significantly improve the fit of the model, according to AIC.

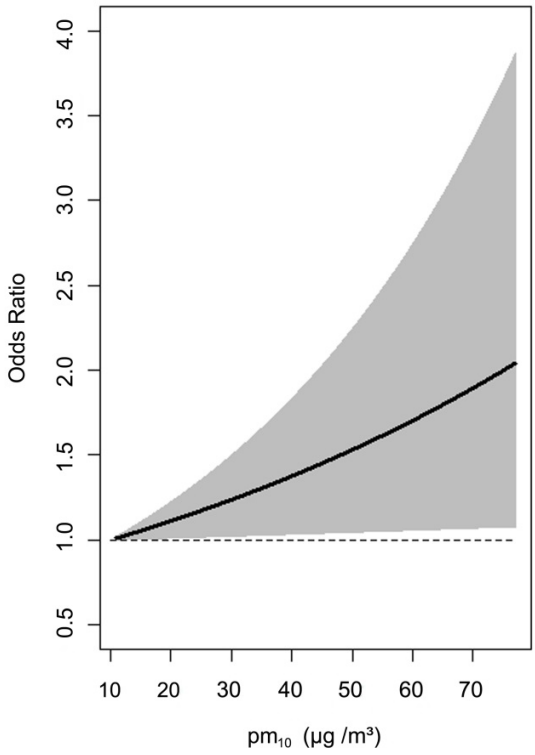

Figure 3. Shape of the association between exposure to PM_{10} and risk of mortality in late neonates, expressed as estimated OR (with 95% CI, the grey area), using fractional polynomials and 10 µg/m³ as reference; 77 µg/m³ is the 99th percentile of exposures during the study period.

DISCUSSION

The key finding of our study was that PM air pollution, expressed as PM_{10}, is associated with late neonatal mortality, thus suggesting that airborne particles act as a rapid trigger of infant death. On days with average PM_{10} levels exceeding the EU limit value of 50 µg/m³ – which is allowed to be exceeded on 35 days per year – the odds for late neonatal mortality was 1.74 times higher than on days below that value. Assuming causality, these results imply that on days above the EU limit value of 50 µg/m³, 43% (the AF) of late neonatal mortality could be triggered by an acute increase in fine PM air pollution levels on the same day. The shape of the association between the risk of late neonatal mortality and PM_{10} (**Figure 3**) gives no evidence for a threshold, thus suggesting the risk exists even at < 50 µg/m³. Analyses of lagged exposures suggested that exposure on the event day was more important than exposure during the 3 days preceding the event.

Most publications on infant mortality and PM air pollution have used a time-series approach. The case-crossover design represents a relatively novel approach to study acute health effects. It was developed in the early 1990s by Maclure (1991) to study effects of brief exposures on the change in risk of acute and discrete events, such as myocardial infarction. Recently, the case-crossover design has been applied to assess effects of short-term changes in exposure to air pollution (e.g. Romieu et al. 2004; Son et al. 2008; Tsai et al. 2006; Yang et al. 2006; Zanobetti and Schwartz 2005; for review, see Carracedo-Martinez et al. 2010).

The major power of the case-crossover approach is the ability to control for confounding. All the study subjects have experienced the event. The hazard period is defined as the average time period that is relevant for the acute event, and this period is compared with control times. Thus, subjects serve as their own controls at an individual level. In contrast, the traditional time-series studies cannot control for varying individual characteristics because the unit of observation consists of daily counts of the event rather than of individuals. By matching for outdoor temperature, we excluded temperature as a potential confounder

in our models, and because control days were close to event days, we controlled for seasonal effects as well (Bateson and Schwartz 1999, 2001; Maclure and Mittleman 2000). The time-stratified design for the selection of control days, as applied in our study, has been shown to be the best selection method to avoid statistical bias (Janes et al. 2005; Mittleman 2005).

So far, only five case-crossover studies on infant mortality and air pollution have been published, conducted in the cities of Seoul (South Korea) (Son et al. 2008), Kaohsiung (Taiwan) (Tsai et al. 2006), Taipei (Taiwan) (Yang et al. 2006), Ciudad Juárez (Mexico) (Romieu et al. 2004), and Mexico City (Mexico) (Carbajal-Arroyo et al. 2010). Apart from the latter, they found no short-term association between postneonatal mortality and air pollution (ORs were 1.00 or 1.01 for an increase of 10 μg/m³). In contrast to these studies, which exclusively dealt with postneonatal mortality (> 1 month of age), we also included neonates in our analysis. We observed no evidence of an association between PM_{10} and postneonatal mortality either, but we estimated a significant positive association between a 10 μg/m³ increase in PM_{10} and mortality on the same day for all age classes combined, that was almost entirely attributable to an association between PM_{10} and mortality during the late neonatal period (2-4 weeks after birth).

In both studies performed in Mexico (Carbajal-Arroyo et al. 2010; Romieu et al. 2004), the risk of death was significantly higher in infants from low - and/or medium - SES areas than in those from high SES areas. We found no difference in ORs among municipalities classified according to SES. Because of privacy restrictions, we were not able to classify SES on an individual level, but for the present, we conclude that SES does not modify the association between PM exposure and infant death in the study region.

We found no indications for a role of PM_{10} in infants who died from cardiorespiratory complications or SIDS. Earlier studies on the association between exposure to PM and SIDS yielded mixed results (Glinianaia et al. 2004; Tong and Colditz 2004), although our results for cardiorespiratory deaths may be unreliable because of the very small sample size. In the present study, we estimated the highest ORs for deaths attributed to congenital malformations and perinatal circumstances, but only the latter

proved to be significant for the whole study population and only the former for deaths among late neonates.

We did not find clear evidence of differences between term and preterm births, and we did not detect a significant association between air pollution and early neonatal mortality during the first week of life. Reasons for this might be that the most susceptible children die during the first week of life because of conditions that do not need to be triggered by air pollution, or that the measured outdoor air pollution does not reflect actual exposure during the first week of life (or during the first month for premature infants), because most of these newborns probably would have remained in the hospital during this time. However, we had no access to data on the duration of hospitalization after birth to verify this hypothesis.

In this context, a limitation of our study is the use of outdoor measurements of air pollution with interpolations at the municipality level in order to estimate partly indoor personal exposures. However, recent studies (Janssen et al. 2005; Williams et al. 2000) comparing personal and ambient exposure have reported good correlations among day-to-day changes in central measurement stations of PM and personal exposure. In addition, we found very high correlations (ranging from 0.87 to 1) among municipalities for the interpolated PM_{10} levels. In other words, spatial variability in PM_{10} (which is rather low in our small study area) appeared to be less important than temporal variability, which is driven largely by weather conditions. During stable meteorological conditions with low wind speeds, and in the presence of a temperature inversion, locally produced pollution accumulates in the lower parts of the atmosphere, which results in a cloud of dust inhaled by humans.

In their comprehensive review, Pope and Dockery (2006) discuss several plausible biological pathways for the relationship between exposure to PM and health. They derived evidence for these pathways mainly from observations on adults or experiments on animals, but at least some of the proposed mechanisms, such as pulmonary or systemic inflammation and modulated immunity, are likely to explain adverse health effects in infants as well, because their lungs, heart and immune system are

immature and fragile. In particular, there is growing evidence that ambient air pollution is associated with decreased heart rate variability (HRV) in adults (Pope and Dockery 2006) and reduction in HRV is a plausible biological mechanism in infant deaths as well (Patzak 1999).

We did not find significant associations between PM_{10} and cardiorespiratory diseases as the official cause of death, but the number of children in this group was very low, which in turn might be the consequence of misclassification on death certificates [subtle mechanisms as systemic inflammation or HRV are presumably more easily overlooked than perinatal or congenital abnormalities, see Nembhard et al. (2008) and references therein for examples of misclassification of cardiovascular diseases]. Hence, there is clearly a need for further research in order to understand the underlying mechanisms of the observed associations between air pollution and mortality in infants, as well as a better differentiation between acute and chronic effects of air pollution in this segment of the population.

CONCLUSIONS

Our study shows that air pollution standards have to be taken more seriously. We estimated that 43% of mortality during the late neonatal period may be triggered by peaks of $PM_{10} > 50$ µg/m³. We do not claim that air pollution was the major, let alone the only cause of death in these infants, but our data suggest that air pollution may precipitate death in infants with pre-existing conditions. A trigger is not necessarily the primary cause of death, but it may increase the risk of death in susceptible infants, such as infants with perinatal complications or other pre-existing conditions.

European regulation, which currently uses standards that are considerably higher than those of the WHO (2006), stipulates that we may have a maximum of 35 days/year with a mean $PM_{10} > 50$ µg/m³ [comparable to the U.S. Environmental Protection Agency (2011) standard for $PM_{2.5}$ of 35 µg/m³ (~46 µg/m³ PM_{10})]. In Belgium, this standard is barely met at present, and although minor improvements due to emission reduction measures are expected, the limit value of 50 µg/m³ will continue to be exceeded frequently in the coming decade. The same is largely true for other European regions, including northern

France, the southern part of the Netherlands, the German Ruhr area and the Po valley in Italy. The argument that it is difficult to meet standards in densely populated areas ignores the fact that the importance of a factor with respect to public health increases in proportion to the number of people who are exposed to it.

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CHAPTER 2

LYMPHOCYTIC BRONCHIOLITIS AFTER LUNG TRANSPLANTATION IS ASSOCIATED WITH DAILY CHANGES IN AIR POLLUTION

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[*] Hans Scheers and Stijn Verleden share first authorship for the paper entitled 'Lymphocytic bronchiolitis after lung transplantation is associated with daily changes in air pollution'. Hans Scheers was responsible for data management, statistical analysis and writing of the study. Stijn Verleden was responsible for the concept, data collection and writing of the study.

ABSTRACT

Acute rejection represents a major problem after organ transplantation, being a recognized risk for chronic rejection and mortality. Recently, it became clear that lymphocytic bronchiolitis (LB, B-grade acute rejection) is more important than previously thought, as it predisposes to chronic rejection. We aimed to verify whether daily fluctuations of air pollution, measured as particulate matter (PM) are related to histologically proven A-grade rejection and/or LB and bronchoalveolar lavage (BAL) fluid cellularity after lung transplantation.

We fitted a mixed model to examine the association between daily variations in PM_{10} and A-grade rejection/LB on 1,276 bronchoscopic biopsies (397 patients, 416 transplantations) taken between 2001 and 2011. A difference of 10 $\mu g/m^3$ in PM_{10} 3 days before diagnosis of LB was associated with an OR of 1.15 (95%CI 1.04-1.27; p=0.0044) but not with A-grade rejection (OR=1.05; 95%CI 0.95-1.15; p=0.32). Variations in PM_{10} at lag day 3 correlated with neutrophils (p=0.013), lymphocytes (p=0.0031) and total cell count (p=0.024) in BAL. Importantly, we only found an effect of PM_{10} on LB in patients not taking azithromycin. LB predisposed to chronic rejection (p<0.0001).

The risk for LB after lung transplantation increased with temporal changes in particulate air pollution, and this was associated with BAL neutrophilia and lymphocytosis. Azithromycin was protective against this PM effect.

INTRODUCTION

Survival after lung transplantation (LTx), a last resort for selected patients with end-stage lung disease, has tremendously improved since 1963 (1). Survival, however, is still hampered by the development of chronic rejection or its clinical correlate bronchiolitis obliterans syndrome (BOS), as the lung experiences one of the highest rejection rates among solid organ transplantations (2). The higher susceptibility of lungs to be rejected could conceivably follow from its direct contact with the environment. Recently, we demonstrated that chronic exposure to (traffic-related) air pollution, indirectly measured by proximity of the address of residence of each patient to a major road, increases the risk of chronic rejection and mortality in LTx patients (3).

As a consequence, we hypothesized that daily variations in air pollution ('acute exposures') may also affect the outcome of LTx. Various studies demonstrated that exposure to ambient particulate matter (PM) can contribute to pulmonary and systemic inflammation, causing the release of inflammatory mediators, and resulting in oxidative stress and accumulation of inflammatory cells like neutrophils (4), comparable to what happens during lymphocytic bronchiolitis (LB), the pathological correlate of acute graft rejection. In the early years, not much attention was given to LB until 2008 when Glanville reported a significant association between the occurrence of LB and the prevalence of chronic rejection (5).

A-grade rejection, pathologically characterized by perivascular lymphocytic inflammation, is a common phenomenon, especially in the early phase after LTx: up to 40 % of the patients experience at least one episode within the first year (1). Some established risk factors for A-grade rejection include human leukocyte antigen mismatching, low immunosuppressive trough levels, recipient age and genetics (6). External parameters, such as gastroesophageal reflux (7) may also impact on A-grade rejection, but the exact trigger remains unknown. In LTx patients, both A-grade rejection and LB (B-grade rejection) are associated with higher total cell count and an increased bronchoalveolar lavage (BAL) neutrophilia compared to transplanted controls (8).

We, therefore, evaluated all lung biopsies taken from patients in our LTx cohort since 2001 up to May 2011 and correlated histological features indicative of A-/B-grade rejection with the daily PM_{10} values at the home address up to 7 days preceding the biopsy. In a further analysis, we included systemic and airway inflammation, the potential protective effect of azithromycin and, finally, we verified the effect of possible covariates such as socio-economic status (SES), age, gender, underlying disease, daily temperature, ischemic time, postoperative day (POD), date of transplantation and immunosuppressive regime. We speculated that daily changes in air pollution are associated with lymphocytic airway inflammation and BAL neutrophilia.

MATERIALS AND METHODS

Study design

In a prospective, observational study, we analyzed all bronchoscopic procedures with transbronchial/endobronchial biopsies (TBB/EBB) and BAL, performed between October 2001 and May 2011, in 397 LTx recipients (416 transplantations). This led to a total final study cohort of 1,276 biopsies. The study was approved by the Hospital Ethical Board (S51577). All patients gave written informed consent.

Patient based data

Scheduled bronchoscopy with TBB and BAL was performed 1 and 3 months post transplantation and diagnostic bronchoscopy was done whenever acute rejection, infection or chronic rejection was suspected. In addition, EBB has been introduced in the follow-up at day 180, 360, 540 and 720 since January 2011.

The term lymphocytic airway disease (LAD) was introduced to describe LB diagnosed with TBB as well as LB identified on EBB. Diagnosis of A-grade rejection/LAD was based on evaluation of TBB/EBB. When airways were not adequately sampled on TBB, EBB was used to score airway inflammation. When both were available and there was a discrepancy in degree of airway

inflammation, the highest score was used. Upon diagnosis of both A-grade rejection and LAD on one TBB, both events were interpreted as separate events and hence separately analysed.

TBB and BAL were performed according to ISHLT guidelines (9). EBB were taken at the level of the segmental carina of the lower lobe, middle lobe or lingula. For BAL, classical methodology was used for total and differential cell counting. IL-6/IL-8 were measured using ELISA (3). Plasma C-reactive protein (CRP) levels were routinely measured at the University Hospital laboratory (Tina-quant CRP latex assay, Roche, Vilvoorde, Belgium).

BOS was defined as a decrease in forced expiratory volume in 1 second (FEV_1) of at least 20% in absence of other identifiable causes, according to ISHLT guidelines (9,10). Spirometry was performed according to ATS criteria (11). Patients with < 6 months of follow-up were excluded from this analysis.

Exposure measurements

Air pollution was quantified by estimating the daily mean values for PM_{10} at each participant's home address, using a validated model (12,13) that provides interpolated values for PM_{10} in 4x4 km grids based on the Belgian telemetric air quality network. The model has shown good agreement between daily model-derived values and measured air pollution.

We used PM_{10} data up to 7 lag days before the biopsy procedure, together with averages of several days as peaks in air pollution lasting for more than one day might provoke more pronounced biological responses. In first instance, we aimed to look at immediate effects of exposure to PM_{10}, that is, PM_{10} levels up to 3 days before the biopsy. We further analyzed PM_{10} levels up to 7 days before the biopsy to check the importance of earlier exposures. When patients were hospitalized during days preceding the biopsy, the PM_{10} levels at the hospitals residence were used for analysis.

Statistical analysis

Data management and statistical analyses were performed in SAS 9.2 (SAS Institute, Cary, NC,USA). For A-grade rejection and LAD, we created binary outcome variables, by contrasting grade 0 to presence of rejection.

Number of cells/concentrations (BAL cell counts; plasma CRP; IL-6 and IL-8) were log-transformed and percentages (% neutrophils and % lymphocytes) were logit-transformed to attain normality.

The relationship between A-grade rejection/LAD and short-term exposure to PM_{10}, and between physiological parameters and PM_{10}, was evaluated by statistical models accounting for repeated biopsies within a patient. For binary outcome variables (A-grade rejection and LAD), we used General Estimating Equations (GEE) in PROC GENMOD with a logit link function, and for continuous outcomes (physiological parameters), we applied a General Linear Mixed Model (GLMM) in PROC MIXED. Results are expressed as odds ratios (OR) with corresponding 95% confidence intervals (CI) per 10 $\mu g/m^3$ increment in PM_{10} concentration or as x-fold increases per doubling PM_{10} concentration. All tests were two-sided with α=0.05, except for the initial analyses for PM_{10} on lag days 0 to 3, where we applied the Holm-Bonferroni test to correct for possible type 1 errors. We additionally performed the analysis with A- grade rejection and LAD as ordinal variables using proc GLIMMIX with a cumulative logit link for an ordinal distribution of the response variable.

We accounted for important covariates in the regression models: age, gender, SES, underlying disease, ischemic time, date of transplantation, type of transplantation, daily temperature, POD, type and trough blood levels of immunosuppressives, azithromycin treatment. We coded SES (1-3) on the basis of occupation and education (3). We defined three types of LTx (single, double and heart-lung), and we studied effect-modification by azithromycin therapy.

A contingency table was used to identify an association between LAD and BOS. Cox regression was used to determine a time dependent effect of LAD on development of BOS.

RESULTS

Patients were excluded if their home address was not situated in Flanders (n=19). Four biopsies were excluded because they were not gradable (n=3) or PM values were not available (n=1). In 19 patients, the first and second (reLTx) transplantation were included as two separate events. In total, 1,276

biopsies were analyzed, of which 273 (21.4%) showed A-grade rejection and 193 (15.1%) displayed LAD; 65 biopsies displayed both A-grade rejection and LAD (5.1%).

Association between A-grade rejection/LB and PM_{10}

Characteristics of biopsies are presented in **Table 1**: of 1,276 biopsies, 431 had been taken as part of routine assessment and 845 for diagnostic purposes. Diagnostic biopsies displayed significantly more A-grade rejection/LAD ($p<0.0001$). Of all biopsy procedures (1,276), 775 were TBB (60.7%), 293 were combined TBB and EBB (23.0%) and 208 biopsies were exclusively EBB (16.3%). TBB and EBB showed in general good agreement regarding the bronchial inflammation ($R=0.70$, $p<0.0001$), although 13 of 293 biopsies (5.8%) showed disagreement in scoring (8 between grade 0 and 1, 4 between grade 0 and 2 and 1 between 1 and 2; EBB scored higher in 8 of 13 cases).

We analyzed daily PM_{10} fluctuations from the day of biopsy (lag0) up to 7 days before biopsy (lag7) separately for A-grade rejection and LAD (**Figure 1**). Of the 1,276 biopsies, 1,022 could be histologically evaluated for A-grade (of which 272 were positive, 26.6 %) and 1,179 for LAD (of which 193 were positive, 16.3%). In univariate analysis, an increase of 10 $\mu g/m^3$ PM_{10} three days before biopsy (lag3) was significantly associated with LAD (OR=1.15; 95%CI 1.04-1.26; p=0.0044), which means that an increase in PM_{10} concentration of 10 $\mu g/m^3$ 3 days before a TBB/EBB increases the risk of LB with 15%. PM_{10} exposure on lag day 2 and the average of 2 and 3 was also significantly associated with LAD (OR= 1.14; 95%CI 1.02-1.26, p=0.015 and OR = 1.17; 95%CI 1.05-1.3; p=0.0049 for an increase of 10 $\mu g/m^3$ PM_{10}). After correction for potential type1 errors using a more stringent α-value, these associations remained significant.

In contrast, the analysis of the association between PM_{10} and A-grade rejection demonstrated no significant ORs per 10 $\mu g/m^3$ PM_{10} increment at lag2 (OR=1.02; 95%CI 0.92-1.12; p=0.76), lag3 (OR=1.05; 95%CI 0.95-1.15; p=0.32), or lag23 (OR=1.04; 95%CI 0.94-1.15; p=0.47).

PM_{10} and BAL cellularity

A doubling in PM_{10} lag3 was associated with a 1.29-fold increase in number of BAL neutrophils (95%CI 1.06-1.59; p=0.013) and with a 1.20-fold increase in their percentage (95%CI 1.01-1.42; p=0.042). For number and percentage of lymphocytes, and BAL total cell count, these numbers were a 1.24-fold increase (95%CI 1.08-1.43; p=0.0031), a 1.10-fold increase (95%CI 0.99-1.22; p=0.063), and a 1.12-fold increase (95%CI 1.01-1.24; p=0.026), respectively (**Figure 2**). There was no association between PM_{10} exposure and total number of macrophages, IL-6, IL-8 and CRP.

Effect of azithromycin treatment

Because neutrophils are important in both A-grade rejection and LAD and seem to be involved in the inflammatory reaction caused by PM_{10}, and because azithromycin is known to reduce BAL neutrophils (14), we performed subgroup analysis with on the one hand patients on azithromycin therapy and on the other hand patients that were not taking azithromycin. In that respect, 300 biopsies were obtained from patients taking azithromycin (23.5%), of which 71 (23.6%) were positive for A-grade rejection and 63 for LAD (21%).

Subdividing the total population according to azithromycin treatment, we found no association between PM_{10}lag3 and LAD (OR=1.04; 95%CI 0.86-1.24; p=0.71) in the cohort taking azithromycin. However, in patients without azithromycin, the association between PM_{10} and LAD was stronger than in the entire cohort (OR=1.19; 95%CI 1.06-1.34; p=0.0030) (**Table 2**). There was no effect of azithromycin on A-grade rejection.

Table 1. Characteristics of 416 transplantations in 397 transplanted patients (19 retransplantations).

	Average (SD)	**N (%)**
Age at transplantation, years	48.4 (13.9)	
Date of first biopsy, dd/mm/yyyy, (SD of year)	23/05/2006 (3.5)	
Female gender		208 (50%)
Underlying disease		
Emphysema (COPD)		201 (48%)
Pulmonary Fibrosis		76 (18%)
Cystic Fibrosis		64 (15%)
Eisenmenger		10 (2%)
Pulmonary Arterial Hypertension		21 (5%)
Obliterative Bronchiolitis		21 (5%)
Others		23 (6%)
Double lung transplantation		334 (80%)
Ischemic time, min	400 (80)	
Socioeconomic status		
Low		194 (47%)
Middle		193 (46%)
High		28 (7%)
Number of biopsies per patient	3.1 (1.6)	
A-grade rejection		
Nr. of positive scores per patient	0.7 (0.9)	
Patients with at least one A≥2		86 (21%)
LAD		
Nr. of positive scores per patient	0.5 (0.8)	
Patients with at least one B≥2		61 (15%)

Data are means (SD) or numbers (%). COPD, chronic obstructive pulmonary disease. In case of retransplantation, both transplantations were considered as separate events.

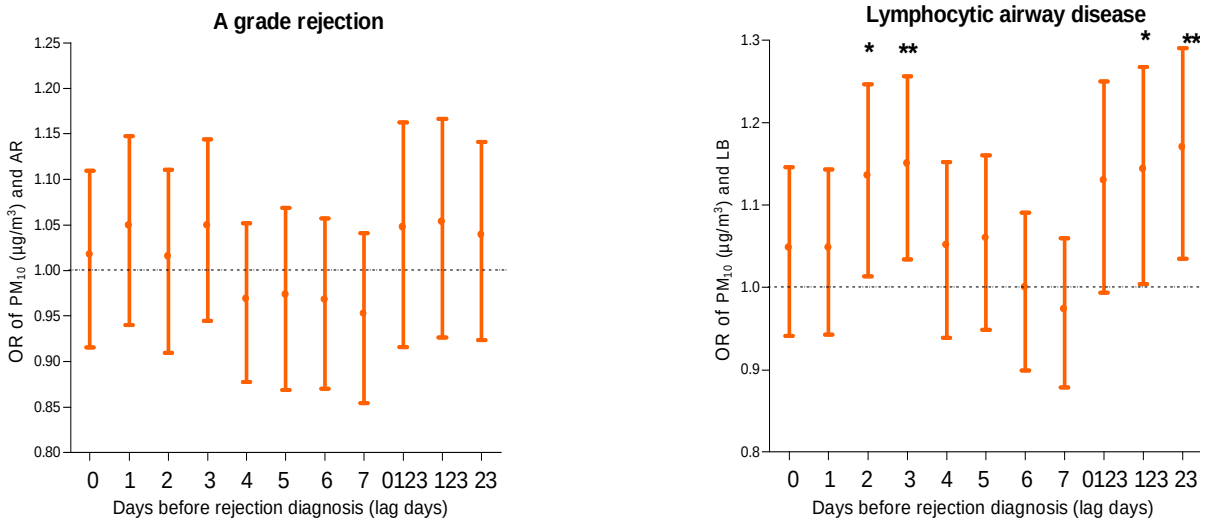

Figure 1. Association between A-grade rejection or lymphocytic airway disease (LAD) and PM_{10}.
A) Odds ratios (±CI) to develop A-grade rejection per difference of 10 µg/m³ PM_{10} as a function of days preceding the biopsy (n=1022).
B) Odds ratios (±CI) to develop LAD per difference of 10 µg/m³ PM_{10} as a function of days preceding the biopsy (n=1179).

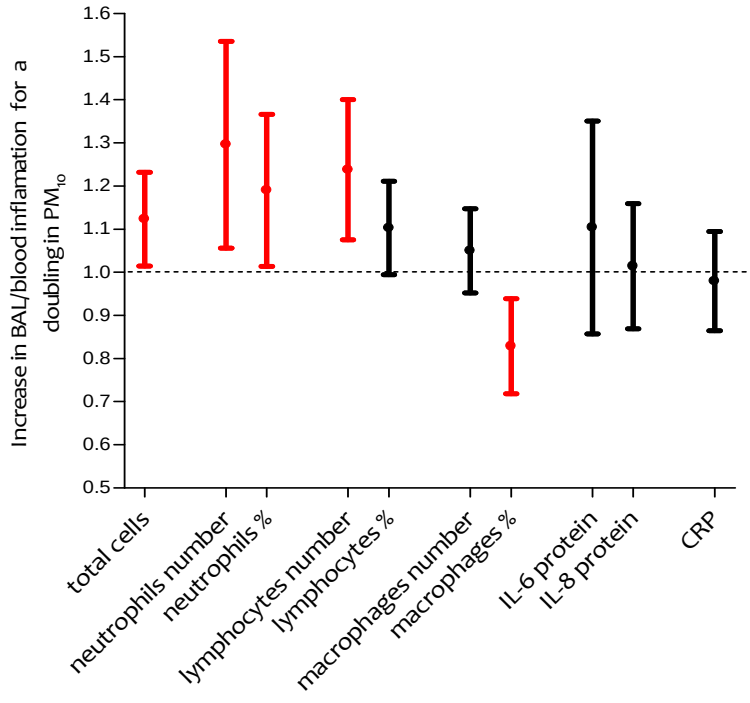

Figure 2. Association between PM_{10} and different BAL cellular characteristics. Multiplicative effect of doubling in PM_{10} concentration on the different studied variables (x-fold increase ± CI). The dotted line indicates no change in concentration or percentage. Red parameter estimates demonstrate a significant association, black a non-significant association.

Covariates

In univariate analysis, type of transplantation (single, double, heart lung; p=0.90 and p=0.23), ischemic time (p=0.06 and p=0.19), SES (p=0.75 and p=0.17), and underlying disease (p=0.14 and p=0.16) were not associated with A-grade rejection and LAD, respectively.

Earlier date of transplantation and longer interval between transplantation and biopsy (POD) were significantly associated with both a higher risk of A-grade rejection and LAD (all p<0.001) and higher levels of PM_{10} (both p<0.001). Moreover, in LAD but not in A-grade rejection, immunosuppressive trough levels of tacrolimus (p=0.016 and p=0.22) and cyclosporine (p=0.05 and p=0.67) proved to be important. More details about the covariate analysis can be found in **Table 3**.

We entered gender, age and the significant covariates (immunosuppressive trough level, time of transplantation and POD) in a multivariate mixed model, in addition to PM_{10} and atmospheric temperature. The significant association between PM_{10} and LAD that we found in the univariate analysis, was borderline significant in the adjusted analysis. When stratified for azithromycin treatment, the association remained apparent in patients who did not take azithromycin (**Table 2**). For A-grade rejection, adjustment for covariates did not alter the results of the crude analyses: no association between PM_{10} and A-grade rejection was found, neither in the whole patient population, nor when stratified for azithromycin treatment.

When analyzing LAD as an ordinal variable instead of a binary value, the association between LAD and PM_{10} remained significant in the patients not taking azithromycin after adjustment for all covariates (p=0.033, OR 1.16; 95%CI 1.01-1.32).

TBB versus EBB

We performed a subgroup analysis and compared the effect of PM_{10} on TBB (734 biopsies) and EBB (361 biopsies) separately. The observed effect seems to be predominantly present when analyzing the B-grade on TBB, as in these biopsies from patients not taking azithromycin, we found a significant association after adjustment for covariates (p=0.032; OR 1.16; 95%CI 1.01-1.34), however, this was not significant when analyzing EBB seperately (p=0.54; OR 1.10; 95%CI 0.81-1.49).

Table 2. Relation between exposure to PM_{10} three days before biopsy (lag 3) and acute rejection.

	A- grade rejection				LAD			
All observations	N	OR	95% CI	p	N	OR	95% CI	p
Unadjusted	1022 (272) †	1.05	0.95-1.15	0.32	1179 (193)	1.15	1.04-1.27	0.004
Adjusted for covariates *	1003 (265) ‡	0.99	0.89-1.11	0.91	1162 (189)	1.12	1.00-1.25	0.049
Patients on azithromycin	N	OR	95% CI	P	N	OR	95% CI	P
Unadjusted	223 (71)	1.04	0.85-1.28	0.67	285 (63)	1.04	0.86-1.24	0.71
Adjusted for covariates *	221 (70)	1.02	0.81-1.29	0.87	282 (63)	1.01	0.80-1.27	0.96
Patients without azithromycin	N	OR	95% CI	P	N	OR	95% CI	P
Unadjusted	799 (201)	1.04	0.94-1.15	0.44	894 (130)	1.19	1.06-1.34	0.003
Adjusted for covariates *	782 (195)	0.98	0.87-1.10	0.68	880 (126)	1.16	1.02-1.32	0.028

OR is the odds ratio for a change of 10 $\mu g/m^3$ PM_{10}

* Adjusted for gender, age, temperature, transplantation date, post-operative day and trough levels of immunosuppressives.

† Numbers are total numbers of biopsies (biopsies with positive rejection score)

‡ Numbers in adjusted analyses are slightly smaller due to some missing data in immunosuppressive trough level

Table 3. Univariate analysis to determine covariates for multivariate analysis

	A- grade rejection				LAD			
Patient-related covariates	N	OR	95% CI	p	N	OR	95% CI	p
Age (per year increase)	1023 (273)	0.98	0.97-0.99	0.0004	1180 (193)	0.99	0.97-1.00	0.053
Gender (M vs. F)	1023 (273)	0.92	0.68-1.25	0.59	1180 (193)	1.06	0.75-1.50	0.73
SES (3 classes)	1023 (273)	Several ORs *		0.96	1180 (193)	Several ORs*		0.44
Tx-related covariates	N	OR	95% CI	P	N	OR	95% CI	P
Underlying disease (7 classes)	1023 (273)	Several ORs*		0.14	1180 (193)	Several ORs*		0.17
Type of Tx (double vs. single)	1023 (273)	1.03	0.70-1.50	0.90	1180 (193)	1.40	0.80-2.45	0.23
Date of Tx (per year increase)	1023 (273)	0.93	0.86-0.96	0.0003	-	0.86	0.83-0.93	<.0001
Ischemic time	737 (200)†	1.17	0.99-1.37	0.058	869 (148)	1.09	0.96-1.25	0.19
Biopsy-related covariates	N	OR	95% CI	P	N	OR	95% CI	P
Cyclosporin (per 10mg/l)	244 (65)‡	0.99	0.96-1.03	0.67	269 (40)	0.97	0.93-1.00	0.047
Tacrolimus (per mg/l)	760 (201)‡	0.98	0.94-1.01	0.22	894 (149)	0.93	0.88-0.99	0.016
POD (per month increase)	1023 (273)	1.00	0.99-1.01	0.65	1180 (193)	0.99	0.98-0.99	<.0001
Daily temperature (per °C)	1023 (273)	1.00	0.98-1.02	0.91	1180 (193)	1.00	0.97-1.02	0.74

* Class variables had n!/2 pairwise comparisons (with n the number of classes). The p-value shown is that of the class variable as a whole.

† N for ischemic time is considerably lower due to missing values.

‡ Patients received either cyclosporine or tacrolimus as immunosuppressive medication.

LAD and infection

Of the 1,276 biopsy procedures, 84 (6.5%) were performed during infectious episodes, characterized by growth of Aspergillus, Pseudomonas, cytomegalovirus (CMV) or less common pathogens in BAL, abnormalities on CT scan and clinical symptoms like fever, combined with an increase in blood CRP level. All these episodes needed appropriate treatment. The mean blood CRP and BAL IL-8 level related to these procedures was significantly higher compared to noninfectious episodes (36.3 mg/L vs 10.6 mg/L, p<0.0001 and 167 pg/mL vs 96 pg/mL, p<0.0001). Of these 84 biopsies, 25 demonstrated LAD (29.6%), indicating that infection might also have contributed to LAD. When we excluded these 84 biopsies from the total analysis, the association between PM_{10} and LAD remained significant (p=0.015, OR 1.14, 95%CI 1.03-1.26)**.**

LAD and BOS

BOS could be evaluated in 381 patients (>6 months of follow up). In total, 130 patients (34%) were diagnosed with BOS of which 50 (38.4%) had a biopsy-proven LAD before BOS diagnosis. In the patients without BOS, only 47 of 251 patients had a biopsy-proven LAD (18.7%). The prevalence of BOS was significantly higher in patients with more LAD compared to the group that experienced no LAD during their follow-up (p<0.0001).

The median BOS-free time period after transplantation was significantly lower in the group with LAD compared to the control group (5.7 year vs. 8.1 year, p=0.0089) (**Figure 3**). The hazard ratio resulting from a cox regression method was 1.57 (95% CI 1.11-2.20; p=0.010), indicating that patients with at least one LAD event were 1.57 times more likely to develop BOS than patients with no LAD events. This association remains significant when looking at the 189 patients who only underwent EBB. Indeed, 24 of 138 (17%) patients without BOS had LAD on EBB, compared to 22 of 51 (43%) patients with later development of BOS (p=0.0005).

Finally, patients with BOS underwent a mean of 3.5 biopsy procedures per transplantation, whereas patients without BOS only underwent a mean of 2.8 biopsy procedures (p=0.0008).

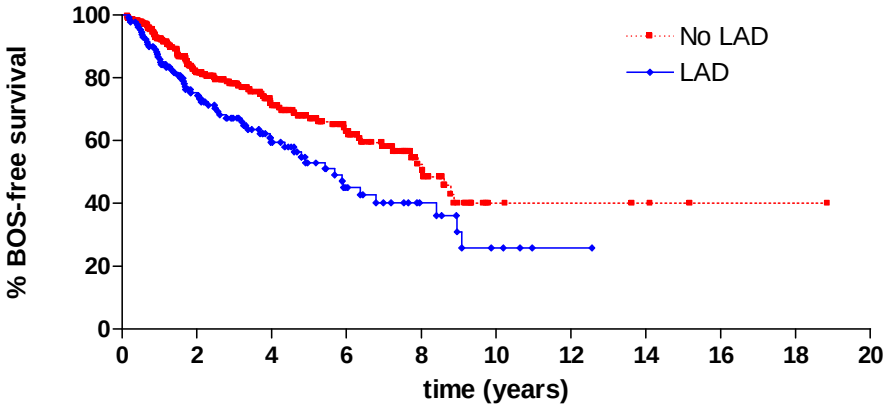

Figure 3. Patients experiencing at least one event of LAD during their follow-up after LTX had a worse BOS-free survival compared to patients that never experienced LAD.

DISCUSSION

In this paper, we found a significant association between LAD after LTx, and recent exposure to particulate air pollution (measured as PM_{10}). We provided evidence that a 10 µg/m³ increase of PM_{10} is associated with a higher chance of LAD (15%), diagnosed 2-3 days after this PM_{10} increase. We found no significant effect of PM_{10} on the development of A-grade rejection.

Moreover, PM_{10} was also associated with increased BAL neutrophils and lymphocytes. This demonstrates the potential role of anti-neutrophilic therapy, confirmed by the results for azithromycin, which appeared to block the effect of PM_{10} on LAD. Indeed, only in patients not taking azithromycin, the effect of PM_{10} was demonstrable as the risk for airway inflammation per 10 µg/m³ PM_{10} increased to 19%. Our results proved to be robust, as the findings remained significant when we took several covariates into account.

The prevalence of LAD was much higher in patients who later on developed BOS compared to those who remained stable, hence confirming the association between LAD and BOS, which has been described previously (5). This might be explained by the high BAL neutrophilia found during LAD (8), which, as already demonstrated in other studies, is an important risk factor towards the development of BOS (15). Our study suggests that at least part of the LAD and BAL neutrophilia is explained by exposure to PM_{10}. Hence, acute exposures to PM_{10} predisposes indirectly towards the development of

BOS. Consequently, these data further strengthen our previous study in which we demonstrated the effect of chronic exposure (=spatial effect) to traffic-related air pollution on the development of BOS and mortality in LTx patients (3). Patients with BOS underwent a higher number of biopsies compared to patients without BOS. It is well known that acute rejection and LAD (5,16) are major risk factors for the development of BOS (also corroborated by this study), hence patients probably had more clinical indications to undergo an extra biopsy.

It is remarkable that only 14.5% of the biopsies displayed LAD, while other centers found a positive score in almost 50% of their biopsies (5, 17). This could be explained by our clinical routine follow up, as we only take routine biopsies on day 21 and day 90, otherwise only when clinically indicated. In this way, it is very much possible that we miss a lot of LAD as it might manifest subclinically. Moreover, many patients are already under routine azithromycin treatment at day 90, which might interfere with the development of LAD as it decreases (neutrophilic) airway inflammation.

Time of transplantation proved to be important as patients transplanted more recently, experienced less rejection. This time trend may be due to the increased expertise gained over time, as well as the introduction of azithromycin. However, another possible explanation is the slightly declining PM_{10} level over the last years. Age was also an important predictor, especially for A-grade rejection, in agreement with previous data (18).

Epidemiological research already demonstrated clear associations between acute and chronic exposure to particulate air pollution and respiratory symptoms like cough, wheezing, shortness of breath, exacerbations of asthma/chronic obstructive pulmonary disease, increased use of rescue medication, respiratory infections and reduction of peak flow rate (19). Biomarkers of airway and systemic inflammation and oxidative stress have already been linked with exposure to PM, but direct sampling in the lung (using BAL and biopsies) remains problematic as it is not always acceptable and much more invasive. Up to now, an association between PM_{10} exposure and histological diagnosis, as we did in this study, has never been demonstrated. Only animal studies and some human studies, including only low numbers of subjects, attempted to link PM with airway neutrophilia in healthy adults

(20,21). In most cases the investigators used very high, acute exposures to PM, whereas we investigated the effect of real life levels in Flanders, Belgium, over several years.

Admittedly, our lung transplant population is a very specific one and a healthy individual can react differently from a vulnerable one (22). The LTx setting is unique to study the possible effects of PM_{10}, as the routine follow-up in our center includes repeated bronchoscopies (with biopsies and BAL), thus providing an opportunity to study the PM_{10} exposure in relationship to histological signs of inflammation in a large cohort. Moreover, we also have the results of BAL data, which provides us with information about the cellular inflammation within the airway lumen.

For many respiratory diseases, including chronic rejection after LTx, persistent airway neutrophilia is one of the most important risk factors (23,24). Therapies that inhibit/prevent this neutrophilic inflammation may have a very important role in these diseases. We and others recently demonstrated in observational and interventional trials that azithromycin is capable of preventing and treating chronic rejection and related mortality via its inhibitory effect on airway neutrophilia (23). In this study, we observed that PM_{10} was no longer associated with LAD if azithromycin was part of the maintenance therapy. Hence, the present results are in favor of a preventive strategy with azithromycin, from discharge from the hospital on. Our recent placebo-controlled study in LTx patients indeed demonstrated that azithromycin, compared to placebo, prevents the development of BAL neutrophilia and chronic rejection (23).

The lag period of 2 to 3 days between exposure to PM_{10} and diagnosis of LAD seems immunologically plausible as a full innate inflammatory response with attraction of neutrophils and some types of lymphocytes, ultimately resulting in a histological diagnosis of LAD, needs time to develop. However, we could not detect a direct mechanistic link between PM_{10} and airway neutrophilia as IL-8 was not associated with PM_{10}. This discrepancy may be explained by the introduction of azithromycin, as 300 biopsies (23.5%) are taken from patients on active azithromycin therapy. It has already been demonstrated that azithromycin decreases IL-8 and CRP levels, both in a therapeutic and preventive manner (14,23). An additional explanation could be that part of the lavages and biopsies,

as mentioned in the literature, are taken to diagnose infection, which is associated with high IL-8 and CRP levels as shown in the results. When we only looked at biopsy procedures during infectious episodes, we indeed found an increase of blood CRP and BAL IL-8.

Our study has some limitations. This study spans a period of 10 years, and therapies and insights changed a lot during this period. We, however, tried to take this into account by correcting our analyses for time after transplantation and azithromycin treatment. It is a single center study including a cohort of patients restricted to a relatively small geographical area in Belgium (Flanders: 13,521 km^2). It would have been ideal to study a larger area, with larger spatial contrasts in pollution. Moreover, we did not look at acute effects of air pollution on FEV_1, although some studies report an association between FEV_1 and PM (25). Also, A-grade rejection/LAD is diagnosed via biopsies, which are very small and could give a skewed image. Various chemical compounds in ambient PM, including transition metals and aromatic organic compounds, may contribute to adverse effects through intrinsic generation of reactive oxygen species (13). We did not investigate these specific compounds. A further limitation of our study is the use of outdoor measurements of air pollution with interpolations at residential level in order to estimate partly indoor personal exposures. However, studies comparing personal and ambient exposure have reported good correlations among day-to-day changes in central measurement stations of PM and personal exposure (26,27).

We used both EBB and TBB in our analysis, to exclude selection bias. Although generally there is good agreement, the effect of PM_{10} on LAD seems to be only present when analyzing TBB and not EBB. It is, however, important to remark here that there are twice as many events in the TBB group compared to the EBB group. Importantly, LAD diagnosed on EBB, remained a major risk factor towards the later development of BOS. We also have no clear idea whether the pathology of LAD in our study truly represents airway rejection (B-grade acute rejection). We can only argue in favor of this by the fact that patients who develop LAD are indeed more prone to later on develop BOS, which may suggest that LAD in our study represents acute airway rejection (9). Moreover, when excluding all patients

experiencing infection (both in the group with or without LAD), the association between LAD and PM_{10} remained present, clearly proven that infection does not interfere with our results.

In conclusion, daily exposure to PM smaller than 10µm independently increased the risk of LAD in LTx patients. An increase in PM_{10} by 10 µg/m³ was associated with a 19% higher risk for LAD 3 days later. Mechanistically, airway inflammation was identified, and a protective effect of azithromycin was observed. The observed impact of acute exposure to air pollution adds up to the effect of chronic exposure to traffic-related air pollution which was previously reported by our group (3). This may have substantial health implications for LTx patients. A reduction of 10 µg/m³ is feasible in large parts of the world (28) and based on our estimates, this would be associated with a significant reduction in the risk of LAD and ultimately, in the prevalence of BOS.

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CHAPTER 3

CHANGING PLACES TO STUDY ACUTE AND SUBACUTE EFFECTS OF AIR POLLUTION ON CARDIOVASCULAR HEALTH

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ABSTRACT

Background: Short-term and long-term exposure to air pollution is associated with cardiovascular disease, but intermediate timeframes have remained relatively unexplored. In a panel study, we evaluated effects of deliberate exposure for ten days to varying levels of ambient pollution on several indicators of cardiovascular health.

Methods: Exposure to air pollution and cardiovascular endpoints were assessed in 20 healthy elderly volunteers in three locations during one year: in Leuven (Belgium – their country of residence; intermediate air pollution) and during 10-days stays in Milan (Italy, high pollution) and Vindeln (Sweden, low pollution). We measured blood pressure, carotid arterial stiffness, endothelial function, C-reactive protein (CRP), and blood cell counts. We used mixed-effects linear regression models adjusted for potential confounders to evaluate associations between these endpoints and exposure to particulate matter (PM), black carbon and NO_2 during one week before each health assessment.

Results: Exposure to pollutants was higher in Milan and lower in Vindeln than in Leuven, with the highest contrast found for NO_2 (averages: Milan 64 μg/m³; Vindeln 4 μg/m³; Leuven 26 μg/m³). 7-days exposure to air pollution was associated with arterial stiffness, e.g. a -4.8% [95% confidence interval (CI): -7.1;-2.5%] decrease in compliance for a 10 μg/m³ increment in PM_{10} (adjusted for covariates). In contrast, endothelial function 'improved' with elevated air pollution: e.g. the reactive hyperemia index increased by 0.36 (CI: 0.19;0.54) points for a 10 μg/m³ increment in PM_{10}. No inflammatory effects, measured as plasma CRP and white blood cell counts, were detected.

Conclusions: In a real life intervention study, we demonstrated in a healthy elderly population that short to medium term exposure to higher or lower levels of air pollution is associated with an increase or decrease, respectively, in carotid arterial stiffness.

INTRODUCTION

Numerous epidemiological studies have identified exposure to ambient air pollution as an important cause of respiratory and cardiovascular morbidity and mortality.[1,2] Short-term (hours to a few days of exposure before the adverse health event) 'triggering' effects of air pollution on all-cause mortality[3] and acute events, such as myocardial infarctions,[4] have been demonstrated by time-series and case-crossover (CCO) studies, whereas long-term effects (several years of exposure) on both the onset of acute events and the development of chronic disease have typically been explored in ecological studies or large prospective cohorts.[5,6]

Although these epidemiological studies have contributed to an increased understanding of the detrimental impact of air pollution on human health in real-world settings at ambient levels, they have limitations as well. By design, short-term studies are inappropriate to capture effects of persistent exposure or to explain the prevalence and development of chronic diseases. Moreover, time-series and CCO designs require rigorous modeling techniques in order to avoid confounding by time trends and weather variables,[7] or bias by selection of control days, respectively.[8] Long-term studies either lack information on important confounding risk factors, such as smoking status or socio-economic status (SES) (retrospective ecological approach), or are time-consuming and expensive (prospective cohort studies). Additionally, population or cohort-based studies cannot measure personal exposure to air pollution directly, because of the large number of subjects involved (needed to obtained sufficient statistical power), but they use data from central monitoring stations or from interpolation models as a proxy. Finally, intermediate effects and pathophysiological pathways usually remain undetected due to the 'black box' approach of epidemiological studies.

In contrast, controlled-exposure studies in animals and humans are able to capture individual exposure with greater precision and have provided more insight in possible physiological pathways of the relationship between inhalation of pollutants and cardiovascular and respiratory disease. However, in the case of human volunteers, this is only possible for very brief periods of exposure

(minutes to hours) and hence, a response that emerges only after a longer exposure (e.g. one week) to a given pollutant, can remain undetected.

In this study, bearing characteristics of an observational study and an intervention study at the same time, we aimed to combine the advantages of epidemiological and experimental studies, and to measure health effects of both reduced and elevated exposure to air pollution, compared to the usual level of exposure. We moved a panel of study volunteers to three locations with varying real-world ambient air pollution levels: Vindeln (Sweden, low pollution), Leuven (Belgium, intermediate pollution, home town), and Milan (Italy, high pollution). Exposure to different pollutants was measured at an individual level, and obtained from local monitoring stations. Possible physiological pathways of the relationship between exposure to air pollution and life-threatening cardiovascular disease have been reviewed recently.[9-11] We quantified several health-related endpoints that have been identified as intermediate steps between exposure and disease: systemic oxidative stress and inflammation,[12,13] endothelial function,[12,14] arterial stiffness,[15] and coagulation.[16]

We hypothesized that a decrease or increase in air pollution exposure, compared to the study persons' place of residence, during one to two weeks would be associated with detectable changes in biomarkers of cardiovascular health. With an eye to future public health policy, the possible beneficial effects of lowering exposure are as important as the adverse effects of increased exposure.

METHODS

Study design

We set up a panel study, in which we measured multiple health endpoints and personal exposure to air pollution at regular time points and in locations with widely differing ambient air pollution levels during one year in 20 healthy elderly volunteers. We selected 10 male-female couples with both partners fulfilling the inclusion (60-75 years of age; never-smoking or having quit smoking at least one year before the start of the study; willing and available to travel during the study period; good mental condition) and exclusion criteria (history of serious cardiovascular disease or cancer; presence of other

diseases that could interfere with the measurements). All participants were given detailed oral and written information on the study and gave written informed consent. The study was approved by the Ethical Committee of KU Leuven (S55482).

From September 2013 to September 2014, we collected data over 11 measurement periods: approximately every two months in Leuven, Belgium (seven periods); twice during a 10-day stay in Milan, Italy (one halfway and one at the end of the stay); twice during a similar 10-day stay in Vindeln (a rural area near Umeå, northern Sweden) (see **Figure 1**). These locations are representative for the highest (Milan, >50 µg/m³) and lowest (Vindeln, <10 µg/m³) yearly averages in PM_{10} that can be found in Western Europe, with intermediate values for Leuven (30 µg/m³)[17-19]. During the trips, on days with no health assessments, participants had ample time for touristic activities, as long as these took place in the targeted exposure environment (urban or rural, respectively). To reduce differences in temperature between the two study trips, we stayed in Milan in autumn (October 2013) and in Vindeln in summer (June 2014).[20] Clinical measurements were performed in appropriate study rooms at the UZ Leuven, the Ospedale Maggiore in Milan, and Umeå University.

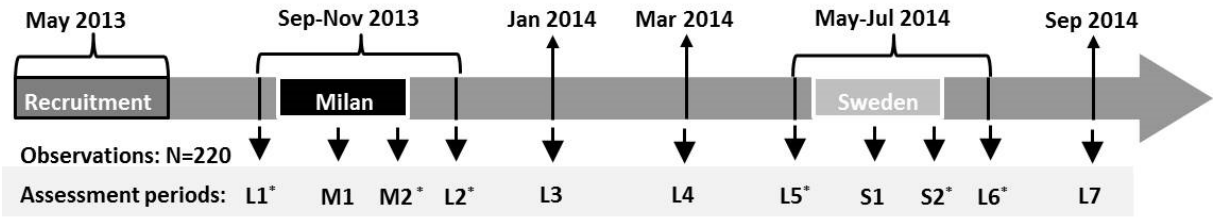


Figure 1. Timeline of the study. L1 to L7: health assessment periods in Leuven; M1-2: stay in Milan; S1-2: stay in Sweden. All variables mentioned in the text were measured in 20 study volunteers in all 11 periods, except for endothelial function (six periods, indicated with *) and plasma levels of cholesterol and glucose (only L1, baseline)

Collection of environmental data

Exposure to air pollution was assessed during each of the 11 study periods, and we combined our own measurements with data obtained from central monitoring stations.

In Belgium, we estimated daily residential exposure to PM_{10}, $PM_{2.5}$ and NO_2 using a land use regression model[21] that provides interpolated values in 4 by 4 km grids, based on the Belgian telemetric air quality network. In the absence of such a model for black carbon (BC), exposure to this pollutant was estimated by using daily averages of the station nearest to the participant's home address (average distance: 13 km).[22] In Milan, we used the online database of the Regional Agency for the Protection of the Environment in Lombardy (ARPA Lombardia) to estimate exposure to PM_{10}, $PM_{2.5}$, BC and NO_2, by averaging values from the different monitoring stations in the city.[23] In Vindeln, we averaged data from the nearest measurements stations in Umeå, Skellefteå and Strömsund to estimate regional levels of PM_{10}, $PM_{2.5}$, and NO_2.[24]

To validate the results obtained from the monitoring stations, we regularly sampled outdoor concentration of pollutants by using two portable laser-operated aerosol mass analysers: an Aerocet 53 (Met One Instruments Inc, Grants Pass, OR, USA) for PM_{10} and $PM_{2.5}$, and a microAeth Model AE51 (AethLabs, San Francisco, CA, USA) to measure BC concentration.

Finally, personal exposure to NO_2 was estimated using Radiello diffusive samplers (Sigma-Aldrich, Bellefonte, PA, USA). Six to 10 study volunteers wore the clip-on device during six days prior to each health assessment day in Leuven or prior to the second (and last) health assessment days in Milan and Vindeln. After the six-day sampling period, samplers were sent to the lab of the Fondazione Salvatore Maugeri (Padova, Italy) for quantification of average exposure to NO_2 during the sampling period.

Daily temperature and relative humidity during the study period were obtained from local meteorological websites for Belgium[25] and Milan[26] and an international website for Umeå.[27]

Cardiovascular measurements

During each sampling period, we measured blood pressure and carotid arterial stiffness, and collected non-fasted blood samples from each study volunteer. Endothelial function was measured once during each trip (on day 9-10) and in Belgium only in control periods immediately before and after trips, resulting in six time points with endothelial function assessments.

Blood pressure

Systolic (SBP) and diastolic blood pressure (DBP) were measured according to guidelines of the European Society of Hypertension,[28] with an automated device (Stabilograph, Stolberg, Germany). After the subject had rested for at least 5 min, blood pressure was measured five times consecutively in sitting position. We used the average of the last two measurements for analyses, and we calculated pulse pressure (ΔP) as average SBP - DBP, and mean arterial pressure as DBP + ΔP/3.

Carotid arterial stiffness

We measured carotid arterial stiffness by using an ultrasound device with automatic boundary detection software in RF-mode (MyLabOne, Esaote Benelux, Maastricht, The Netherlands) according to previously reported protocols.[29] Participants were at rest for 10 min in a supine position before starting the measurements. All measurements were performed by the same trained investigator.

We determined carotid intima-media thickness (CIMT) of the right common carotid artery at 2 cm proximal of the carotid bulb under three different angles; i.e. 90, 135 and 180° with the participant's head slightly turned to the left, according to the recommendations of the Mannheim Carotid Intima-Media Thickness Consensus.[30] Results obtained from measurements at the three angles were averaged.

We quantified carotid arterial stiffness by averaging diastolic artery diameter (D) and systolic increase in diameter (ΔD) over three consecutive ultrasound measurements, each spanning eight cardiac cycles. We subsequently used D and ΔD to calculate four parameters related to arterial stiffness. Carotid distensibility (DC) and compliance (CC) coefficients are inversely related to arterial

stiffness, and pulse wave velocity (PWV) is a direct measure of arterial stiffness. Young's Elastic Modulus (YEM) combines measures of arterial wall elasticity with wall thickness (CIMT). These parameters were calculated as follows:[31,32]

$DC = (2*D*\Delta D + \Delta D^2)/(D^2*\Delta P)$ (1/kPa)

$CC = (2*D*\Delta D + \Delta D^2)/\Delta P$ (mm^2/kPa)

$PWV = (\rho*DC)^{-0.5}$ (m/s)

$YEM = D / (CIMT*DC)$ (kPa)

with D = diastolic artery diameter (mm); ΔD = systolic increase in diameter (mm); ΔP = pulse pressure (kPa, converted from mm Hg by multiplication with conversion factor 0.133); ρ = density of blood (1.060 kg/dm^3).

Intra-observer coefficients of variation ranged from 5.2% to 10.1% for the different stiffness parameters, indicating good reproducibility of the measurements.[15]

Endothelial function

We measured endothelial function with a non-invasive device that uses pneumatic probes to record finger arterial pulse-wave amplitude in a beat-to-beat manner (EndoPAT, Itamar Medical Ltd, Caesarea, Israel), according to the study protocol described by Axtell et al. 2010.[33] In brief, the EndoPAT finger probes were placed on the index fingers of the study subject sitting on a chair with the lower arms on an armrest. Baseline measurements were recorded during 5 minutes, followed by 5 minutes of occlusion of the left upper arm by inflating a cuff up to 50 mm Hg above systolic pressure, and another 5 minutes of measuring reactive hyperemia after deflating the cuff. The Reactive Hyperemia Index (RHI) was calculated by the device; values lower than 1.67 indicate impaired endothelial function. Participants were fasted for at least 4 hours prior to the measurements.

Blood cell count

We collected non-fasted blood in EDTA and heparin vacutainer tubes for blood cell counts and measurement of plasma C-reactive protein (CRP), respectively. At baseline, plasma levels of cholesterol

and glucose were also determined in fasted blood samples. Blood cell counts (including platelet counts) and differential leukocyte counts were determined within 4 hours after sampling, using automated cell counters with flow differential (in Leuven and Milan: Cell Dyn 3500, Abbott Diagnostics, Abott Park, IL USA; in Umeå: XE-5000, Sysmex Corporation, Kobe, Japan). Plasma samples from heparin tubes were kept frozen at -80°C for subsequent analysis of plasma CRP, cholesterol and glucose levels at the UZ Leuven laboratory (Tina-quant CRP latex assay, Roche, Vilvoorde, Belgium).

To verify whether blood cell variables were quantified similarly by the three different devices, we divided ten fresh samples of whole blood among three new EDTA tubes each. Each batch of 10 identical samples was analyzed in either Milan, Umeå or Leuven with the same devices as were previously used in the main experiment. Because in this validation experiment, values for mean volume of red blood cells (MCV, equal to Hct/RBC*10) were on average 7% higher when measured in Umeå than in Leuven or Milan, we decided to exclude MCV results obtained in Sweden from the analyses.

Covariates

Information on smoking status (never or former), having a cold and medication use for hypertension was obtained by face-to-face interviews. Since physical activity, diet, alcohol consumption, and perceived mental health were assumed to differ between the home situation and a 10-day trip abroad, these variables were assessed as well.

During one complete week preceding each health assessment day, study subjects recorded their physical activity by wearing a SenseWear Pro Armband (BodyMedia, Inc., Pittsburgh, PA), a validated multisensory activity monitor combining a triaxial accelerometer with different sensors.[34] We used the number of steps walked per day and physical activity duration (PAD) [i.e. the number of minutes per day that the subject had an energy expenditure >3.0 metabolic equivalents of tasks (METs)], both averaged over one week, as covariates in the analyses. During one week preceding three selected health assessments periods (L1, M2, S2), participants kept a food diary, which allowed us to estimate weekly alcohol consumption (in g/week) at baseline and during trips abroad. At the start of each health assessment, participants filled in the Positive and Negative Affect Schedule (PANAS), which

comprises 20 items on instantaneous mental condition and results in a calculated positive affect (PA) and negative affect (NA) score. [35]

Because of too many missing values, physical activity, alcohol consumption, and instantaneous mental condition were omitted from the final models, but they were included in sensitivity analyses. More details on these variables will be reported separately.

Data management and analysis

Data management and statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). We investigated associations between health parameters and exposure to air pollution by using linear mixed models with random intercept and random slope, accounting for the repeated-measures design of the study. We evaluated different lag structures for the exposure variables: 'acute' effects of air pollution were estimated by using lag day 0 and 1 (exposure on the day of measurement and the day before, respectively) and 'subacute' effects by calculating the average of lag days 0 to 6 (average exposure during the week preceding the measurement day, av06), corresponding to the duration of exposure with the Radiello NO_2 sampler. We performed sensitivity analysis with different lag structures for the subacute exposure (av02 and av04).

We included the following covariates in statistical models, as appropriate: age at baseline, sex, date of measurement, external temperature, relative humidity, heart rate, mean arterial pressure, having a cold (y/n), medication use for hypertension (y/n), smoking status (former/never), mental health (PA and NA), and physical activity (PAD). We tested the assumption of normal distribution of the error terms by visual inspection of the Q-Q plots of residuals. For DC, CC, YEM, white blood cells (WBC) and differential WBC counts, this assumption was only met after log10-transformation. For the sake of consistency, PWV outcomes were log-transformed as well. Results for these outcomes are presented as % change, whereas parameter estimates of other analyses are unit changes.

RESULTS

Description of study population

Twenty study volunteers, consisting of 10 male-female couples, started the study in September 2013, and all of them completed the study in September 2014, without any dropout or missed measurement period.

Table 1 summarizes the main characteristics of the study population at baseline. No differences were observed between males and females, except body height and (borderline significant) DBP, which were both higher in males than in females. Five female volunteers took hypertension medication during the whole study period, one male started taking medication after period L2.

Environmental data

We obtained complete data from the local measurements stations in Belgium, Milan, and northern Sweden for PM_{10}, $PM_{2.5}$ and NO_2. Daily values of BC were not measured by any of the monitoring stations near the study area in Vindeln (Sweden). However, we continuously sampled BC in Vindeln with our own device, and our BC results correlated well with data from central monitoring stations for those days when we had obtained both measures (in Leuven or Milan, N = 57 days, Pearson's r = 0.76, $p<0.001$). Therefore, we used our own results for Vindeln to fill the gap in the BC dataset from the monitoring stations.

Individual exposure levels to PM_{10}, $PM_{2.5}$, NO_2 and BC are presented in **Figure 2**. Levels of ambient NO_2 (own measurements) and BC were clearly highest in Milan and lowest in Vindeln with intermediate values for Leuven (Belgium), whereas concentrations of PM_{10}, $PM_{2.5}$ and NO_2 (monitoring stations) did not differ between Leuven and Vindeln. In general, standard deviations (SD) were much smaller in Milan and Vindeln because the exposure windows were more uniform in time and space than in Leuven.

Table 1. Baseline characteristics of the study participants.[*]

Characteristic	All participants (N= 20)	Males (N=10)	Females (N=10)	P-value[†]
Age (y)	65 (58-76)	68 (58-76)	64 (59-70)	0.29
Height (m)	1.71 (1.58-1.96)	1.76 (1.69-1.96)	1.66 (1.58-1.71)	<0.001
Body-mass index (kg/m²)	24.3 (18.9-29.4)	25.2 (18.9-29.4)	23.5 (19.2-29.1)	0.73
Smoking status: N (%)				
Former	10 (50%)	6 (60%)	4 (40%)	
Never	10 (50%)	4 (40%)	6 (60%)	0.66
Blood pressure (mm Hg)				
Systolic	132 (109-165)	133 (113-165)	127 (109-155)	0.53
Diastolic	80 (65-105)	85 (67-105)	76 (65-89)	0.06
Plasma cholesterol (mg/dL)[‡]				
Total	206 (144-282)	206 (160-238)	207 (144-282)	0.72
LDL	133 (57-212)	133 (93-150)	130 (57-212)	0.91
Plasma glucose (mg/dL)[‡]	99 (86-131)	100 (88-131)	99 (86-112)	0.37
Medication for hypertension: N (%)	6 (30%)[#]	1 (10%)[#]	5 (50%)	0.14

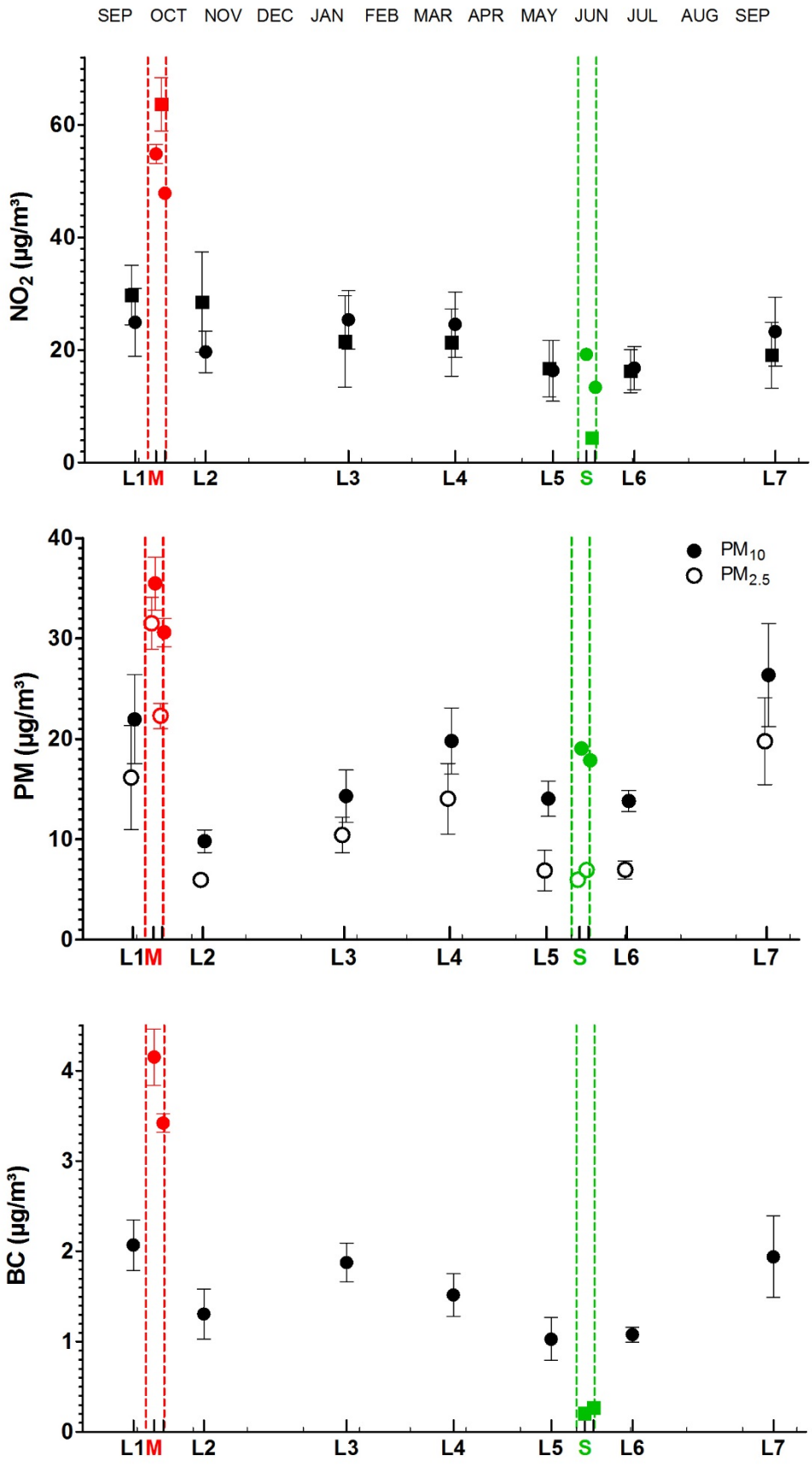
[*]All values are medians (range).

[†]P-value for t-test comparing males to females (except smoking status and medication use: Fisher exact test).

[‡]plasma cholesterol and glucose levels mere measured in fasted blood samples

[#]Male study subject started taking medication during the course of the study (after period M2).

Figure 2 (next page). Personal exposure to NO_2, PM_{10}, $PM_{2.5}$, and BC during the study period. All symbols and error bars represent means with SD, obtained from values averaged over one week preceding the day of health assessment ('av06' lag structure). Circles indicate data from central monitoring stations, squares are our own measurements (NO_2: Radiello device; BC: Aethlab device). N=20 for each data point, except Radiello NO_2 (N=6 to 10, depending on the period).


SEP OCT NOV DEC JAN FEB MAR APR MAY JUN JUL AUG SEP
NO2 (µg/m³)
PM (µg/m³)
BC (µg/m³)
PM10
PM2.5
L1 M L2 L3 L4 L5 S L6 L7

Exposure to air pollution and arterial stiffening

The associations of blood pressure and biomarkers of carotid arterial stiffness with ambient concentrations of PM_{10}, $PM_{2.5}$, NO_2 and BC are presented in **Table 2**. Results shown are those obtained by models adjusted for age at baseline, sex, HR, smoking status, having a cold, medication use for blood pressure, date, temperature, relative humidity. Crude individual patterns of the association between arterial stiffness and exposure to PM_{10} are shown in **Figure 3** and unadjusted coefficients can be found in supplemental **Table S1**.

We found no changes in blood pressure variables related to changes in pollutant concentrations, regardless of the time window.

We detected no short-term associations (lag0) between pollutant concentrations and indicators of arterial stiffness, except a 2.0% (0.4;3.5%) decrease in CC related to a 10 µg/m³ increase in PM_{10}, and a similar association with $PM_{2.5}$. In contrast, we found effects of subacute exposure (av06 lag structure) to air pollution on all biomarkers of arterial stiffness: increases in pollutant concentrations were associated with increasing PWV and YEM, and decreasing DC and CC. These associations were strongest for PM_{10} and $PM_{2.5}$ [e.g. a 4.8% (2.5;7.1%) decrease in CC for a 10 µg/m³ increment in PM_{10}]. Analyses with different lag structures (av04 and av02) produced very similar results (supplemental **Table S2**).

Exposure to air pollution and endothelial function

Endothelial function was positively associated with both 24 hours and 7 days averages of exposure to different pollutants, e.g. RHI was 0.36 (95% CI 0.19;0.54) points higher for a 10 µg/m³ increment in PM_{10} (av06), indicating an improvement in endothelial function with increasing air pollution exposure (**Table 2**). Similarly, when using a binary RHI outcome variable with 1.67 as the cut-off value, the risk for having endothelial dysfunction decreased with increasing pollutant concentrations (results not shown).

Exposure to air pollution and markers of inflammation

Adjusted results for plasma CRP and blood cell counts are summarized in **Table 3.** Results from crude analyses can be found in supplemental **Table S2**. CRP concentration was related with air pollution exposure in the crude models, but this association disappeared in the adjusted models, due to the influence of the covariate 'having a cold'. Outcomes for WBC and neutrophil counts suggest a negative association with BC (lag0) and NO_2 (lag0) exposure, but these results were not confirmed by using PM as the pollutant, nor by using a one-week timeframe. We found no associations between air pollution exposure and lymphocyte counts.

We detected no effects of acute exposure (lag0) to air pollution on red blood cells (RBC) or hemoglobin (Hb) concentration, but all pollutant variables representing subacute exposure (av06) were negatively related to RBC levels. Hematocrit (Hct) and MCV were both negatively associated with all indicators of short-term (lag0) and medium-term (av06) exposure. Results for MCV shown in **Table 3** are those obtained from the analyses without the Sweden data.

Mean cell hemoglobin (MCH, calculated as Hb/RBC*10), mean cell hemoglobin concentration (MCHC = Hb/Hct*100), and mean platelet volume (MPV) were higher with increasing pollutant concentration for most pollutants in both time windows (results not shown in **Table 3**)**.** Number of platelets did not change with short-term exposure to air pollution, and it was negatively related to one measure of medium-term exposure (NO_2).

Table 2. Adjusted[†‡] changes (with 95% CI) in blood pressure and indicators of carotid wall stiffness and endothelial function, associated with a 10 μg/m³ increase in PM10 or NO2, a 5 μg/m³ increase in PM2.5 or a 1 μg/m³ increase in BC.

Acute exposure (lag0)	**PM10**	**PM2.5**	**BC**	**NO2 (stations)**	**NO2 (portable)**
Systolic BP (mm Hg) [*]	-0.16 (-1.47;1.14)	0.11 (-0.57;0.78)	-0.02 (-0.99;0.94)	-1.02 (-2.11;0.06)	n/a
Diastolic BP (mm Hg) [*]	-0.47 (-1.34;0.40)	-0.15 (-0.61;0.30)	-0.02 (-0.72;0.69)	-0.39 (-1.12;0.34)	n/a
Pulse pressure (mm Hg) [*]	0.26 (-0.67;1.19)	0.25 (-0.23;0.73)	-0.04 (-0.73;0.65)	-0.66 (-1.44;0.12)	n/a
PWV (%) [†]	0.7 (-0.1;1.6)	**0.4 (0.0;0.9)**[*]	0.3 (-0.3;0.9)	0.3 (-0.5;1.0)	n/a
DC (%) [†]	-1.5 (-3.2;0.3)	**-0.9 (-1.8;0.0)**[*]	-0.7 (-2.0;0.6)	-0.6 (-2.1;0.9)	n/a
CC (%) [†]	**-2.0 (-3.5;-0.4)**[*]	**-1.1 (-1.9;-0.3)**[*]	-0.8 (-2.0;0.3)	-1.0 (-2.3;0.3)	n/a
YEM (%) [†]	1.2 (-0.8;3.2)	1.0 (0.0;2.0)	0.8 (-0.7;2.2)	0.5 (-1.2;2.1)	n/a
RHI [†]	**0.20 (0.10;0.30)**[**]	**0.19 (0.06;0.32)**[*]	**1.67 (0.76;2.57)**[**]	**0.12 (0.03;0.21)**[*]	n/a
Subacute exposure (av06)	**PM10**	**PM2.5**	**BC**	**NO2 (stations)**	**NO2 (portable)**
Systolic BP (mm Hg) [*]	0.23 (-1.8;2.26)	0.25 (-0.66;1.15)	-0.12 (-1.57;1.34)	-1.28 (-2.53;-0.04)	-0.14 (-1.10;0.81)
Diastolic BP (mm Hg) [*]	-0.90 (-2.23;0.43)	-0.24 (-0.85;0.37)	-0.17 (-1.17;0.82)	-0.78 (-1.65;0.10)	-0.28 (-0.95;0.39)
Pulse pressure (mm Hg) [*]	1.11 (-0.36;2.59)	0.47 (-0.17;1.11)	0.03 (-1.00;1.06)	-0.55 (-1.44;0.34)	0.11 (-0.58;0.79)
PWV (%) [†]	**2.0 (0.8;3.3)**[**]	**0.9 (0.4;1.5)**[**]	0.9 (-0.1;1.9)	0.7 (-0.1;1.6)	**0.6 (0.0;1.3)**[*]
DC (%) [†]	**-4.6 (-7;-2.2)**[**]	**-2.1 (-3.3;-1.0)**[**]	**-2.4 (-4.3;-0.4)**[*]	**-1.8 (-3.4;-0.1)**[*]	-1.3 (-2.5;0.0)
CC (%) [†]	**-4.7 (-6.9;-2.5)**[**]	**-2.1 (-3.2;-1.1)**[**]	**-2.5 (-4.3;-0.7)**[*]	**-2.0 (-3.5;-0.5)**[*]	**-1.4 (-2.6;-0.3)**[*]
YEM (%) [†]	**3.8 (0.8;6.9)**[*]	**1.9 (0.5;3.3)**[*]	**2.3 (0.2;4.5)**[*]	1.5 (-0.4;3.5)	1.4 (0.0;2.8)
RHI [†]	**0.36 (0.19;0.54)**[**]	**0.20 (0.08;0.31)**[**]	**0.27 (0.12;0.42)**[**]	**0.19 (0.09;0.30)**[**]	0.07 (-0.02;0.15)

Legend to Table 2. Coefficients are in mm Hg for the BP variables, and % changes for the carotid stiffness variables because we used log-transformed data in these analyses. For all results, N=218 (11 time points), except for RHI, where N = 118 (6 timepoints).

Statistically significant results are highlighted in bold. * P<0.05; ** P<0.01; *** P<0.001

† Adjusted for age at baseline, sex, HR, smoking status, having a cold, medication use for blood pressure, date, temperature, relative humidity.

‡ Additionally adjusted for arterial pressure.

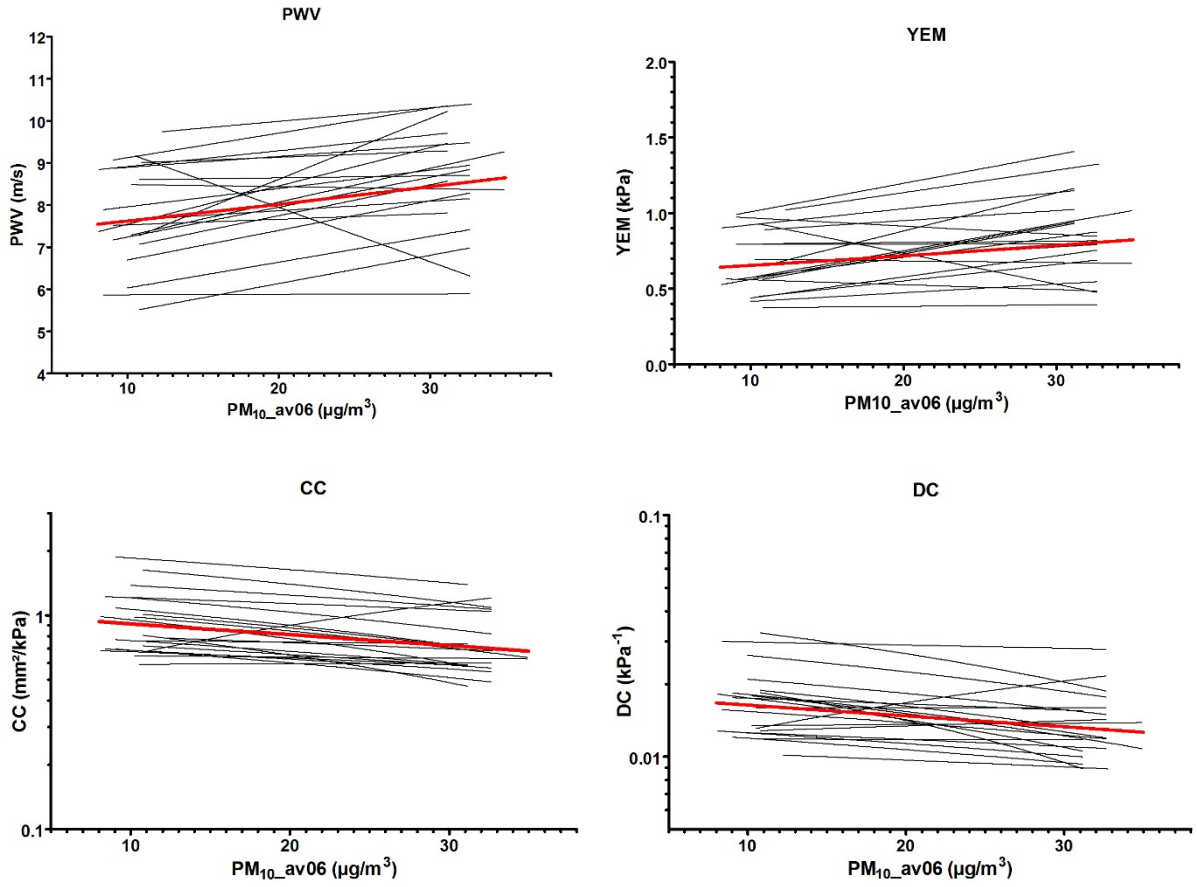


Figure 3. Crude individual patterns of 20 study volunteers for the association between PWV, CC, DC, or YEM and exposure to PM_{10}. Black lines are the linear regression lines for 11 data points, thick red lines represent the crude associations from the mixed model. One individual has a divergent association between stiffness biomarkers and PM_{10}, this is the same individual for all biomarkers.

Table 3. Adjusted* changes (with 95% CI) in plasma CRP and blood cell counts associated with a 10 µg/m³ increase in PM_{10} or NO_2, a 5 µg/m³ increase in $PM_{2.5}$ or a 1 µg/m³ increase in BC.

Acute exposure (lag0)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (portable)**
Plasma CRP (%)	2.3 (-2.3;7.0)	1.1 (-1.4;3.5)	0.0 (-3.4;3.5)	-1.1 (-4.6;2.5)	n/a
WBC (%)	-0.5 (-1.5;0.5)	-0.5 (-1.0;0.0)	**-0.9 (-1.6;-0.1)**	**-1.2 (-2.0;-0.3)**	n/a
Neutrophils (%)	-0.4 (-1.9;1.1)	-0.5 (-1.3;0.2)	**-1.2 (-2.4;0.0)**	**-1.5 (-2.8;-0.3)**	n/a
Lymphocytes (%)	-0.4 (-1.4;0.7)	-0.2 (-0.7;0.3)	-0.2 (-1.0;0.6)	-0.4 (-1.3;0.5)	n/a
RBC (10^6/µL)	-0.019 (-0.047;0.009)	-0.011 (-0.027;0.006)	-0.011 (-0.037;0.015)	-0.022 (-0.053;0.008)	n/a
Hb (g/dL)	0.007 (-0.090;0.104)	-0.009 (-0.059;0.041)	-0.031 (-0.111;0.049)	-0.007 (-0.098;0.085)	n/a
Hct (%)	**-0.28 (-0.56;-0.01)***	**-0.22 (-0.37;-0.07)****	**-0.32 (-0.55;-0.09)***	**-0.45 (-0.71;-0.19)****	n/a
MCV (fL)	**-0.16 (-0.30;-0.02)***	**-0.11 (-0.19;-0.04)****	**-0.17 (-0.30;-0.05)***	**-0.23 (-0.39;-0.07)***	n/a
Platelets (10^3/µL)	-2.5 (-6.7;1.8)	0.4 (-2.2;2.9)	3.0 (-0.6;6.6)	-1.4 (-5.5;2.7)	n/a
Subacute exposure (av06)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (portable)**
Plasma CRP (%)	2.3 (-4.6;9.1)	0.9 (-2.5;4.3)	-0.2 (-5.5;5.1)	-0.7 (-5.3;3.8)	-2.5 (-5.9;0.9)
WBC (%)	-0.4 (-1.9;1.1)	-0.4 (-1.2;0.3)	**-1.3 (-2.5;-0.1)**	-0.9 (-2.0;0.2)	-0.4 (-1.2;0.3)
Neutrophils (%)	-0.3 (-2.6;2)	-0.5 (-1.7;0.6)	-1.7 (-3.5;0.2)	-1.1 (-2.7;0.5)	-0.7 (-1.8;0.5)
Lymphocytes (%)	0.0 (-1.7;1.7)	0.0 (-0.8;0.8)	-0.2 (-1.4;1.1)	-0.4 (-1.5;0.8)	0.2 (-0.5;1.0)
RBC (10^6/µL)	**-0.054 (-0.098;-0.010)***	-0.025 (-0.050;0.000)	-0.032 (-0.072;0.008)	**-0.039 (-0.074;-0.003)***	**-0.024 (-0.047;0.000)***
Hb (g/dL)	0.019 (-0.132;0.169)	-0.019 (-0.097;0.060)	-0.072 (-0.197;0.052)	0.005 (-0.107;0.118)	0.002 (-0.074;0.078)
Hct (%)	**-0.66 (-1.09;-0.23)****	**-0.39 (-0.62;-0.16)****	**-0.68 (-1.03;-0.32)****	**-0.59 (-0.91;-0.27)****	**-0.42 (-0.63;-0.20)****
MCV (fL)	-0.24 (-0.52;0.05)	-0.15 (-0.30;0.00)	**-0.37 (-0.64;-0.1)***	**-0.23 (-0.44;-0.02)***	**-0.17 (-0.32;-0.01)***
Platelets (10^3/µL)	-4.4 (-10.4;1.7)	0.1 (-3.1;3.2)	**2.8 (-2.2;7.9)**	**-4.6 (-9.0;-0.2)***	-1.2 (-4.2;1.8)

Legend to Table 3. Coefficients are % changes for CRP, WBC and differential cell count because we used log-transformed variables in the analyses of these variables. For all results, N=219 (11 time points), except for MCV, where N=180 (9 time points), because we excluded the Sweden data. Statistically significant results are highlighted in bold. * P<0.05; ** P<0.01; *** P<0.001

* Adjusted for age at baseline, sex, HR, smoking status, having a cold, medication use for blood pressure, date, temperature, relative humidity.

DISCUSSION

In a panel study with 10 male-female couples of healthy elderly volunteers, we investigated the association between exposure to air pollution and various relevant cardiovascular endpoints in a quasi-experimental way by deliberately exposing study volunteers to the range of ambient pollution levels that can be found in Europe.

We evaluated several indicators of cardiovascular health that have been linked to short-term or long-term exposure to air pollution.[9,11] Our analyses revealed mixed results: some biological endpoints were indeed associated to variation in air pollution exposure, whereas others showed no effects of subacute exposure to pollution, and one outcome was associated with air pollution in the opposite direction than hypothesized.

Carotid arterial stiffness

We found evidence for a link between carotid arterial stiffness, indicated by increased PWV and YEM and decreased DC and CC, and short-to- medium-term exposure to several pollutants. Arterial stiffness is an important determinant of increased blood pressure and pulse pressure, and therefore of acute cardiovascular events such as myocardial infarction and stroke.[31,36,37] Since effects of short-term elevated air pollution on myocardial infarction and stroke have repeatedly been demonstrated,[1,2,4,38] our results provide a possible pathway for this trigger effect, although pulse pressure itself was not linked to air pollution exposure in our study. Similar associations between short-term air pollution exposure and arterial stiffness were found in recent intervention and epidemiological studies.[15,39-41]

The small average changes that we found are not clinically relevant for an individual, but the entire population is exposed to air pollution, including more vulnerable individuals. Small average effects in a population may reflect substantial changes in the most susceptible portion of the population.[42-44]. Moreover, the effects were considerably larger for the 7-days averaged pollutant concentrations than for one-day values, indicating that medium-term exposure increases the detrimental effect of air pollution.

Endothelial function

Contrary to our hypothesis, RHI was positively associated with pollutant concentration, and the risk of having endothelial dysfunction (binary approach of RHI with 1.67 as the cut-off value) was lower with increasing air pollution. The effect was strongest for the 7-days averaged concentrations. This result was unexpected, since endothelial dysfunction, a marker of atherosclerotic processes,[45] has repeatedly been associated with increased air pollution exposure levels.[9,11,12,14]

Endothelial function was measured six times in this study, and the highest average and median value were recorded in Milan (session M2), which had also the highest levels of air pollution. Measurements in Milan took place in the afternoon and evening (between 16:00h and 20:00h), whereas those in Leuven were always in the morning (between 8:00h and 12:00h), and those in Vindeln were spread over the whole day. There are some indications that endothelial function sustains a circadian rhythm, with a lower RHI in the morning.[46] Moreover, the same authors question the suitability of EndoPAT to measure endothelial function in small panels, such as those used in clinical pharmacology studies (and ours).[46]

Whatever the case may be, when removing the M2 results from the analysis, no positive or negative association between any of the pollutants and endothelial function could be detected. Therefore, our results on endothelial function and air pollution exposure have to be interpreted with care.

Systemic inflammation

We found no evidence of systemic inflammation, quantified as concentrations of WBC and plasma CRP. Either by a release of inflammatory cytokines into the circulation, or by direct translocation of particles through the lung-blood barrier into the circulation,[10] systemic inflammation has been held responsible for noxious processes such as endothelial dysfunction, development of atherosclerosis, reduced HRV, coagulation, and thrombosis.[9-11]

In our research group, we already found that concentrations of WBC and differential cell counts (neutrophils and lymphocytes) were associated with short-term air pollution exposure in susceptible populations such as patients with diabetes[16] and lung-transplanted patients.[47] However, in general, controlled-exposure studies at relatively low exposure levels in healthy humans, such as the present study, did not demonstrate a robust inflammatory response.[9]

Red blood cells

We detected a decrease in RBC count, Hct and MCV for short- and medium-term exposure to all pollutants. Although RBC and related parameters are generally not discussed in review papers on cardiovascular health effects of air pollution,[2,9-11] there is growing evidence for decreased oxygen-carrying capacity of the blood resulting from exposure to elevated levels of air pollution. RBC count, Hb concentration, and Hct were lower for increased PM_{10} exposure in elderly,[48] after exercise in polluted air by healthy sportsmen,[49,50] or in individuals cooking with biomass fuels,[51] for time windows ranging from less than an hour,[50] over days,[48] to years[49,51] of increased exposure.

However, in our study, we found no changes in Hb concentration related to air pollution, because MCH and MCHC were higher with increased air pollution. In other words, total Hb concentration was unaltered by changes in air pollution, but it was more densely concentrated in smaller and fewer RBC. It is unclear whether this is a reliable result or an artifact caused by the use of three different cell count devices.

Clotting

Rapid platelet activation is a well-documented physiological response to inhalation and translocation of pollutants,[9,11] but it is not necessarily linked to an increased number of platelets in the blood.[16]

Increased MPV has also been recognized as a predictor of thrombotic events and, moreover, as a marker of inflammation.[52] We found strong positive associations between MPV and exposure to all pollutants in both time windows. However, MPV was not measured by the device used in Sweden and we found a poor correlation between Leuven and Milan values in the validation experiment. When removing Milan data from the analysis, the positive association disappeared.

Therefore, it is not clear whether our results reflect a true effect of air pollution exposure on MPV, or were caused by a difference in MPV quantification by the devices used.

Strengths and limitations

We expected to find ambient PM_{10} concentrations as low as 10 μg/m³ in rural Sweden and as high as 50 μg/m³ in Milan during several days in a row. Although this was true for some days during each stay, we also recorded daily averages higher than 20 μg/m³ in Sweden and lower than 20 μg/m³ in Milan. This resulted in average one-week exposures (av06) of 19.8 μg/m³ in Sweden (S1) and only 30.6 μg/m³ in Milan (M2), which was considerably higher and lower than their respective yearly average.[17,18] No less than three assessment periods in Leuven, intended as intermediate exposure occasions, had lower one-week averages for PM_{10} than our stay in Sweden. A similar pattern was found for exposure to $PM_{2.5}$, but not for BC and NO2, which had both much higher values in Milan and lower values in Sweden than in Leuven. Nevertheless, despite the lower range in PM exposures than aimed at, we found significant results for PM_{10} and $PM_{2.5}$, just as for BC and NO_2.

In addition to the unpredictable air quality at the time of our study trips, the 10-day stays abroad also posed some logistic challenges. For example, we learned by experience that blood cell counts can differ among automated cell counters, even when devices have been calibrated and validated. As a consequence, results for MCV, MCH, MCHC, and MPV have to be interpreted with care.

We used interpolated data from central monitoring stations to estimate personal exposure to air pollution. For those days we measured PM and BC ourselves, the correlations between own values and those from monitoring stations were very high. Moreover, the NO_2 measurements with the clip-on passive samplers were successful, and they clearly indicated an enormous difference between an urban (Milan) and a rural area (Vindeln) in real-life exposure to NO_2, which is a typical traffic-related pollutant with much more spatial variation in ambient concentration than PM. [53]

A 10-day group travel abroad is very different from the common home situation in many aspects that can confound the association between biological endpoints and exposure to air pollution. We quantified physical activity, alcohol use, and mental state and adjusted all analyses for these covariates, but due to missing values, we eventually removed them from the list of covariates in the final analyses, as shown in the tables. Including PAD, steps, alcohol use, PA and NA did not produce substantially different results. We still may have overlooked other, real confounders of the associations found. However, when we totally excluded a possible "trip effect" by analyzing only Leuven data or by just comparing Milan to Sweden results, the parameter estimates, especially those for carotid stiffness, were still similar to those when we analyzed the whole dataset.

Our longitudinal study includes 11 health assessment periods during one year in a panel of 20 healthy elderly volunteers, without any missing measurements, drop-out or important changes in health status. Moreover, we used a large battery of objective health and exposure measurements, including personal exposure measures of NO_2. This strongly increased the statistical power of the analyses, allowing us to find subtle, but significant changes in cardiovascular health parameters related to changes in air pollution in only 20 subjects.

Public health relevance

The changes we found in carotid arterial stiffness and hematology, in relation to short-to-medium-term exposure to air pollution, were small and probably of little clinical relevance for the healthy individual study participants. However, since ambient air pollution is ubiquitous, the whole population is exposed, including more susceptible subgroups such as children, patients with preexisting diseases,

and elderly.[54] As a consequence, small individual risks result in a large global burden. Moreover, the time window of exposure in our study was relatively short. Many people living in urban environments are continuously exposed to much higher levels of air pollution than those our study subjects were exposed to during 10 days.[55] Long-term exposure to air pollution induces pathophysiological processes, eventually causing cardiovascular events and chronic diseases. Thus, it increases the risk for mortality to an even greater extent than the triggering effect of short-term exposures.[2,9]

According to the Global Burden of Disease (GBD) 2010 study, 3.7 million deaths and 3.1% of disability-adjusted life years (DALYs) worldwide were attributed to air pollution, placing it in the top 10 of risk factors.[56] In our study, we found that decreases in air pollution exposure, compared to the 'normal' level of exposure, were associated with decreases in biomarkers of cardiovascular health. Our result is in line with follow-up analyses of the Harvard Six Cities cohort study, showing a reduction in mortality risk in association with a decrease in ambient PM concentration.[57,58] These observations clearly demonstrate that measures leading to a reduction in exposure to air pollution are likely to have beneficial public health effects worldwide.

Conclusion

In a panel study of 20 healthy elderlies, exposed to different ambient air pollution levels typical for Europe, we found evidence for effects of subacute exposure to PM, BC and NO_2 on carotid stiffness. Oxygen-carrying capacity and coagulation showed some association with air pollution as well, but these results need to be interpreted with care.

The small individual effects that we found in healthy subjects are relevant for public health policy, since the whole population is exposed to air pollution, often to much higher concentrations than those in our study, and many individuals can be considered as more susceptible to the effects of air pollution than our healthy study volunteers. Finally, our intervention study suggests that decreasing exposure leads to fewer adverse health effects.

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DISCLOSURES

None.

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Supplemental Table S1. Crude changes (with 95% CI) in blood pressure and indicators of carotid wall stiffness and endothelial function, associated with a 10 µg/m³ increase in PM_{10} or NO_2, a 5 µg/m³ increase in $PM_{2.5}$ or a 1 µg/m³ increase in BC.

Acute effects (lag0)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (portable)**
Systolic BP (mm Hg)	-0.05 (-1.28;1.18)	0.37 (-0.25;0.99)	0.69 (-0.15;1.53)	0.32 (-0.57;1.20)	n/a
Diastolic BP (mm Hg)	-0.45 (-1.34;0.44)	0.07 (-0.39;0.54)	0.51 (-0.11;1.13)	0.42 (-0.19;1.03)	n/a
Pulse pressure (mm Hg)	0.39 (-0.46;1.24)	0.30 (-0.13;0.73)	0.18 (-0.41;0.76)	-0.10 (-0.72;0.51)	n/a
PWV (%)	**1.0 (0.2;1.8)***	**0.4 (0.0;0.8)***	0.3 (-0.3;0.9)	0.3 (-0.3;0.9)	n/a
DC (%)	**-2.0 (-3.6;-0.4)***	**-0.9 (-1.7;-0.1)***	-0.7 (-1.8;0.5)	-0.6 (-1.8;0.6)	n/a
CC (%)	**-2.6 (-4.0;-1.1)****	**-1.1 (-1.9;-0.4)****	-0.9 (-2.0;0.1)	**-1.1 (-2.1;0.0)***	n/a
YEM (%)	1.5 (-0.3;3.3)	**1.0 (0.1;1.9)**	1.0 (-0.2;2.3)	0.8 (-0.5;2.1)	n/a
RHI	**0.11 (0.02;0.19)***	0.07 (-0.03;0.17)	0.51 (-0.1;1.12)	0.05 (-0.02;0.12)	n/a
Subacute effects (av06)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (portable)**
Systolic BP (mm Hg)	0.51 (-1.09;2.11)	0.58 (-0.10;1.26)	**1.08 (0.11;2.05)***	**0.32 (-0.56;1.20)***	0.62 (0.04;1.19)
Diastolic BP (mm Hg)	-0.28 (-1.36;0.79)	0.18 (-0.30;0.66)	**0.77 (0.06;1.49)***	**0.31 (-0.37;0.98)***	0.44 (-0.04;0.92)
Pulse pressure (mm Hg)	0.84 (-0.41;2.09)	0.40 (-0.07;0.87)	0.31 (-0.37;0.98)	0.03 (-0.59;0.65)	0.18 (-0.22;0.58)
PWV (%)	**2.0 (1.0;3.0)*****	**0.9 (0.4;1.3)****	**0.8 (0.2;1.5)***	**0.7 (0.1;1.3)***	**0.5 (0.1;0.8)***
DC (%)	**-4.1 (-6.1;-2.1)*****	**-1.8 (-2.7;-0.8)****	**-1.7 (-3;-0.4)***	**-1.4 (-2.6;-0.3)***	**-1.0 (-1.7;-0.2)***
CC (%)	**-4.7 (-6.4;-3.1)*****	**-2.0 (-2.8;-1.2)*****	**-2.2 (-3.4;-1.0)****	**-1.9 (-2.9;-0.8)****	**-1.2 (-1.9;-0.5)****
YEM (%)	**3.7 (1.4;6.0)****	**1.8 (0.8;2.9)****	**2.1 (0.7;3.5)****	**1.4 (0.1;2.7)***	**1.0 (0.1;1.8)***
RHI	0.12 (-0.01;0.25)	0.03 (-0.04;0.11)	0.05 (-0.05;0.15)	0.07 (-0.01;0.14)	0.02 (-0.03;0.07)

Legend to Table S1. Coefficients are in mm Hg for the BP variables, and % changes for the carotid stiffness variables because we used log-transformed data in these analyses. For all results, N=218 (11 time points), except for RHI, where N = 118 (6 timepoints).

Statistically significant results are highlighted in bold. * P<0.05; ** P<0.01; *** P<0.001

Supplemental Table S2. Adjusted* changes (with 95% CI) in indicators of carotid wall stiffness, associated with a 10 µg/m³ increase in PM_{10} or NO_2, a 5 µg/m³ increase in $PM_{2.5}$ or a 1 µg/m³ increase in BC for other lag structures of subacute exposure.

Subacute effects (av04)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (portable)**
PWV (%)	**2.1 (0.9;3.4)**	**1.0 (0.4;1.5)**	0.8 (-0.1;1.7)	0.8 (0.0;1.6)	0.6 (-0.1;1.2)
DC (%)	**-4.3 (-6.8;-1.7)**	**-1.9 (-3.1;-0.7)**	-1.7 (-3.5;0.2)	-1.6 (-3.2;0.0)	-1.2 (-2.5;0.1)
CC (%)	**-4.7 (-6.9;-2.4)**	**-2.1 (-3.1;-1.0)**	**-2.1 (-3.8;-0.5)**	**-2.1 (-3.4;-0.7)**	**-1.3 (-2.5;-0.1)**
YEM (%)	**4.2 (1.3;7.1)**	**2.1 (0.7;3.4)**	**2.2 (0.3;4.2)**	1.6 (-0.2;3.3)	1.0 (-0.4;2.5)
Subacute effects (av02)					
PWV (%)	**1.5 (0.5;2.5)**	**0.8 (0.2;1.3)**	0.6 (-0.1;1.4)	0.6 (-0.1;1.3)	0.6 (-0.1;1.2)
DC (%)	**-3.0 (-5.1;-0.9)**	**-1.5 (-2.5;-0.5)**	-1.3 (-2.8;0.2)	-1.3 (-2.7;0.2)	-1.2 (-2.5;0.1)
CC (%)	**-3.4 (-5.2;-1.5)**	**-1.6 (-2.5;-0.7)**	**-1.6 (-3.0;-0.2)**	**-1.6 (-2.9;-0.3)**	**-1.3 (-2.5;-0.1)**
YEM (%)	**3.2 (0.9;5.5)**	**1.8 (0.7;3.0)**	**1.7 (0.1;3.4)**	1.2 (-0.4;2.9)	1.0 (-0.4;2.5)

av04: average exposure on five consecutive days (day of health measurement and four days before); av02: analogously for three consecutive days.

* Adjusted for age at baseline, sex, HR, arterial pressure, smoking status, having a cold, medication use for blood pressure, date, temperature, relative humidity.

Supplemental Table S3. Crude changes (with 95% CI) in plasma CRP and blood cell counts associated with a 10 µg/m³ increase in PM_{10} or NO_2, a 5 µg/m³ increase in $PM_{2.5}$ or a 1 µg/m³ increase in BC.

Acute effects (lag0)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (portable)**
Plasma CRP (%)	3.4 (-1.4;8.1)	2.2 (-0.3;4.6)	3.1 (-0.2;6.3)	**3.7 (0.6;6.8)**	n/a
WBC (%)	-0.3 (-1.3;0.6)	-0.3 (-0.8;0.2)	-0.4 (-1.1;0.2)	-0.4 (-1.1;0.2)	n/a
Neutrophils (%)	-0.2 (-1.6;1.2)	-0.5 (-1.2;0.3)	-0.9 (-1.9;0.1)	-0.9 (-1.9;0.1)	n/a
Lymphocytes (%)	-0.5 (-1.5;0.5)	0.0 (-0.5;0.5)	0.3 (-0.4;0.9)	0.2 (-0.5;0.9)	n/a
RBC (10^6/µL)	-0.031 (-0.057;-0.004)	-0.009 (-0.023;0.006)	-0.004 (-0.027;0.02)	-0.012 (-0.039;0.015)	n/a
Hb (g/dL)	0.011 (-0.071;0.093)	0.002 (-0.04;0.043)	-0.011 (-0.085;0.063)	0.001 (-0.083;0.086)	n/a
Hct (%)	**-0.40 (-0.66;-0.14)**	**-0.22 (-0.36;-0.09)**	**-0.27 (-0.48;-0.06)**	**-0.35 (-0.58;-0.12)**	n/a
MCV (fL)	**-0.19 (-0.32;-0.07)**	**-0.13 (-0.2;-0.06)**	**-0.19 (-0.3;-0.09)**	**-0.21 (-0.35;-0.07)**	n/a
Platelets (10^3/µL)	**-4.2 (-8.0;-0.4)**	0.1 (-2.1;2.2)	2.7 (-0.2;5.6)	-1.1 (-4.2;1.9)	n/a
Subacute effects (av06)					
Plasma CRP (%)	2.6 (-3.2;8.3)	2.1 (-0.8;5.0)	**4.4 (0.2;8.7)**	**4.2 (0.6;7.8)**	2.2 (-0.3;4.8)
WBC (%)	-0.3 (-1.5;0.9)	-0.2 (-0.9;0.4)	-0.4 (-1.2;0.5)	-0.2 (-1.0;0.6)	0.1 (-0.5;0.6)
Neutrophils (%)	-0.2 (-2.0;1.6)	-0.3 (-1.2;0.6)	-0.8 (-2.1;0.5)	-0.5 (-1.6;0.7)	-0.2 (-0.9;0.6)
Lymphocytes (%)	-0.7 (-2.1;0.6)	-0.1 (-0.7;0.6)	0.4 (-0.5;1.3)	0.1 (-0.7;0.9)	0.4 (-0.1;0.8)
RBC (10^6/µL)	**-0.080 (-0.117;-0.043)**	**-0.031 (-0.054;-0.009)**	-0.023 (-0.057;0.010)	**-0.030 (-0.060;-0.001)**	-0.016 (-0.034;0.003)
Hb (g/dL)	0.027 (-0.087;0.141)	0.004 (-0.061;0.070)	-0.009 (-0.113;0.095)	0.016 (-0.078;0.109)	0.008 (-0.045;0.061)
Hct (%)	**-0.86 (-1.22;-0.49)**	**-0.43 (-0.63;-0.23)**	**-0.53 (-0.82;-0.23)**	**-0.49 (-0.76;-0.22)**	**-0.28 (-0.45;-0.12)**
MCV (fL)	**-0.26 (-0.50;-0.01)**	**-0.16 (-0.29;-0.03)**	**-0.29 (-0.51;-0.06)**	**-0.22 (-0.40;-0.04)**	**-0.12 (-0.25;0.00)**
Platelets (10^3/µL)	**-8.6 (-13.4;-3.8)**	-1.9 (-4.4;0.6)	0.6 (-2.9;4.1)	**-3.8 (-6.9;-0.7)**	**-1.2 (-3.0;0.6)**

Legend to Table S3. Coefficients are % changes for CRP, WBC and differential cell count because we used log-transformed variables in the analyses of these variables. For all results, N=219 (11 time points), except for MCV, where N=180 (9 time points), because we excluded the Sweden data. Statistically significant results are highlighted in bold.

CHAPTER 4

LONG-TERM EXPOSURE TO PARTICULATE MATTER AIR POLLUTION IS A RISK FACTOR FOR STROKE: META-ANALYTICAL EVIDENCE

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ABSTRACT

Background and Purpose: Epidemiological studies suggest an association between stroke incidence and stroke mortality and long-term exposure to particulate matter (PM) air pollution. However, the magnitude of the association is still unclear.

Methods: We searched the Pubmed citation database for epidemiological studies and reviews on stroke and PM exposure. Then, we carried out a meta-analysis to quantify the pooled association between stroke incidence and mortality and long-term exposure to PM. Meta-analyses were performed for stroke events and stroke mortality and for PM_{10} and $PM_{2.5}$ separately and jointly.

Results: We identified 20 studies, including a total of >10 million people, on long-term PM exposure and stroke event or stroke mortality. For exposure to PM_{10} (including estimated exposure to PM_{10} from studies using $PM_{2.5}$), the pooled hazard ratio for each 10 $\mu g/m^3$ increment in PM_{10} was 1.061 (95% confidence interval 1.018-1.105) and 1.080 (0.992-1.177) for overall stroke events and stroke mortality, respectively. A stratified analysis by continent revealed that the association between stroke and long-term PM_{10} exposure was positive in North America [1.062 (1.015-1.110)] and Europe [1.057 (0.973-1.148)], but studies in Asia [1.010 (0.885-1.153)] showed a high degree of heterogeneity. Considering exposure to $PM_{2.5}$ (Europe and North America combined), the hazard ratios for a 5 $\mu g/m^3$ increment were 1.064 (1.021-1.109) and 1.125 (1.007-1.256) for stroke events and mortality, respectively.

Conclusions: The scientific evidence of the past decade identifies long-term exposure to PM, and $PM_{2.5}$ in particular, as a risk factor for stroke. However, we found some currently unexplained geographical variability in this association.

INTRODUCTION

With an incidence rate of 40 to > 300 cases per 100,000 inhabitants, depending on the region, and a global death rate of 110 per 100,000 inhabitants, stroke is one of the most prominent causes of mortality, accounting for 12% of all deaths worldwide.[1,2] Stroke, an acute event itself, can be triggered by acute events occurring a few hours or days before the stroke onset, such as alcohol abuse[3] or an outburst of anger.[4] However, long-term underlying conditions are even more important predictors of stroke. In a recent study[5], 90% of all ischemic and hemorrhagic strokes could be attributed to 10 major risk factors, with history of hypertension and current smoking as the most prominent causes.

From the past decade of the 20th century on, numerous epidemiological studies found that respiratory and cardiovascular diseases, as well as general morbidity and mortality, could be associated with increased levels of air pollutants, especially particulate matter (PM).[6-8] Biological pathways that have been proposed to explain the association between PM and cardiovascular diseases[6,7,9,10], are plausible mechanisms for a link between PM exposure and certain cerebrovascular events as well.[11]

Studies investigating the triggering effect of recent exposure to peak concentrations of PM_{10} or $PM_{2.5}$ (PM with an aerodynamic diameter of <10 µm or <2.5 µm, respectively) on stroke, were summarized in 3 recent meta-analyses on the short-term effects of PM on stroke hospitalization and mortality.[12-14] These meta-analyses suggested a small but significant effect of recent PM exposure and the risk of stroke in general and ischemic stroke in particular. In contrast to our knowledge about short-term exposure to air pollution being a trigger of stroke, the record for long-term effects of PM on cerebrovascular disease is much less extensive. Three comprehensive narrative reviews[6,11,15] provided a summary of the literature on the topic, but no meta-analyses were conducted. Although studies discussed in these review papers reported fairly mixed results, the overall size and importance of the effect is still unclear. Therefore, we conducted a meta-analysis of the existing literature to quantify the association between the risk of stroke event and stroke mortality and long-term exposure to PM air pollution. A better understanding of the magnitude of the effect of air pollution on a common cause of death, such as stroke, is important in the light of public health.

METHODS

Literature search

A bibliographic search was carried out by 2 independent reviewers (HS and LJ) in the Pubmed database (last accessed on July 20, 2015) to identify original studies analyzing the associations of long-term exposure to PM_{10} or $PM_{2.5}$ with stroke events (both fatal and non-fatal). Details on the search terms used can be found in the online-only Data Supplement. Study designs could be ecological or cohort studies. Experimental studies, case reports, studies on short-term associations between PM and stroke, and publications with no or incomplete results were excluded. Articles not written in English were considered for inclusion. Reviews and the reference lists of eligible studies were screened for additional data. Our meta-analysis complies with the preferred reporting items of the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) statement for meta-analyses of observational studies.[16]

Data management

Study results were classified according to the endpoint of the analysis: stroke event or stroke mortality. When available, preference was given to results obtained by models fully adjusted for covariates. We assessed quality of the selected studies taking into account the following aspects: study design, number and nature of covariates in the analysis, definition of the endpoint, and estimation of the exposure (details can be found in the online-only Data Supplement).

We needed to standardize reported results to hazard ratios (HRs) for a 10 $\mu g/m^3$ increment of PM_{10}, because HRs of individual studies have been reported for increments other than 10 $\mu g/m^3$ (e.g. an interquartile range increment) or in comparison with a reference category. Results for $PM_{2.5}$ were converted to estimated results for PM_{10} to be included in the overall analysis. Details on calculations and conversions made to standardize the data can be found in the online-only Data Supplement.

Meta-analysis

For those studies that provided results on both stroke event and stroke mortality, the most comprehensive data set (i.e. on stroke event) was selected for the overall analysis. When a study presented results for both PM_{10} and $PM_{2.5}$ exposure, we selected PM_{10}. However, we also performed separate analyses for PM_{10} and $PM_{2.5}$, and additional analyses for stroke mortality only. We performed sensitivity analyses per continent and according to the result of the quality assessment. For articles with independent subgroups within 1 study (e.g. different cities or different types of stroke), we used the general HR resulting from a meta-analysis by the authors, or, if no general HR was provided, we treated each subgroup as a separate study.

The overall HR and 95% confidence interval were estimated using a random-effects model, which is more conservative than a fixed-effect model and accounts for heterogeneity between studies in terms of population and methodology.[17] Heterogeneity and publication bias were tested with the I^2 statistic and Egger linear regression method, respectively (details can be found in the online-only Data Supplement).

All tests were two-sided with α=0.05. Meta-analyses, including tests for heterogeneity and publication bias, were performed with StatsDirect statistical software (StatsDirect Ltd, Altrincham, United Kingdom).

RESULTS

Selection and characteristics of studies

A flow chart of the selection procedure is given in **Figure S1** in the online-only Data Supplement. We included 20 publications on stroke and long-term PM exposure in our meta-analysis.[18-37] They are listed by region and then chronologically in **Table 1**. Fourteen studies were cohort studies and included covariates at an individual level; the other 6 made use of registered-based entries of stroke mortality or hospital admission and provided covariates on an ecological scale. Eight studies were conducted in Europe, 7 in North America and 5 in Eastern Asia.

The exposure levels reported by the 20 selected studies are shown in **Figure 1**. The exposure measure was PM_{10} in 9 studies and $PM_{2.5}$ in 7 studies; 4 publications investigated the association of stroke with exposure to both $PM_{2.5}$ and PM_{10}. The endpoint was stroke event (8 studies), stroke mortality (7 studies), or stroke event with separate results for mortality (5 studies). More details on the definition of the outcome can be found in **Table S1** in the online-only Data Supplement.

All publications displayed adjusted results for at least age and, when applicable, sex. Other covariates varied among studies, but the most common variables included in the adjusted models were body mass index, smoking status, alcohol use, and a measure of socio-economic status (SES) at an individual or area level (**Table S1** in the online-only Data Supplement).

Main analyses on long-term exposure and stroke

The overall meta-analysis, including >10 million people and >200,000 stroke events from 20 scientific articles, showed a pooled HR with 95% confidence interval of 1.061 (1.018-1.105) for a 10-$\mu g/m^3$ increment in long-term PM_{10} or (converted) $PM_{2.5}$ exposure (**Table 2**, **Figure 2**). Twelve of 20 publications presented results on stroke mortality. The result of the analysis was similar to that of stroke event, with a slightly higher HR of 1.080 (0.992-1.177). A stratified analysis by continent revealed that the association between long-term PM exposure and stroke event was positive in North America and Europe (but not statistically significant for the latter) and null in Asia (**Table 2**).

Table 1. Characteristics of the selected studies on stroke and long-term exposure to PM

ID	First author (year of publication)	Area	Study period	Pollu-tant	Pollutant concentration (μg/m³)	Study design‖	Population (number)	Stroke type (as in publication)	Official classification	Endpoint	Number of cases
1	Ueda (2012)[35]	Japan	1985-2004	PM_{10}	27.3-43.1‡	COH	≥30y (7,250)	Stroke	ICD-9 430-438, ICD-10 I60-69	Mortality	250
2	Nishiwaki (2013)[29]	9 cities, Japan	1990-2008	PM_{10}	17.2-43.7§	COH	>40y (78,057)	Stroke	ICD-10 I60-69	Mortality	unknown
		7 cities, Japan			17.2-28.7§		>40y (62,142)	Stroke	ICD-10 I60-69	Incidence	2,181
								Ischemic stroke	unknown	Incidence	unknown
								Subarachnoid hemorr.	unknown	Incidence	unknown
								Intracerebral hemorr.	unknown	Incidence	unknown
3	Zhang (2014)[37]	4 cities, China	1998-2009	PM_{10}	144 (36)*	COH	All (39,054)	Cerebrovascular disease	ICD-10 I60-69	Mortality	295
										Hosp. adm.	5,122
4	Qin (2015)[32]	3 cities, China	2006-2008	PM_{10}	123.1 (14.6)*, 123 (19)†	COH	18-74y (24,845)	Stroke	Self-reported	Incidence	589
5	Wong (2015)[36]	Hong Kong, China	1998-2011	$PM_{2.5}$	35.3 (33.8-37.2)†	COH	>65y (66,820)	Cerebrovascular disease	ICD-10 I60-69	Mortality	1,621
6	Maheswaran (2005)[26]	Sheffield, UK	1994-1998	PM_{10}	18.8 (16.8-20.6)‡	ECO	≥45y (199,682)	Stroke	ICD-9 430-438, ICD-10 I60-69	Mortality	2,979
7	Beelen (2009)[19]	The Netherlands	1987-1996	$PM_{2.5}$	Unknown	COH	55-69y (111,391)	Cerebrovascular disease	ICD-9 430-438, ICD-10 I60-69	Mortality	1,175
8	Huss (2010)[21]	Switzerland	2000-2005	PM_{10}	18.8 †	ECO	≥30y (4,580,311))	Stroke	ICD-10 I60-64	Mortality	25,231
9	Maheswaran (2012)[27]	London, UK	1995-2004	PM_{10}	25.1 (1.2)*	ECO	All (267,839)	Stroke	unknown	1st / Mort.	2,610 / 179
								Ischemic	unknown	1st / Mort.	1,832 / 41
								Hemorrhagic	unknown	1st / Mort.	348 / 64

ID	First author (year of publication)	Area	Study period	Pollu-tant	Pollutant concentration (μg/m³)	Study design‖	Population (number)	Stroke type (as in publication)	Official classification	Endpoint	Number of cases
10	Atkinson (2013)[18]	England, UK	1982-2000	PM_{10}	19.7 (2.3)*	ECO	40-89y (819,370)	Stroke	ICD-10 I61, I63-64	First stroke	13,012
11	Beelen (2014)[20]	22 cohorts in 13 countries, Europe	1985-2012	$PM_{2.5}$ PM_{10}	6.6-31.0§ 13-50§ (estimated)#	COH	All (367,383)	Cerebrovascular disease	ICD-9 430-438, ICD-10 I60-69	Mortality	2,484
12	Katsoulis (2014)[23]	Athens, Greece	1994-2011	PM_{10}	39.4 (4.0)*	COH	All (2,752)	Stroke	ICD-10 I60-69	Incidence	60
13	Stafoggia (2014)[33]	11 cohorts in 5 countries, Europe	1992-2010	$PM_{2.5}$ PM_{10}	7-31§ 14-48§	COH	All (99,446)	Stroke	ICD-9 431-436, ICD-10 I61-64	First stroke	3,086
14	Pope (2004)[30]	50 states, USA	1982-1998	$PM_{2.5}$	17.1 (3.7)*	COH	≥30y (319,000)	Cerebrovascular disease	ICD-9 430-438, ACS-CPS-II 6	Mortality	21,692
15	Miller (2007)[28]	36 cities, USA	1994-1998	$PM_{2.5}$	13.5 (3.7)*	COH	Women 50-79y (65,893)	Cerebrovascular disease	unknown	First stroke Mortality	600 122
16	Johnson (2010)[22]	Edmonton, Canada	2003-2007	$PM_{2.5}$	5.0 (0.2)*	ECO	All (103,4945)	Stroke Hemorrhagic Non-hemorrhagic stroke TIA**	ICD-10 I60-68, G45 ICD-10 I60-62 ICD-10 I63-68 G45	First hosp. admission	7,336
17	Lipsett (2011)[25]	California, USA	1996-2005	$PM_{2.5}$ PM_{10}	15.64 (4.48)* 29.21 (9.73)*	COH	Female teachers ≥30y (73,489)	Cerebrovascular disease Stroke	ICD-9 430-438, ICD-10 I60-69 ICD-9 431-434,436, ICD-10 I61-64	Mortality Incidence	486 1,179

ID	First author (year of publication)	Area	Study period	Pollu-tant	Pollutant concentration (μg/m³)	Study design‖	Population (number)	Stroke type (as in publication)	Official classification	Endpoint	Number of cases
18	Puett (2011)[31]	13- states, USA	1989-2003	$PM_{2.5}$ PM_{10}	17.8 (3.4)* 27.9 (5.8)*	COH	M health professionals 40-75y (17,545)	Ischemic Hemorrhagic	unknown unknown	Incidence Incidence	230 70
19	Kloog (2012)[24]	New England, USA	2000-2006	$PM_{2.5}$	9.65 (0.81)*, 9.65 (9.16-10.14)†	ECO	>65y (1,963,293)	Stroke	ICD-9 430-438	Hosp. adm.	125,382
20	To (2015)[34]	Ontario, Canada	1980-2013	$PM_{2.5}$	12.5 (2.4) *	COH	Female 40-59y at baseline	Stroke	ICD-9 433-436, ICD-10 G45-46, I63-64	Incidence	5,993

*average (SD); †median (IQR); ‡median (20%-80%); §lowest and highest average of all cities in study; ‖COH = cohort study; ECO = ecological study; #estimated from graph in publication; **TIA = transient ischemic attack

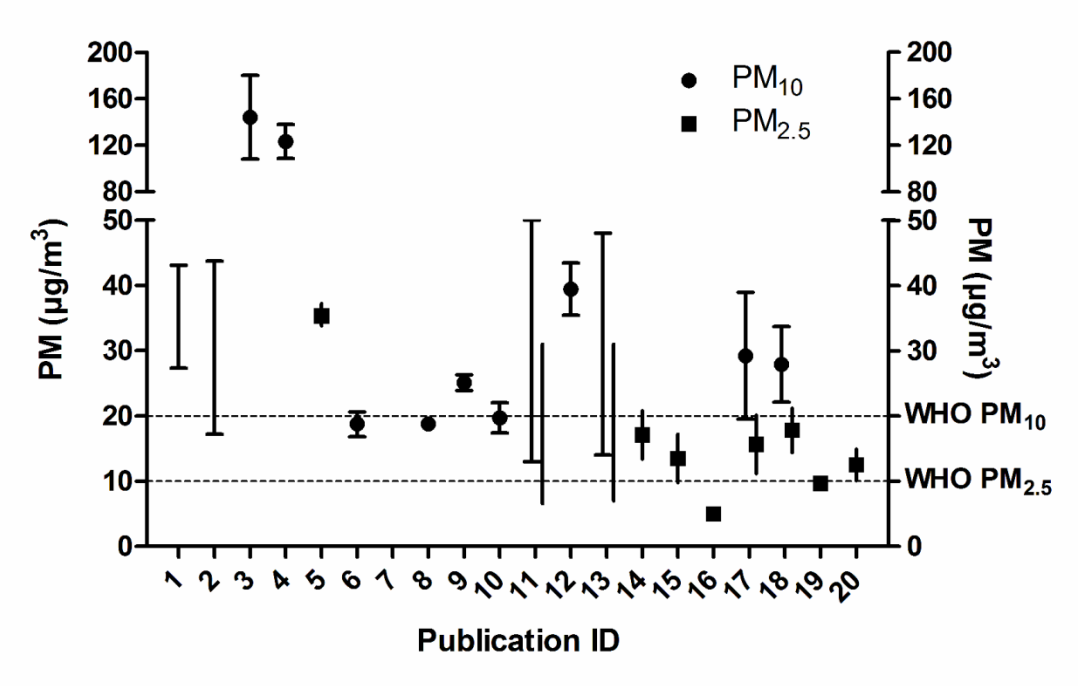

Figure 1. Exposure levels of 20 publications included in the meta-analysis. Publication IDs correspond to those in **Table 1**. Values are mean ± SD, median (IQR), or range, as reported by the respective authors. In order to distinguish PM_{10} from $PM_{2.5}$ when no central value is given, the error bars are presented as **T** for PM_{10} and **I** for $PM_{2.5}$. The dotted lines at 10 µg/m³ and 20 µg/m³ represent the WHO air quality guidelines for long-term exposure to $PM_{2.5}$ and PM_{10}, respectively.[38]

Sensitivity analyses

The 3 studies conducted in China[32,36,37] reported high ambient PM concentrations (3 to 6x the respective World Health Organization air quality guideline values for long-term exposure to $PM_{2.5}$ or PM_{10}[38]). A meta-analysis of these study results revealed a highly significant association between stroke onset and PM exposure [HR, 1.123 (1.010-1.248)]. Because the Chinese studies reported such exceptional exposure levels and the 2 Japanese studies were deviant with respect to circumstances, as well as study design and results (see Discussion), we excluded the 5 Asian studies from subsequent sensitivity analyses.

For PM_{10} alone (n=9 studies), that is, without the converted $PM_{2.5}$ results from 6 studies, the association between stroke event and PM_{10} exposure disappeared, with a HR of 1.021 (0.975-1.069) for a 10-µg/m³ increment in PM_{10}. In contrast, the estimate for $PM_{2.5}$ exposure only was significantly

higher than 1: HR, 1.064 (1.021-1.109) for a 5-μg/m³ increment in $PM_{2.5}$ (n=10 studies; **Figure 3**). A similar difference between PM_{10} and $PM_{2.5}$ exposure, but with generally higher HRs, was found for stroke mortality (**Table 3**).

A subanalysis including only studies with a high-quality score (more than the median overall quality score; **Table S1** in the online-only Data Supplement) resulted in a pooled HR of 1.087 (1.023-1.154) for stroke event (n=8) and 1.056 (0.957-1.165) for stroke mortality (n=4), for a 10-μg/m³ increment in PM_{10} or converted $PM_{2.5}$. The corresponding HRs for a 5-μg/m³ increase in $PM_{2.5}$ exposure alone were slightly higher (**Table 3**). All high-quality studies had a prospective cohort design, and all but one estimated personal exposure by using spatial interpolation models instead of raw data from monitoring stations.

Results for analyses with converted $PM_{2.5}$ data proved to be robust against changes in the conversion factor for $PM_{2.5}$ (**Table S2** in the online-only Data Supplement). Our a priori choice for random effects models was justified, given the considerable heterogeneity. We found no indications of publication bias in most analyses (more details can be found in the online-only Data Supplement; **Figures S2-S4**).

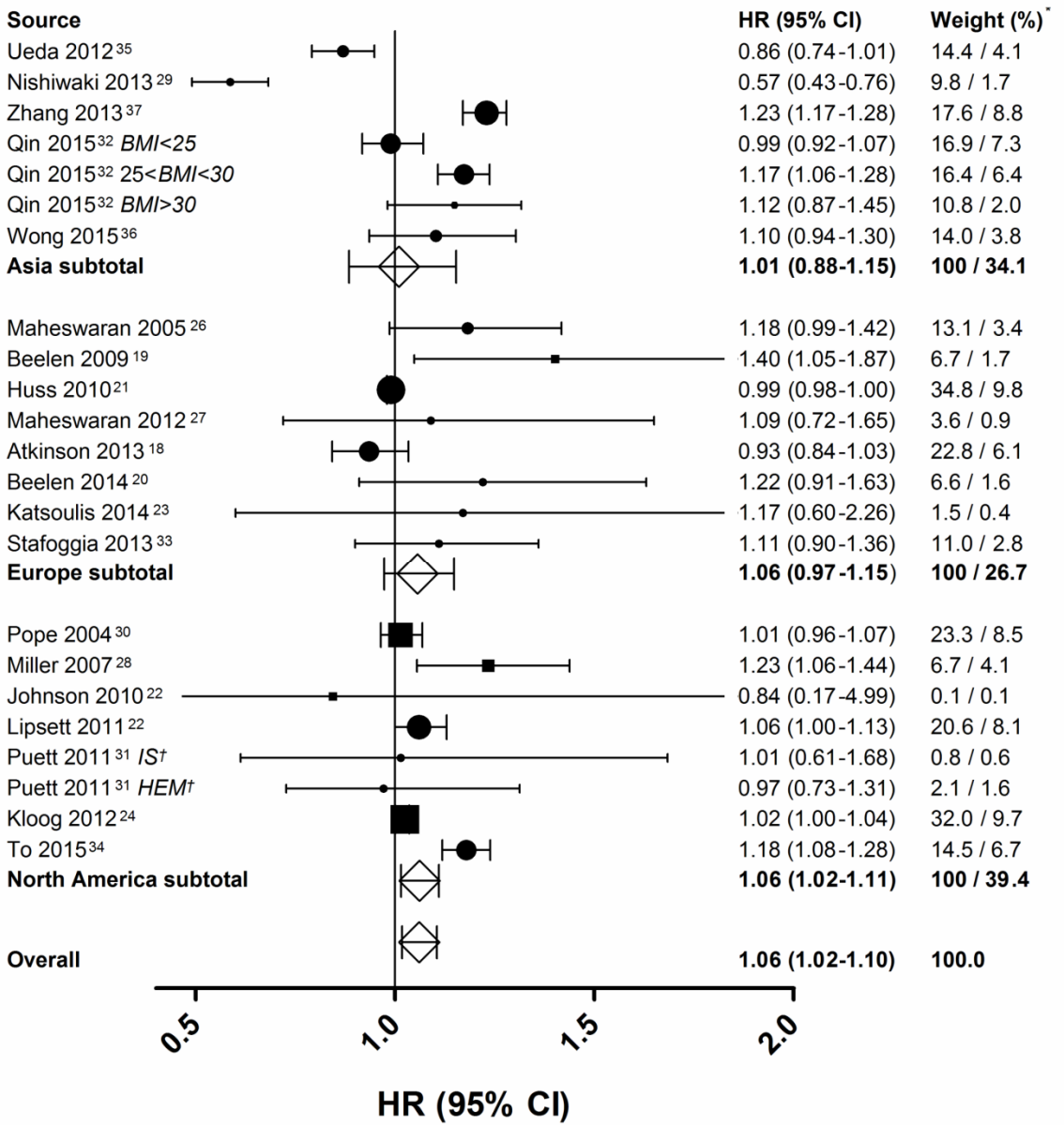

Figure 2. Forest plot of the overall analysis on stroke event and long-term PM exposure. Hazard ratios (HR) with 95% confidence intervals (CI) for a 10 µg/m³ increment in PM_{10} are presented (including converted HRs from $PM_{2.5}$ exposure). Circles are for PM_{10}, squares for converted $PM_{2.5}$; symbol size is proportional to the weight of the study in the meta-analysis. Open diamonds represent pooled results from meta-analysis.

*Weights are calculated as $(1/SE^2) / (\Sigma\ 1/SE^2) * 100$ (with SE^2 the variance of each study effect, and $\Sigma\ 1/SE^2$ the sum of inverted variances for all study effects): within continent / overall (sum may differ from 100 due to rounding).

†IS = ischemic stroke; HEM = hemorrhagic stroke

Table 2. Results of overall meta-analyses and stratified analyses by continent

				Meta-analysis		Tests of heterogeneity		Test of publ. bias
Pollutant	Endpoint	Stratum	Nr. of studies	Combined HR [*] (95% CI)	P value (model)	P value[†]	I^2 in % (95% CI)	P value[‡]
PM_{10} + converted $PM_{2.5}$	Stroke event	All	20	1.061 (1.018-1.105)	0.005	0.004	85.8 (80.2-89.3)	0.11
		Asia	5	1.010 (0.885-1.153)	0.88	<0.001	89.9 (81.9-93.5)	0.079
		Europe	8	1.057 (0.973-1.148)	0.19	0.050	50.2 (0-75.9)	0.066
		North America	7	1.062 (1.015-1.110)	0.009	0.030	54.9 (0-77.8)	0.26
		Europe + North America	15	1.045 (1.011-1.081)	0.010	<0.001	68.6 (41.7-80.1)	0.018
	Stroke mortality	All	12	1.080 (0.992-1.177)	0.077	<0.001	90.9 (86.6-93.4)	0.21
		Asia	4	0.986 (0.788-1.234)	0.90	<0.001	90.8 (78.2-94.8)	0.091
		Europe	5	1.213 (0.955-1.541)	0.11	<0.001	81.2 (42.9-90.2)	0.16
		North America	3	1.041 (0.932-1.162)	0.48	0.063	63.8 (0-87.6)	n/a[§]
		Europe + North America	8	1.085 (1.004-1.172)	0.039	<0.001	74.8 (38.5-85.9)	0.040

[*]HR for a 10 µg/m³ increment in PM_{10} or converted $PM_{2.5}$; [†]P for Cochran's Q test; [‡]P for Egger's test; [§]Too few studies for calculation of bias indicator.

DISCUSSION

Our meta-analysis on risk of stroke event and fatal stroke in association with long-term exposure to PM air pollution includes 20 epidemiological studies, comprising > 10 million people and 200,000 stroke events on 3 different continents. We found a positive association between the risk of stroke and PM exposure, with a 2% to 21% excess risk, depending on the definition of exposure, outcome, and population.

Considerable geographical variation was observed, with the highest combined HR found in Europe and high heterogeneity in Asia. The 5 studies conducted in Asia were remarkable in various ways. The reported average PM concentrations in Chinese cities[32,36,37] were 3- to 10-fold higher than those found in European and North American cities (**Figure 2**). In addition, the authors found strong associations between stroke and long-term PM exposure. The 2 Japanese studies[29,35] reported contrasting findings. According to the authors of both publications, stroke incidence in Japan is more prominent in rural areas than urban areas, and it has been attributed to high salt intake in those low-polluted but socioeconomically lower-rated rural areas. These 2 publications did not adjust their analysis for diet, and they were the only studies in our systematic review not adjusting for SES indicators. Moreover, they did not account for Asian dust storms (ADS). During ADS, a common weather phenomenon in Eastern Asia, dust from the deserts in Mongolia and China is transferred through the atmosphere to countries like Japan and Taiwan. Composition of PM in Japan, particularly during ADS, is likely to be different (containing more crust elements and sea salt) from that in Europe and North America. Adverse health effects of ADS have been reviewed in 2010,[39] and many additional epidemiological and experimental studies have confirmed the importance of ADS on respiratory and cardiovascular health in more recent years. Therefore, we decided that the 3 Chinese and 2 Japanese studies were too dissimilar from those conducted in North America and Europe to include them in the sensitivity analyses.

Recently, 3 meta-analyses on stroke mortality and hospitalization in association with recent PM exposure have been published.[12-14] The pooled HR for stroke mortality was 1.014 (1.009-1.019)[12]

or 1.013 (1.003-1.024)[13] for a 10-μg/m³ increment in $PM_{2.5}$. These increases in risk per 10-μg/m³ increment are smaller than those we obtained for a 5-μg/m³ increment in long-term exposure, but it should be noted that daily variation in ambient PM levels is usually substantially higher than spatial variation within a region. Recent exposure and long-term exposure to PM are different concepts and deserve equal attention. For short-term variation in ambient PM levels, the research question is *when* adverse events, such as strokes, are most likely to occur, whereas for long-term exposure, the question is rather *where* people are most at risk. However, although different in concept, the effects of short-term and long-term exposure to PM are not entirely independent from each other because peak elevations of PM (the exposure measure for short-term effects) are likely to occur more frequently in locations with higher long-term ambient PM concentrations.

Other studies on stroke and air pollution

Two studies on stroke and long-term PM exposure were not included in our meta-analysis because the study cohort was not representative for the general population. Koton et al.[40] found no association between stroke and $PM_{2.5}$ exposure in a cohort of myocardial infarct survivors. Similarly, Maheswaran et al.[41] studied a cohort of stroke survivors and found a 52% increased risk of all-cause death for a 10-μg/m³ increase in PM_{10} concentration.

We restricted our meta-analysis to publications on exposure to PM, but we also found studies quantifying (traffic-related) air pollution by using other pollutants, residential proximity to a major road, or noise as the exposure variable. Most of these studies were reviewed by Ljungman and Mittleman,[15] and they reported positive associations between stroke and long-term exposure to NO_2 or NO_x[22,26,42], SO_2[18], or CO[26]. However, null results for NO_x[43] and ozone[18,42] were found as well. In addition, Maheswaran and Elliott[44] reported higher stroke mortality for living within 200m of a main road compared with >1000m; Finkelstein et al[45] published similar results using 50m for an urban road and 100m for a highway as the exposure cut-off value.

Overall, these findings support those of our meta-analysis.

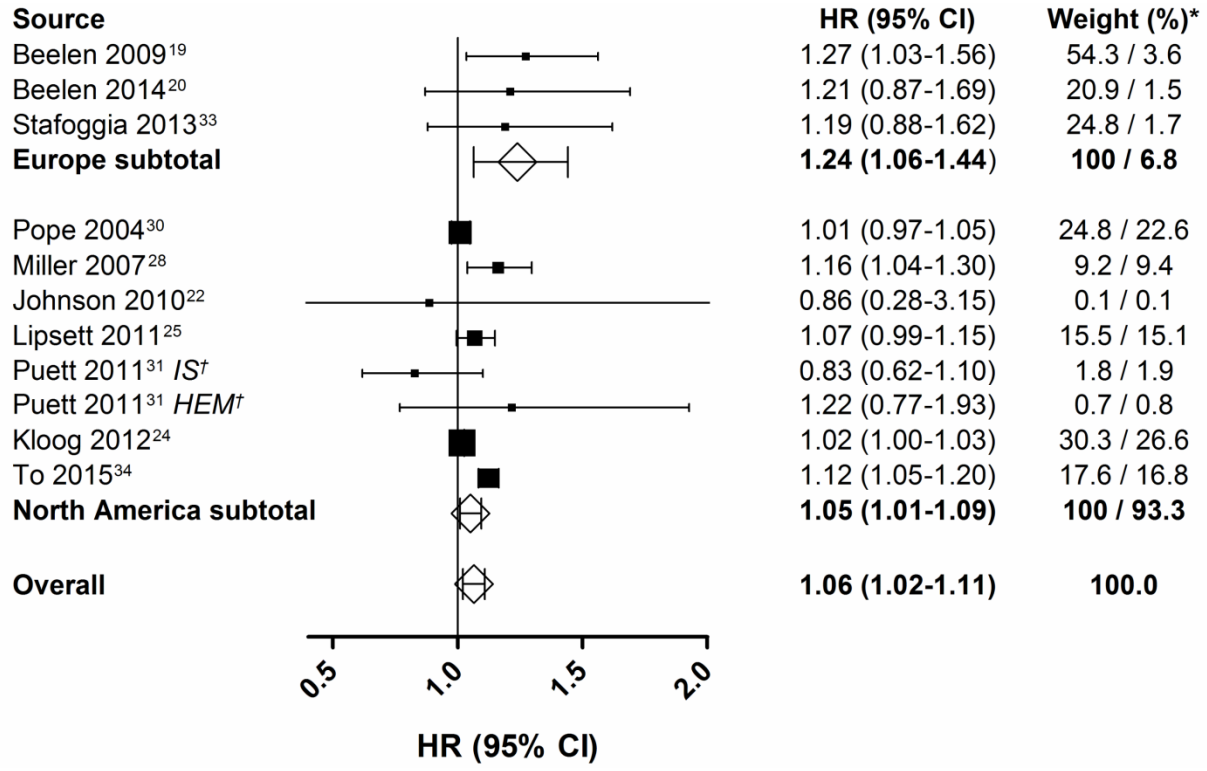

Figure 3. Forest plot of the subanalysis on stroke event and long-term $PM_{2.5}$ exposure. Hazard ratios (HR) with 95% confidence intervals (CI) for a 5 µg/m³ increment in $PM_{2.5}$ are presented. Symbol size is proportional to the weight of the study in the meta-analysis. Open diamonds represent pooled results from meta-analysis.

*Weights are calculated as $(1/SE^2) / (\Sigma\ 1/SE^2) * 100$ (with SE^2 the variance of each study effect, and $\Sigma\ 1/SE^2$ the sum of inverted variances for all study effects): within continent / overall (sum may differ from 100 due to rounding).

†IS = ischemic stroke; HEM = hemorrhagic stroke

Table 3. Results of sensitivity analyses[*]

				Meta-analysis		Tests of heterogeneity		Test of publ. bias
Stratum	Pollutant	Endpoint	Nr. of studies	Combined HR[†,‡] (95% CI)	P value (model)	P value[§]	I^2 in % (95% CI)	P value[‖]
All (Europe + North America)	PM_{10}[†]	Stroke event	9	1.021 (0.975-1.069)	0.38	0.16	31.3 (0-66.3)	0.09
		Stroke mortality	5	1.091 (0.958-1.242)	0.19	0.011	74.5 (3.5-87.8)	0.33
	$PM_{2.5}$[‡]	Stroke event	10	1.064 (1.021-1.109)	0.003	0.006	59.8 (1.7-77.7)	0.070
		Stroke mortality	5	1.125 (1.007-1.256)	0.037	0.024	64.5 (0-84.4)	0.016
High quality score (Europe + North America)	PM_{10} + converted $PM_{2.5}$[†]	Stroke event	8	1.087 (1.023-1.154)	0.007	0.10	39.6 (0-70.8)	0.23
		Stroke mortality	4	1.056 (0.957-1.165)	0.28	0.09	53.9 (0-82.9)	0.18
	$PM_{2.5}$[‡]	Stroke event	5	1.094 (1.038-1.153)	0.001	0.36	8.2 (0-64.1)	0.88
		Stroke mortality	4	1.081 (0.981-1.190)	0.12	0.09	54.0 (0-82.9)	0.065

[*]The five Asian studies[29,32,35-37] were excluded from the sensitivity analyses; [†]HR for a 10 µg/m³ increment in PM_{10}; [‡]HR for a 5 µg/m³ increment in $PM_{2.5}$; [§]P for Cochran's Q test; [‖]P for Egger's test

Biological mechanisms

Ambient air pollution is a mixture of several pollutants, but epidemiological and experimental evidence suggests that PM explains the harm caused by air pollution best. By selecting PM_{10} as a common indicator, we may capture all effects of different sources and components of PM.

Four recent reviews[6,7,9,10] summarized the literature concerning biological pathways of the relationship between exposure to PM air pollution and cardiovascular disease. Chronic inhalation of pollutants may cause chronic pulmonary and systemic oxidative stress and inflammation that are critical and well-documented factors leading to the manifestation of endothelial dysfunction, vasoconstriction and atherosclerosis at the vasculature level and coagulation and thrombosis at the blood tissue level.[9,10,46,47] These processes in turn are key factors in the development of chronic or acute cardiovascular diseases, and similarly, they are critical for the onset of cerebrovascular events, such as stroke, especially ischemic stroke. Moreover, the neural cells of the brain are also vulnerable to long-term PM exposure. Particulates can impair the blood-brain barrier, either directly (after having penetrated into the circulatory system) or through the inflammatory processes mentioned above, and subsequently cause chronic inflammation and oxidative stress within the neural cells.[11]

Strengths and limitations

In this comprehensive literature review, we pooled data of 20 different studies from several geographical regions in 1 meta-analysis, thus increasing the statistical power and allowing an investigation of regional patterns. Furthermore, all these studies were published in the past decade (and even 14 of 20 in the past 4 years), indicating that data on both the exposure and the outcome are recent and relevant. By recalculating results for $PM_{2.5}$ to estimated results for PM_{10}, we were able to pool studies using $PM_{2.5}$ and those using PM_{10} as the exposure measure in the main analysis, in addition to separate analyses for both fractions.

This recalculation implies the use of a conversion factor. We opted for a conversion factor of 0.7[48] because $PM_{2.5}/PM_{10}$ ratios in the range of 0.5 to 0.8 have been reported, depending on region or city.[38] Although the estimated values may not reflect the true PM_{10} concentration for the studies in

question, changing the conversion factor to 0.5, 0.8, or a region-specific value did not at all influence the overall result (**Table S2**). In our subanalyses of $PM_{2.5}$ data only, the estimated effects were always higher than those in the corresponding analyses using PM_{10} and converted $PM_{2.5}$, indicating the importance of measuring $PM_{2.5}$ directly and confirming the hypothesis that the $PM_{2.5}$ fraction is more hazardous than the coarse fraction ($PM_{2.5-10}$) of PM_{10} .[8]

By pooling data for ischemic stroke and hemorrhagic stroke, we might have underestimated the true association between PM exposure and the onset of ischemic stroke. Indeed, evidence found in the literature suggests that the PM-related risk of ischemic stroke is higher than the risk of hemorrhagic stroke.[11,15] This is not surprising because ischemic stroke is related to general cardiovascular disease, whereas hemorrhagic stroke has a different pathogenesis. Unfortunately, only 4 of 20 publications in our meta-analysis published results for ischemic and hemorrhagic stroke separately.

Two other potential limitations concern the methodology of the original studies. First, the estimation of exposure was based on data obtained by central monitoring stations. All authors made efforts to approach the personal exposure by using data from the monitoring station closest to the home of the study subject, sometimes excluding subjects living too far from a station, or by applying spatial interpolation models. However, it is clear that the true exposure, taking into account time spent outdoors versus indoors, in traffic, at work, or in other regions can never be measured at an individual level in large-scale cohort or population-based studies. Moreover, 3 studies[21,27,28] extrapolated air pollution data recorded in 1 year to an estimate for the whole study period, hereby neglecting possible long-term trends.

Second, the ecological nature of register-based studies makes it difficult to account for confounding factors, such as smoking status and SES, because these data are generally not provided in the databases from which stroke events are retrieved. The 6 register-based studies included an estimate of SES on the area level (eg. a deprivation index), but only 1 included data on smoking status. In contrast, all 14 cohort studies adjusted for smoking and 9 adjusted for individual SES, by using

educational level, household income, employment status, or a combination of these factors as an indicator of SES. Notably, the 2 studies not adjusting for the important confounder SES were those conducted in Japan[29,35], which is another reason to interpret their study results with greater care.

Because of these important differences in study design and methodology, we created a quality index based on the design, exposure measurement and inclusion of important covariates. All high-quality studies had a prospective cohort design and measured air pollution exposure over the whole study period. In addition, all but one used spatial interpolation models to estimate personal exposure instead of raw data from monitoring stations. Including only high-quality studies resulted in higher pooled estimates for stroke event, but lower HRs for stroke mortality than the corresponding overall analyses.

Implications for public health

The Global Burden of Disease (GBD) 2010 study[49] provided global statistics on attributable deaths and disability-adjusted life years (DALYs) for 67 risk factors, including environmental air pollution. Worldwide, 3.7 million deaths and 3.1% of global DALYs were attributed to air pollution, placing it in the top 10 of risk factors. Cardiovascular and circulatory diseases (including stroke) accounted for the majority of deaths attributed to air pollution. According to the subsequent GBD 2013 study[1], stroke was the third cause of death, with a death rate of 110 per 100,000 inhabitants, resulting in > 6 million deaths worldwide in 2013. A recent paper,[50] based on the GBD 2013 study and exclusively dealing with stroke, presented new figures on DALYs due to stroke for selected risk factors. The authors reported 16.9% of DALYS attributed to air pollution, which is much higher than the 3.1% mentioned above, but here, percentages are not mutually exclusive. There was considerable geographic variation in the impact of air pollution. Globally, stroke-related burden of ambient air pollution increased in the period 1990-2013, but in high-income countries there was a significant reduction in DALYs due to air pollution (10.2% of DALYs, compared to 18.4% for low-income and middle-income countries). These values are similar to the PAFs for alcohol use, diabetes mellitus, and psychological stress, published in the INTERSTROKE study, a global case-control study on risk factors for stroke.[5]

Given the high incidence of stroke and stroke-attributed mortality[1,51], a substantial reduction of exposure to PM may result in an equally substantial decrease in stroke incidence and stroke mortality, not only in areas with extremely high exposures to PM, such as many cities in China, but also in areas with substantially lower ambient concentrations (although still higher than the World Health Organization guideline values), such as many regions in Western Europe and North America. A reduction of ambient PM concentrations requires urgent attention in many areas of the world. Indeed, in large cities worldwide, annual mean PM_{10} concentrations of 30 (Los Angeles), 60 (Sofia, Bulgaria), 70 (Santiago, Chile), 100 (Johannesburg, South Africa), or even 120 µg/m³ (Beijing, China) have been reported.[2] The argument that it is difficult to meet standards in densely populated areas ignores the fact that the importance of a factor with respect to public health increases in proportion to the number of people who are exposed to it. Several cities in North America, Scandinavia and the United Kingdom prove that ambient PM_{10} concentrations of < 20 µg/m³, as recommended by the World Health Organization[38], are realistic, even in an urban environment.

Measures taken to reduce the emissions of PM will not only decrease the risk of cerebrovascular disease but also, and to an even greater extent, that of cardiovascular and pulmonary disease[6,8]. Furthermore, such measures will lead to a decline in the occurrence of peak days with high levels of air pollution and, hence, to a decrease in acute effects caused by short-term exposure, such as stroke[12], cardiovascular and respiratory events and all-cause mortality[52].

Conclusion

In addition to the recognition of PM air pollution as a causal factor in the progression and triggering of cardiovascular disease, our meta-analysis provides evidence for a positive association between the risk of stroke and long-term PM exposure. Given the fact that the whole population is exposed, air pollution is an important risk factor for stroke, and among other diseases, stroke incidence and stroke mortality would substantially decrease when measures are taken to reduce ambient air pollution levels.

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SUPPLEMENTAL METHODS AND RESULTS

Literature search and selection of studies

We used the following search terms in the Pubmed database, without the use of quotation marks:

air pollution stroke

air pollution cerebrovascular disease

air pollution stroke mortality

air pollution cerebrovascular disease mortality

air pollution stroke hospital admissions

air pollution cerebrovascular disease hospital admissions

particulate matter stroke

particulate matter cerebrovascular disease

particulate matter stroke mortality

particulate matter cerebrovascular disease mortality

particulate matter stroke hospital admissions

particulate matter cerebrovascular disease hospital admissions

A flow chart of the selection procedure is given in **Figure S1**. Of the 596 studies identified after our initial search, 572 were excluded for various reasons. Of 24 included publications, six were further excluded because they reported findings based on the same population as another included study. Two initially undetected studies were added on the basis of references and reviews. Thus, we eventually included 20 publications on stroke and long-term PM exposure in our meta-analysis.[1-20]

The definition of the endpoint varied slightly among publications. Both terms 'cerebrovascular disease' and 'stroke' were found. All but four studies[10,11,14,15] defined stroke type based on the International Classification of Diseases (ICD) provided by the World Health Organization (WHO). Hence, it was possible to distinguish between all strokes (ICD-9 430-438 or ICD-10 I60-I69), ischemic strokes (ICD-9 434 or ICD-10 I63) and hemorrhagic strokes (ICD-9 430-432 or ICD-10 I60-I62). However, too few studies reported results for ischemic stroke or hemorrhagic stroke separately to allow analyses by stroke type. On the other hand, one study[14] did not show combined results for all strokes, but only for ischemic and hemorrhagic stroke separately, and we included both HRs in the meta-analysis. Seven studies published results on stroke mortality only. The other 13 papers included non-fatal strokes and defined the outcome as 'stroke incidence' (6), 'first stroke' (4 publications), 'hospital admission' (2), or 'first hospital admission' (1). Five out of these 13 studies conducted a subanalysis on stroke mortality (see **Figure S1**).

Variation in $PM_{2.5}$ values in the Edmonton study[5] was low, which resulted in a HR with a very large CI, thus having virtually no weight in the meta-analysis. Qin et al.[15] presented no overall HR, but only results for stratified analyses by body weight class (normal, BMI<25; overweight, 25<BMI<30; obese, BMI>30). We included these stratified analyses as separate study results in the meta-analysis.

Quality of studies and standardization of results

We assessed quality of the selected studies taking into account the following aspects: study design, number and nature of covariates in the analysis, estimation of the exposure, and definition of the endpoint. Scores for each of these aspects and the overall quality score are presented in Table . Studies with an overall quality score above the median were included in the subanalyses of high quality studies. After removing the five Asian studies[12,15,18-20] from the list, the cut-off was a score of 7 for overall stroke event (n=15 studies) and 6.75 for the mortality subset (n=8).

We standardized reported results to hazard ratios (HR) for a 10 µg/m³ increment of PM_{10} in three steps. First, whenever the HR or relative risk (RR) was not presented on a continuous scale, but as a HR or RR for each exposure quantile with the lowest quantile as a reference, we calculated the difference (D) between the means of the highest and the lowest quantile and treated the HR for the highest quantile compared to the lowest as a HR for a D µg/m³ increment of PM (HR_D).

Second, each HR_D and each HR for an interquartile range (IQR) increment (HR_{IQR}) was recalculated to a HR per 10 µg/m³ increment ($HR_{10\mu g}$) with the formula $HR_{10\mu g} = HR_D\text{^}(10/D)$ or $HR_{10\mu g} = HR_{IQR}\text{^}(10/IQR)$ for PM_{10} and, analogously, to a HR per 5 µg/m³ increment for $PM_{2.5}$: $HR_{5\mu g} = HR_D\text{^}(5/D)$ or $HR_{5\mu g} = HR_{IQR}\text{^}(5/IQR)$.

Third, since we aimed to insert study results for both PM_{10} and $PM_{2.5}$ in a single meta-analysis, we transformed results for $PM_{2.5}$ to estimated results for PM_{10} by applying a conversion factor assuming that, on average, PM_{10} consists of 70% of $PM_{2.5}$ ($HR_{PM10} = HR_{PM2.5}\text{^}0.7$).[21] However, proportions of 50% to 80% have been reported in studies measuring both fractions in the same environment.[22,23] Therefore, the main analyses on stroke event and stroke mortality were repeated with conversion factors of 0.5 and 0.8. Moreover, we estimated PM_{10} values for each of the six studies concerned by applying a region-specific conversion factor, based on the WHO 2014 air pollution database.[24] This conversion factor was 0.6 for the four studies from the US[7,11,13,17], 0.36 for the study conducted in Edmonton, Canada[5], and 0.67 for the Dutch study[2].

Hazard ratios (HR), 95% confidence intervals (CI), and tests for heterogeneity and publication bias proved to be robust against changes in the conversion factor (**Table S2**).

Heterogeneity and publication bias

The overall HR and 95% confidence interval (CI) was estimated using a random-effects model. To check the assumption of substantial heterogeneity among studies, we applied Cochran's Q test and calculated the I^2 statistic. I^2 values of ≤25%, 25-75% and ≥75% indicate low, moderate and high heterogeneity, respectively.[25] Publication bias was assessed by visual inspection of funnel plots and by formal testing with Egger's linear regression method.[26]

Statistics on heterogeneity among studies and publication bias are shown in **Tables 2** and **3** of the main text. Our *a priori* choice for random effects models was justified, given the considerable heterogeneity. Cochran's Q test was significant in most main and subgroup meta-analyses, and I^2 values indicated moderate to high heterogeneity.

Figures S2 to **S4** show the funnel plots for the inspection of publication bias in the overall analysis (15 studies from Europe and North America, **Figure S2**), the analysis of stroke mortality data only (8 studies, **Figure S3**) and the analysis of $PM_{2.5}$ exposure data only (10 studies, **Figure S4**). Severe departure from symmetry around the meta-estimate (indicated by the vertical axis) suggests publication bias towards negative (left) or positive (right) study results.

We found no indications of publication bias in most analyses. However, Egger's test results and funnel plots of the main analyses for Europe and North America taken together suggest a bias towards positive results for studies with large CIs (P=0.018 for stroke event and P=0.040 for stroke mortality) (**Table 2** in main paper). When considering only the high quality studies, moderate publication bias was found only in the subset of stroke mortality with $PM_{2.5}$ exposure.

Supplemental Tables

Table S1. Quality assessment of studies included

Reference	Study design[a]	Covariates					Exposure measurement		Definition of stroke[i]	Overall quality score[j]
		Age + sex[b]	Lifestyle factors[c]	Health[d]	SES[e]	Other[f]	Period[g]	Method[h]		
Ueda et al. (2012)[18]	2	1	2	1	0	0	1	0	1	8
Nishiwaki et al. (2013)[12]	2	1	2	1	0	0	1	0	1	8
Zhang et al. (2014)[20]	1	1	2	0	1	0	1	0	0.5	6.5
Qin et al. (2015)[15]	1	1	2	0	1	0	1	1	0	7
Wong et al. (2015)[19]	2	1	2	0	1.5	0	1	1	0.5	9
Maheswaran et al. (2005)[9]	0	1	0	0	0.5	0	1	1	0.5	4
Beelen et al. (2009)[2]	2	1	1	0	0.5	0	1	1	0.5	7[*,†]
Huss et al. (2010)[4]	0	1	0	0	1.5	0.5	0	1	0.5	4.5
Maheswaran et al. (2012)[10]	0	1	0	0	0.5	0	0	1	0.5	3
Atkinson et al. (2013)[1]	0	1	1	1	0.5	0	1	1	1	6.5
Beelen et al. (2014)[3]	2	1	2	0	1.5	0	1	1	0.5	9[*,†]
Katsoulis et al. (2014)[6]	2	1	2	1	1	0	1	1	0.5	9.5[*]
Stafoggia et al. (2014)[16]	2	1	1	0	1.5	0.5	0.5	1	1	8.5[*]
Pope et al. (2004)[13]	2	1	2	0	1	0	0.5	0	0.5	7[*,†]
Miller et al. (2007)[11]	2	1	1	1	1	0	0	0	0.5	6.5
Johnson et al. (2010)[5]	0	1	0	0	0.5	0	1	1	0.5	4
Lipsett et al. (2011)[8]	2	1	2	1	0.5	0	1	1	1	9.5[*,†]
Puett et al. (2011)[14]	2	1	2	1	0	0.5	1	1	0.5	9[*]
Kloog et al. (2012)[7]	0	0.5	0	0	0.5	0.5	1	1	0.5	4
To et al. (2015)[17]	2	1	2	0	1.5	0	1	1	0.5	9[*]

[a]Prospective cohort with personal baseline questionnaire of all subjects: 2 points; Cohort study with questionnaire organized later: 1 point; Register-based ecological study: 0 points

[b]Adjusted for age and sex: 0.5 points each (in cohort studies on men or women only, the 0.5 points were awarded)

[c]Adjusted for lifestyle factors: 1 point for smoking status; 1 point for at least two of: alcohol consumption, diet, physical activity, occupational exposure.

[d]Adjusted for health: 1 point for BMI plus at least one of: blood pressure (hypertension, medication), cholesterol, diabetes, family history of cardiovascular disease

[e]Adjusted for SES: 1 point for estimation of SES at an individual level (at least one of: income, education, employment status), 0.5 points for area-level estimation (e.g. deprivation index)

[f]Additionally adjusted for at least one of: noise, distance to major road, temperature, season.

[g]Measured during the whole study period: 1 point; Measured during 1 year and extrapolated to whole period: 0 points; Measured during >1y but less than study period: 0.5 points

[h]Interpolation model used (e.g. land-use regression model): 1 point; Raw data from nearest monitoring station: 0 points

[i]Based on CD-9 or ICD-10 coding: 0.5 points; First stroke (i.e. individuals with stroke history excluded): 0.5 points

[j]Sum of scores

[*]Included in the overall subanalysis on high-quality studies (Europe and North America only)

[†]Included in the mortality subanalysis on high-quality studies (Europe and North America only)

Table S2. Results with other conversion factors for $PM_{2.5}$

Pollutant	Endpoint	Conversion factor for $PM_{2.5}$	Nr. of studies	Meta-analysis		Tests of heterogeneity		Test of publ. bias
				Combined HR* (95% CI)	P (model)	P (Cochran's Q)	I^2 in % (95% CI)	P (Egger's test)
PM_{10} +	Stroke event	0.5	20	1.056 (1.019-1.093)	0.003	<0.001	85.6 (79.8-89.1)	0.13
converted		**0.7**	**20**	**1.061 (1.018-1.105)**	**0.005**	**<0.001**	**85.8 (80.2-89.3)**	**0.11**
$PM_{2.5}$		0.8	20	1.062 (1.017-1.110)	0.006	<0.001	85.9 (80.3-89.3)	0.10
		specific†	20	1.057 (1.016-1.099)	0.005	<0.001	86.3 (80.8-89.7)	0.14
	Stroke mortality	0.5	12	1.073 (0.996-1.156)	0.063	<0.001	90.9 (86.6-93.4)	0.21
		0.7	**12**	**1.080 (0.992-1.177)**	**0.077**	**<0.001**	**90.9 (86.6-93.4)**	**0.21**
		0.8	12	1.082 (0.990-1.184)	0.083	<0.001	90.9 (86.6-93.4)	0.21
		specific†	12	1.075 (0.994-1.161)	0.080	<0.001	90.9 (86.6-93.4)	0.21

In bold the conversion factor and related results chosen by the authors of the present paper

*HR for a 10 µg/m³ increment in PM_{10} or converted $PM_{2.5}$

†For the six studies concerned,[2,5,7,11,13,17] the conversion factor was 0.6, 0.6, 0.36, 0.67, 0.6, and 0.6, respectively.[24]

Supplemental Figures

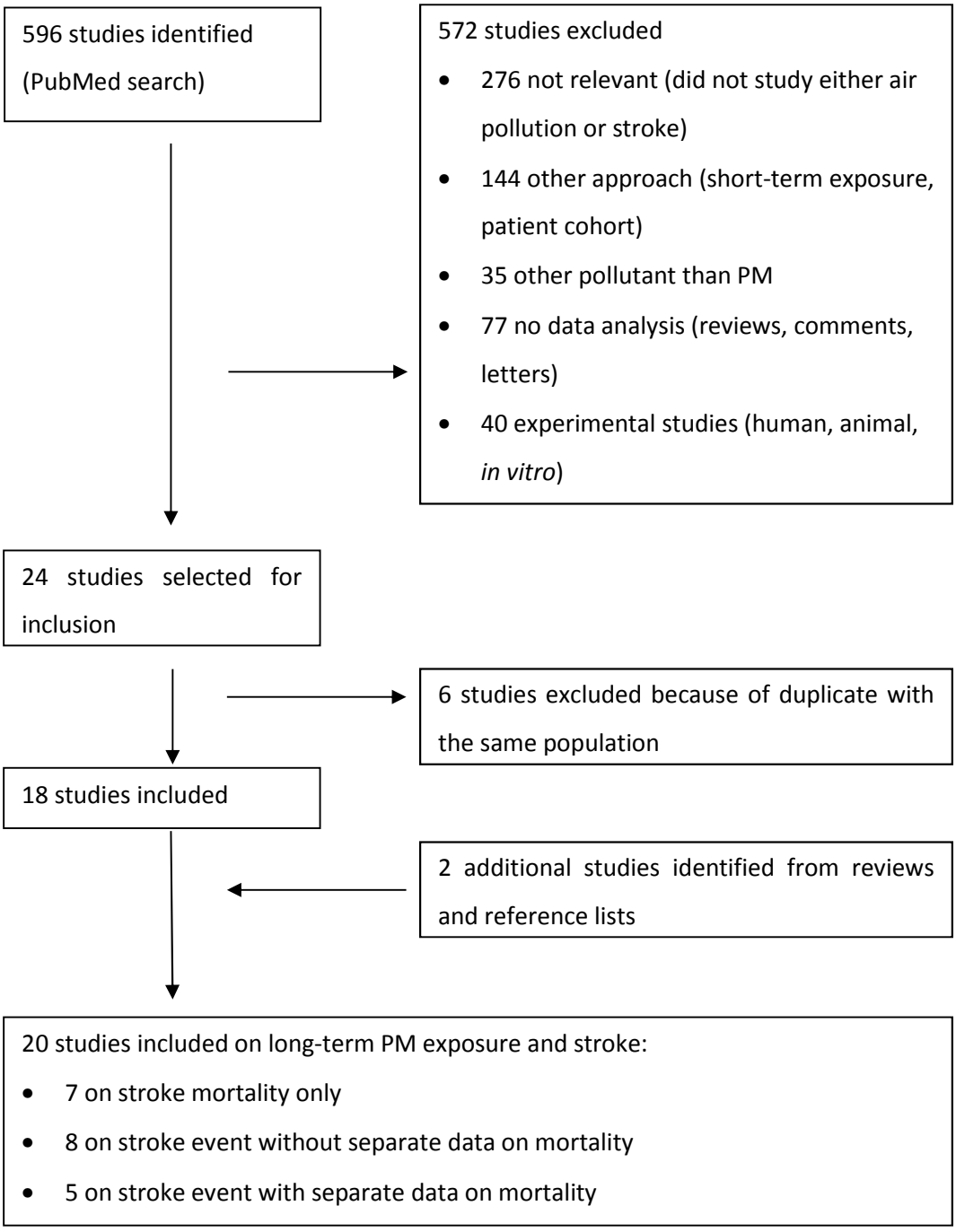

Figure S1. Flowchart of the literature search.

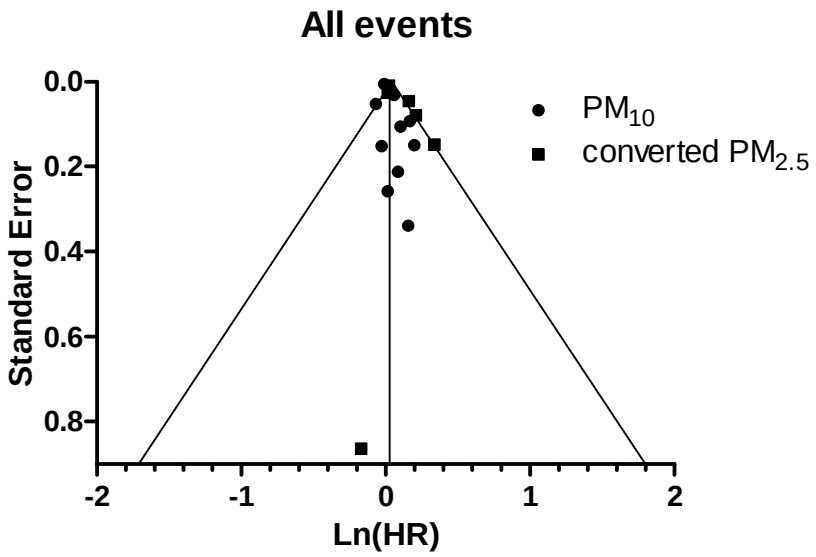

Figure S2. Funnel plot of individual study hazard ratio (presented on a natural log scale) for the association between stroke event and PM_{10} (including converted $PM_{2.5}$) exposure (n=15 studies, North America + Europe).

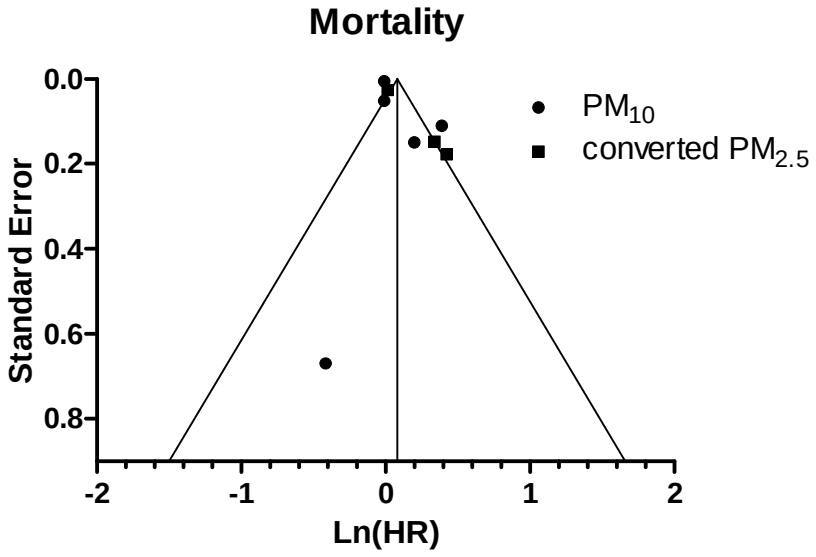

Figure S3. Funnel plot of individual study hazard ratio (presented on a natural log scale) for the association between stroke mortality and PM_{10} (including converted $PM_{2.5}$) exposure (n=8 studies, North America + Europe).

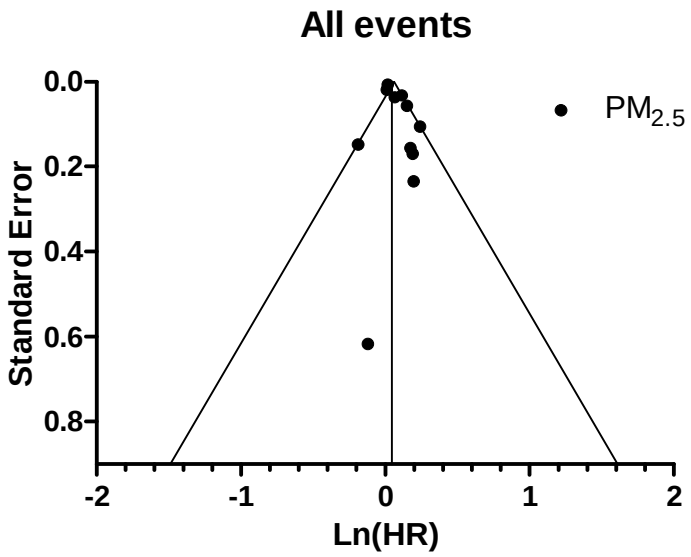

Figure S4. Funnel plot of individual study hazard ratio (presented on a natural log scale) for the association between stroke event and $PM_{2.5}$ exposure (n=10 studies, North America + Europe).

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DISCUSSION

In this PhD project, I investigated adverse effects of exposure to ambient air pollution on human health in four studies. Although these studies differed in their target populations, time windows of the exposure, and health outcomes (**Table 1**), they all established positive associations between air pollution and risk of mortality or morbidity.

In summary, we found that brief periods (1 to 3 days) of elevated particulate matter (PM) concentration can trigger mortality in late neonates (**Chapter 1**) and graft rejection in lung-transplanted (LTx) patients (**Chapter 2**) in Flanders, Belgium. The region of Flanders currently has PM exposure levels around (for daily averages) or below (for yearly averages) the European Union (EU) limit values, but well above the more stringent guideline values proposed by the World Health Organization (WHO). We then found effects of short- to medium-term (up to one week) exposure to air pollution on carotid stiffness, a biomarker of cardiovascular disease, in a study panel of elderly volunteers (**Chapter 3**). These effects were detected in both directions: carotid stiffness decreased when air pollution concentrations were below the volunteers' usually experienced level of exposure, and increased when air pollution levels increased. Finally, I provided meta-analytical evidence for an association between long-term exposure to air pollution and the risk of having a stroke, including stroke mortality (**Chapter 4**).

EXPOSURE ASSESSMENT

In epidemiology, correctly measuring exposure to air pollution is crucial to estimate health effects related to it. However, measuring true exposure is a challenging task: first, especially for PM, not only mass concentration, but also size, chemical composition and oxidative potential of the particles can determine the eventual effects on health.[1-5] Second, air pollution concentrations that are routinely measured by fixed monitor stations do not necessarily reflect accurately levels of exposure for individuals, since these levels are also determined by personal factors and activity patterns.[6,7] Third, the amount of PM that eventually penetrates into the alveoli is not measured directly in epidemiological studies, but is assumed to be proportional to the ambient concentration or to another proxy,

such as distance to major roads. I will discuss these three issues, and how we tackled them, in the following paragraphs.

Characteristics of particles

Size. In our studies on infant mortality and LTx patients, we used PM_{10} mass concentrations as a measure of exposure. PM_{10} levels were obtained from the Belgian telemetric air quality network, which is managed by the Belgian Interregional Environment Agency (IRCEL). During the time periods of exposure in these two studies, $PM_{2.5}$ was monitored by only a limited number of stations. However, in the panel study on carotid stiffness, the growing number of monitor stations measuring $PM_{2.5}$ allowed us to include $PM_{2.5}$ as an exposure variable. Ambient concentrations of ultrafine particles (UFP or $PM_{0.1}$), which are considered the most hazardous fraction of PM,[1] are not being measured by the air quality network.

Table 1. Characteristics of study populations and methodologies used in this PhD thesis.

Chapter	Study population	Time window of exposure*	Health effect	Study type
1	Infants	Acute / short-term (1 to 3 days)	Mortality	Case-crossover
2	LTx patients	Acute / short-term (1 to 3 days)	Graft rejection	Cohort, follow-up
3	Healthy elderly	Subacute / medium-term (1 to 7 days)	Carotid stiffness, markers of inflammation, endothelial function	Cohort, observational and intervention
4	General population	Chronic / long-term (several years)	Stroke	Meta-analysis of literature

*For exposure, both sequences acute-subacute-(subchronic-)chronic and short-medium-long term are used. All these time windows of exposure can be related to acute or chronic health events.

In the carotid stiffness study, we found significant and similar health effects of PM_{10} and $PM_{2.5}$ for most variables (see **Table 2** in **Chapter 3**), and in the stroke meta-analysis, we clearly found stronger effects in the studies using $PM_{2.5}$ than in those using PM_{10} as a measure of exposure (see **Table 3** in **Chapter 4**). These findings are compatible with the idea that more pronounced effects would be found when using a more refined indicator of exposure (e.g. UFP) than those already detected with the crudest indicator, PM_{10}. However, it is clear that this speculation can only be confirmed when UFP concentrations will be monitored on a large scale (as recommended in the "Ten principles for clean air"[8]).

Chemical composition. It was beyond the scope of this thesis to quantify chemical composition of particles. In an epidemiological study conducted in Belgium, investigators from our research group quantified elemental composition of $PM_{2.5}$ and oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) in PM_{10}.[9] They found significant effects of concentrations of vanadium (V), nickel (Ni), iron (Fe), and two different oxy-PAHs on pulse pressure in elderly subjects. These and other chemicals originate from (incomplete) combustion processes (by both industry and vehicles), and have been linked to oxidative stress[10-12] and cardiovascular disease.[13,14] In Belgium, main emission sources of $PM_{2.5}$ are general energy use and road transport.[15] We assume that, in general, PM, as measured in our studies, has a similar chemical composition as the samples analyzed by Jacobs et al.,[9] and that both the sources (*i.e.* incomplete combustion processes in various economic sectors) and health effects of PM can be explained similarly.

In the carotid stiffness study, we estimated exposure to NO_2 and black carbon (BC), in addition to $PM_{2.5}$ and PM_{10}. NO_2 is a good marker of traffic-related exposure, and BC, a component of $PM_{2.5}$, is a good indicator of the combustion-derived and potentially very harmful parts of PM.[16] We found similar effects of PM_{10}, $PM_{2.5}$, NO2 and BC in the carotid stiffness study, again suggesting that using mass concentration of PM_{10} (and $PM_{2.5}$) continues to be a valid tool to investigate health effects of exposure to air pollution.

Estimating individual exposure

In all our studies, we used PM data from the Belgian telemetric air quality network as the measure of exposure. To better estimate residential exposure to PM_{10}, $PM_{2.5}$, and NO_2, a land use regression model combined with a dispersion model was developed to interpolate data from measurement stations to values in 4 by 4 km grids (or to PM_{10} values by municipality of residence in the infant mortality study, as the exact addresses were unknown).[17] For the large-scale epidemiological studies on infant mortality and LTx patients, measuring exposure at a more individual level was unachievable, but in sensitivity analyses, we proved that the effect of spatial variability was negligible, and that only temporal variation is important in studies on short-term exposure to PM.

In the carotid stiffness study, the study population consisted of a panel of 10 elderly couples. During the trips abroad, in Milan (Italy) and Vindeln (Sweden), exposure to air pollution and activity patterns were assumed to be fairly similar among study volunteers. Therefore, we used two portable laser-operated aerosol mass analysers to measure common exposure of the whole panel to PM_{10}, $PM_{2.5}$, and BC. In addition, during each measurement period of this study, we estimated real personal exposure to NO_2 using Radiello diffusive samplers (**Figure 1**).[18] Six to 10 study volunteers wore the clip-on device during six days prior to each health assessment day. Then, we collected the devices and sent them to a specialized lab for the quantification of average exposure to NO_2 during the sampling period.

In general, we had good experiences with the use of these NO_2 samplers. In contrast to the PM and BC mass analysers, they are passive samplers, and hence, much cheaper and not prone to mechanical failure. The devices are easy to clip on a shirt or coat and convenient to wear, as they are small and very light. During stationary activities (e.g. sleeping, other indoor activities, gardening), the samplers can simply be put on a table. The major drawback of this method is that study volunteers are responsible for a correct use of the samplers: when going out, the device can easily be forgotten; the sampler body should not be in contact with water (e.g. heavy rain); and it should not be removed from the triangular support plate, as any contact with the adsorbing cartridges inside the sampler has to be

avoided. Also, analysis of the adsorbed amount of NO_2 produces only one value for the whole sampling period, so any daily variation during the six days of sampling remains undetected.

Analysis of exposure data in the carotid stiffness study revealed that individual NO_2 exposure, as measured by the Radiello samplers, was higher than the exposure estimated from central monitoring stations in Milan, and much lower than that in Sweden (**Figure 2** in **Chapter 3**). This was not surprising because in Milan, study volunteers walked along busy roads and used the metro frequently, and in Sweden, central NO_2 values were measured in the city of Umeå, whereas the volunteers resided in the rural town of Vindeln and frequented the surrounding woods. This observation illustrates the added value of measuring exposure to air pollution at an individual level in panel or cohort studies.

Figure 1. Radiello diffusive sampler consisting of an adsorbing cartridge (brown in this picture), a cylindrical diffusive body, and a triangular clip-on support plate (Sigma-Aldrich, Bellefonte, PA, USA)

Measuring "true" amount of inhaled particles

Even when measured at an individual level, ambient concentrations of air pollutants are not necessarily equal or proportional to the amount of particles that eventually penetrate into the alveoli and beyond. Inhaled particles are phagocytosed by airway macrophages, and the BC core of particles can be visualized using light microscopy. Researchers from our group demonstrated that the amount of BC inside airway macrophages can serve as a marker of individual exposure to PM,[19] and recently wrote a review on the literature on this topic.[20]

We collected induced sputum samples from the volunteers in the carotid stiffness study. These samples are currently being analyzed in our lab in an attempt to estimate "true" exposure to PM in three European locations with different levels of ambient air pollution levels.

HEALTH EFFECTS OF EXPOSURE TO AIR POLLUTION

In the **Introduction**, I wrote an elaborate review on the history and current state of knowledge on health effects of air pollution, including a discussion of biological pathways and susceptible populations. Therefore, here, I will discuss only those aspects that are relevant in regard of this thesis.

Susceptible populations

Infants. Children are considered particularly susceptible to air pollution, because their lungs and immune system are immature during the first few years of life. Using a case-crossover (CCO) approach, we found an elevated risk of mortality in late neonates (2-4 weeks of age) associated with short-term increases in ambient PM_{10} levels.

At the time of publication of this study, the available literature yielded mixed results, and the number of CCO studies and studies conducted in western Europe on infant mortality was rather limited. Recently, a study in Japan found similar results as ours for infant mortality and same-day $PM_{2.5}$, except that they found stronger associations in postneonates (OR = 1.06) than in neonates (OR = 1.02, NS), although not distinguishing between early and late neonates.[21] A German study reported a decrease in infant mortality associated with a decrease of ambient SO_2 levels over an 18-year period.[22]

In addition, other endpoints, such as low birth weight,[23-26] preterm birth,[25,27] impaired lung function,[28] and childhood asthma[28-31] have abundantly been associated to air pollution exposure during pregnancy and early life, providing convincing evidence that infants and young children are indeed particularly susceptible to adverse health effects of air pollution.

Lung-transplanted patients. We found that short-term variation in exposure to PM was associated to an increased risk of graft rejection in LTx patients at the UZ Leuven (**Chapter 2**), and a similar association has been reported for long-term exposure.[32,33] In a multi-center research project involving 13 LTx centers throughout Europe, our colleagues from the Lung Transplantation Unit have recently found evidence for lung allograft dysfunction and mortality among LTx patients associated with long-term exposure to air pollution,[34] thus confirming our single-center study results mentioned above. In addition, protective effects of macrolides, such as azithromycin, were found in both studies. These findings are clinically relevant, since the transplanted lung experiences a higher rejection rate than other solid organ transplantations.

Elderly. A gradual decline in physiological processes over time, and a higher prevalence of pre-existing cardiovascular and other diseases are two factors that make elderly more susceptible for the adverse effects of air pollution.[35] However, in our panel study on carotid stiffness, we deliberately selected healthy elderly with no pre-existing conditions. Still, we detected significant associations between biomarkers of carotid stiffness and air pollution. Associations of a similar magnitude have been found in healthy women averaging 40 years of age,[36] and healthy men averaging only 26 years of age.[37] Therefore, it is not clear whether the effects of air pollution exposure on carotid stiffness that we found in our study panel, were present *because* of their older age.

Susceptibility for hypertension. In the meta-analysis on stroke, there was no supposedly susceptible "target" population, but we found considerable geographical heterogeneity in the results: in Western Europe and North America, we found a positive association between stroke and air pollution exposure, whereas the two Japanese studies[38,39] reported a negative association (for details,

see **Chapter 4**). The reasons for this discrepancy have not entirely been elucidated yet, but there is very likely a role for hypertension.

Hypertension is the most important risk factor for stroke,[40,41] and stroke is more prevalent in the Far East than in Western Europe.[39,42,43] There are indications that Asians are more susceptible for diet-related hypertension (and hence, stroke) than Caucasians.[42-44] This may explain how the apparently protective effect of air pollution in Japan actually might reflect an adverse effect of diet, with higher salt intakes in rural and coastal regions (with lower air pollution) than in urban regions (with higher air pollution).

In other words, diet-related hypertension is such a dominant risk factor for stroke that it may mask the additional risk of air pollution exposure, especially in East Asia. However, this hypothesis requires further investigation and, to complicate the matter even more, blood pressure itself has also been shown to be adversely related to air pollution.[3,45,46]

Biological pathways

Inflammation. Inhalation of particles into the alveoli can cause pulmonary oxidative stress and inflammation, and subsequently, systemic oxidative stress and inflammation, by lung-blood transport of either inflammatory mediators (cytokines, activated WBC, platelets), or the particles themselves.[1,3] We quantified markers of inflammatory responses in two studies, yielding mixed results.

In the cohort of LTx patients, we found positive associations between recent exposure to elevated levels of PM and numbers of neutrophils and lymphocytes in bronchoalveolar lavage (BAL) fluid. However, we could not detect a direct mechanistic link between PM_{10} exposure and airway neutrophilia, since relevant cytokines (IL-6 and IL-8) were not associated with exposure. Concerning the circulatory system, plasma levels of C-reactive protein (CRP), a marker of systemic inflammation, showed no association with PM exposure either, whereas circulatory white blood cells (WBC) were not available in this study.

In the panel of healthy elderly, we measured biomarkers of inflammation in plasma, but not in the airways. We found no evidence of systemic inflammation, quantified as concentrations of WBC

(including differential counts of neutrophils and lymphocytes) and CRP. In general, controlled-exposure studies at relatively low exposure levels in healthy humans, such as ours, have not demonstrated a robust inflammatory response.[3]

Arterial stiffness. In the same study, we found evidence for a link between carotid arterial stiffness, indicated by increased PWV and YEM and decreased DC and CC, and short-to- medium-term exposure to several pollutants. Arterial stiffness is an important determinant of acute cardiovascular events such as myocardial infarction and stroke.[47,48] Since acute effects of elevated air pollution on myocardial infarction and stroke have repeatedly been demonstrated,[49,50] our results provide a possible pathway for this trigger effect. Similar associations between short-term air pollution exposure and arterial stiffness were found in recent intervention and epidemiological studies.[36,37]

Endothelial function. Endothelial dysfunction, a marker of atherosclerotic processes,[51] has repeatedly been associated with increased air pollution exposure levels.[3,52,53] However, in our panel study of healthy elderly, we found a positive association between endothelial function and concentrations of four different pollutants. Possible explanations for this unexpected result, such as the circadian rhythm of endothelial function and the suitability of the device used, are discussed in **Chapter 3**. In brief, endothelial function in Milan, i.e. the location with highest air pollution exposure, was remarkably high. However, it was also the only occasion with measurements of endothelial function in the late afternoon or evening. In Leuven and in Vindeln, we always quantified this parameter in the morning. There are indications that endothelial function sustains a circadian rhythm, with a lower RHI in the morning. Therefore, our results on endothelial function and air pollution exposure have to be interpreted with care.

Shape of the association

From the infant mortality study, we concluded that a linear model adequately described the association between infant mortality and air pollution, with no evidence for a threshold at low levels of PM or a plateau value in the higher regions of exposure. In the carotid stiffness panel study, non-linear exposure terms were tested, but did not show stronger associations with health outcomes than

a linear model. No formal tests of linearity were performed in the study on LTx patients or in the stroke meta-analysis.

Our indications that the association between PM concentration and health endpoints is linear, with no evidence for a threshold or a 'safe level', are in line with those from earlier studies that modeled the shape of the exposure-response relationship.[54-56] This is an important observation when it comes to public health and air pollution policy (see further).

PUBLIC HEALTH RELEVANCE

In four different study settings, we found significant associations between adverse health effects and exposure to air pollution, but calculated risks were always in the order of magnitude of a few percent, which is rather low from an individual point of view. [A notable exception is the 74% increased risk of infant mortality for days with PM_{10} > 50 µg/m³ compared to days below this cutoff value]. However, the whole population is exposed to air pollution and, therefore, small changes in population mean effects, could have substantial public health relevance and are important for prevention.[57,58]

The linear shape of the exposure-response curve means that there is no 'safe level' of air pollution, but also that any decrease in ambient air pollution levels should proportionally result in a decrease in adverse health effects. In our panel study on carotid stiffness, we found indeed that decreases in air pollution exposure, compared to the 'normal' level of exposure, were associated with decreases in stiffness. Follow-up analyses of the Harvard Six Cities cohort study showed a reduction in mortality risk in association with a decrease in ambient PM concentrations.[59,60] Similarly, a Swiss study found reduced rates of respiratory symptoms related to decreased PM_{10} exposure.[61]

FUTURE PERSPECTIVES AND RECOMMENDATIONS

We found adverse health effects of air pollution in susceptible populations (infants, LTx patients, and elderly) and for different time windows (short-term variation in exposure, subacute exposure of one week, long-term exposure). In this final paragraph, I will briefly discuss what these results, combined with an impressive and still growing amount of scientific evidence, could be of relevance for

researchers, policy makers, and the general population. In the **Introduction**, I already discussed many of these items on the basis of the "Ten principles of clean air".[8]

Scientists and health professionals

Among researchers, there is a broad consensus that air pollution is responsible for a wide variety of adverse health effects, but further investigation is still required to fully understand the (public) health impact of air pollution. A current challenge in air pollution research is that subtle changes in health parameters are susceptible to residual error and confounding. To reduce noise and uncertainty in the proposed pathophysiological pathways, continued efforts are needed to estimate true exposure with greater precision. Routine measurements of UFP and BC, and further research toward the magnitude of the health effects of these pollutants are required, since these are considered the most hazardous fractions of PM, and have much steeper gradients close to roadways than the more homogenously distributed coarse fraction of PM.

Clinicians, such as pneumologists and cardiologists, should keep abreast of the newest insights in the mechanisms of pollution-related disease, in order to provide appropriate curative treatment (e.g. administration of macrolides to LTx patients). General practitioners have an even more important role to play. As primary health care providers and confidential advisors, they will need to counsel their patients correctly on prevention strategies, such as avoiding use of indoor combustion, or limiting outdoor exercise at times (e.g. peak traffic periods) or places (e.g. busy roads) with elevated exposure to air pollution.

Policy makers

First, local authorities need to develop *ad hoc* smog alert protocols to minimize the impact of brief episodes of air pollution peaks. Measures can range from warning the population to refrain from certain activities (e.g. physical exercise) to imposing temporary speed limits or even a complete ban of road traffic. Swift communication with clear instructions via different channels is also an important part of the protocol.

Second, more stringent measures concerning emissions of diesel cars are needed, and their use should be discouraged. NO_x and PM emissions by diesel vehicles are much higher than those by cars driven by other fuels,[62] and hence, they largely contribute to ambient air pollution. Unfortunately, there are some recent examples of poor policy making regarding this subject. In 2010, the Belgian federal government implemented an "Eco Premium" for the purchase of small diesel cars because of their (theoretically) low CO_2 emission.[62] In February 2016, in the aftermath of the Volkswagen emissions scandal, the European Commission implemented a temporal relaxation of NOx emission limits for diesel cars.[62,63] On the plus side, the Eco Premium has been abandoned in 2012 and, moreover, road taxes for diesel cars have recently been raised in Flanders. As a result, the share of diesel cars in newly inscribed private cars has decreased from 63% to 23% over the past six years in Flanders.[64]

Third, it is time for a more ambitious long-term planning by the EU. The most recent EU directive, dating from 2008, imposes limit values for ambient PM concentrations that are much more relaxed than those recommended by the WHO (see **Table 2** in the **Introduction**). There is ample recent scientific evidence, including from our own study on infant mortality, that compliance with current EU limit values does not offer complete protection from adverse effects on public health.[65-67] Adopting the WHO guideline values as the new target for EU member states, would be a drastic, but necessary step towards clean air for all citizens. Even though the WHO guideline values are solely based on scientific evidence and ignore technical or economic feasibility, this should not discourage the proper authorities. Indeed, cost-benefit analyses showed that monetized benefits from a more stringent air pollution regulation would outweigh the costs.[68,69]

What can we do?

Not only scientists and policy makers, but also the general population has a role in the pursuit of a cleaner atmosphere. We have to be aware that we are not only victims of air pollution, but also producers. In the course of the 21st century, the overall emission of air pollutants has decreased in Flanders. This reduction is mainly a result of more stringent standards for industrial emissions and a

more efficient energy use. Consequently, the relative share of household and road traffic emissions has increased.[70]

The use of biomass fuel (i.e. wood) for heating is currently being promoted because of its CO_2-neutral image. However, combustion products from ordinary wood stoves and hearths are as toxic as those from fossil fuels. Better options for household heating are natural gas or electricity from solar panels. In any case, burning should be done in modern installations with efficient and clean burning, and only dry and untreated wood should be used. Indoor or outdoor burning of treated or painted wood, paper, cardboard and any other products than untreated wood is forbidden by law in Flanders. Also barbecues and indoor candles are frequently underestimated household contributors to air pollution.

There are plenty of fairly simple measures that we can take to reduce our emission of air pollution in traffic. This is a non-exhaustive list of advices: for short distances, take a bike instead of the car; make use of public transport; when driving, limit tailpipe emissions (implement an economical driving style, do not always turn on the air conditioning); carpool if possible; when purchasing a car, choose wisely in terms of fuel type (no diesel) and size. Be aware that also non-tailpipe emissions, originating from friction of brakes, tyres and road surfaces, contribute significantly to ambient PM at roadside.[8]

Global climate change caused by increasing emissions of greenhouse gases such as CO_2, is a hot topic and a serious problem indeed. Examples such as the eco premium for diesel cars and the promotion of biomass fuels might suggest a conflict of interest between climate-related and air pollution-related measures. However, recent research showed that air pollutants, and ozone and BC in particular, have a positive climate forcing and, as a result, warm the climate.[71,72] Moreover, combined public health effects of air pollution and high temperatures during heat waves, are expected to be more severe than those of air pollution alone.[8] In general, simple behavioral adaptations to reduce one's personal emission of air pollutants, will also result in a smaller climate footprint, and vice versa.[73]

During the past years, the problem of air pollution has gained increasing attention in popular media. Both promotors of this PhD thesis have been interviewed for TV, radio and newspapers. Issues such as the Volkswagen emission scandal in 2015, the Oosterweel Link (a long running proposed project intended to complete the ring road around Antwerp, involving air pollution-related problems), the shifts in taxes on cars driven by different fuels, and the episodes of very poor air quality in cities such as Beijing, are regular news items in the Belgian media. In addition, air pollution is now discussed in biology textbooks for secondary education,[74] so teachers (also in primary schools) can also contribute to raise the awareness of the adults of the future.

There is certainly a need for more scientific research, but we already know a lot about the causes of, and solutions to, the public health impact of air pollution. It is now time to act in accordance with that knowledge.

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SUMMARY

BACKGROUND

In the past few decades, exposure to air pollution has been found to be associated with all-cause mortality, cardiovascular and respiratory morbidity, both in the short term (acute exposure) and the long term (chronic exposure). According to the most recent report by the World Health Organisation (WHO, 2014), 3.7 million deaths worldwide and per year are attributed to ambient air pollution, placing it in the top 10 of risk factors.

Ambient air pollution consists of particulate matter (PM) and gases, such as NO_2, SO_2, and ozone. Of all pollutants, PM is most reliably associated with human disease. It is usually classified according to particle size, with PM_{10} (particles smaller than 10 µm) as the most commonly studied fraction. The European Union (EU) has set two limit values for PM_{10} concentrations: annual mean levels of PM_{10} must not exceed 40 µg/m³ (25 µg/m³ for $PM_{2.5}$), and daily averages must not exceed 50 µg/m³ on more than 35 days/year, for any monitoring station in the EU member states. In contrast, the WHO advises that annual averages of PM_{10} levels should not exceed 20 µg/m³ (10 µg/m³ for $PM_{2.5}$) and that daily averages should not exceed 50 µg/m³ on more than 3 days/year.

OBJECTIVES

The general objective of this PhD project was to gain more insight in the relationship between PM and human health in susceptible subgroups, such as infants, lung-transplanted patients and elderly. Also, in two of the four studies conducted within the scope of this thesis, I investigated the possible biological mechanisms involved in the pathway from PM inhalation to disease. Finally, I aimed to evaluate EU limit values and WHO guidelines for ambient PM concentrations, based on our study results.

MAIN STUDY RESULTS

In a first study (Chapter 1), I investigated effects of daily variation in environmental PM_{10} on risk of infant mortality (<1y of age) in Flanders. 2382 infants died during the study period (1998-2006). The PM_{10} concentration averaged 31.9 µg/m³, and there were 321 days (an average of 35.7 days per year)

with a mean daily concentration exceeding 50 µg/m³. This means that the EU air quality standards were met for the yearly average, but barely for the daily average. It is clear, however, that the more stringent WHO guideline values were not met at all in Flanders.

Using a bidirectional time-stratified case-crossover (CCO) design, I found that PM_{10} was associated with infant mortality, especially in the late neonatal stage (i.e. between one week and one month of age, N=372). For each 10 µg/m³ increment of PM_{10}, the risk of late neonatal mortality increased with 11% (95% CI 1-22%). On days with average PM_{10} levels exceeding the EU limit value of 50 µg/m³, the risk of mortality was 74% higher (95% CI 18-158%) than on days below that value. The current EU limit value for PM_{10} is not protective to prevent triggering infant mortality. Moreover, the linear shape of the association between exposure and mortality gives no evidence for a threshold or a save level.

In a second study (Chapter 2), we investigated whether graft rejection after lung transplantation (LTx) could be linked to recent exposure to PM air pollution, and which underlying mechanisms are involved. In the period 2001-2011, transbronchial biopsies were repeatedly executed in 397 LTx recipients at the UZ Leuven. I linked estimated PM_{10} levels for each patient's home address with symptoms of graft rejection and related physiological parameters.

We found that a 10 µg/m³ increase in PM_{10} concentration 3 days before biopsy increased the risk of lymphocytic airway disease (LAD) with 12% (95% CI 1-25%). LAD is the pathological correlate of acute graft rejection after LTx. Additionally, PM_{10} exposure was positively associated with BAL counts of neutrophils and lymphocytes, indicating that inflammation plays a part in the physiological pathway. Preventive treatment with the antibiotic azithromycin appeared to block the effect of PM_{10} on acute graft rejection.

In chapter 3, I investigated whether a decrease or increase of exposure to air pollution during several days (compared to a person's 'normal' level) can already result in changes in biomarkers of cardiovascular health. During the course of one year, we measured air pollution exposure, carotid

arterial stiffness (a well-established marker of cardiovascular disease), and other biomarkers in a panel of 20 retired and healthy men and women in different locations: during a 10-day stay in Milan (Italy, PM_{10} > 50 μg/m³), during a similar 10-day stay in Vindeln (rural area in northern Sweden, PM_{10} < 10 μg/m³) and at regular time points in Leuven (reference location, PM_{10} ≈ 30 μg/m³).

Compared with Leuven, exposure to pollutants was higher in Milan and lower in Vindeln, with the highest contrast found for NO_2 (averages: Milan 63.7 μg/m³; Vindeln 4.4 μg/m³).We found strong associations between 7-days exposure to air pollution and arterial stiffness, e.g. a 4.4% decrease in compliance (i.e. an increase in stiffness) for a 10 μg/m³ increment in PM_{10}. However, no direct inflammatory effects, measured as concentration of plasma CRP and leukocytes, were detected.

Stroke, or cerebrovascular accident, is a prominent cause of mortality and it has been linked with exposure to air pollution. I performed a meta-analysis of the current literature to quantify the pooled association between stroke and long-term exposure to PM_{10} or $PM_{2.5}$ (Chapter 4).

I identified 20 studies on long-term PM exposure and stroke. The association between PM and stroke was positive in North America (N=7 studies), Europe (N=8), and China (N=3, with extremely high exposures), and negative in Japan (N=2). The estimated effect of $PM_{2.5}$ [6% increase (95% CI 2-11%) in stroke risk for a 5 μg/m³ increment in $PM_{2.5}$] was higher than the corresponding result using PM_{10} [2% increase (95% CI -2 to 7%) in stroke risk for a 10 μg/m³ increment in PM_{10}). This indicates the importance of measuring $PM_{2.5}$ directly and confirms the hypothesis that $PM_{2.5}$ is more hazardous than the coarse fraction ($PM_{2.5-10}$).

CONCLUSIONS

In this thesis, I found detrimental health effects of air pollution in different susceptible populations and for different time windows of exposure. Both short-term exposure (1 to 3 days) and long-term exposure (several months to years) can trigger acute events, such as stroke, lung graft rejection or infant mortality, but long-term exposure can also accelerate the development of chronic cardiovascular or respiratory diseases, such as atherosclerosis or lung cancer. The two panel studies

(LTx and health elderly) provided mixed results concerning the role of inflammation in the process, as measured by levels of leukocytes and plasma CRP.

Compared to other environmental factors, such as smoking, diet and physical activity, individual risk estimates are rather small, but exposure to air pollution is involuntary and ubiquitous. Given the fact that the whole population is exposed, our studies demonstrated that air pollution is an important public health issue. In Belgium (and in Western Europe in general), yearly ambient concentrations of PM_{10} have decreased over the past 10 years to levels well below the EU limit value of 40 µg/m³, but still above the WHO guideline value of 20 µg/m³, especially in urban areas, where most people live. In accordance with other studies, we found no indications for a threshold or 'save' level of air pollution, so public health will benefit from every single µg/m³ decrease in concentration. This is a shared responsibility of policymakers, industry, and the general population (since road traffic and household combustion of wood are important and even increasing sources of air pollution) alike.

SAMENVATTING

ACHTERGROND

Wetenschappelijk onderzoek heeft de afgelopen 20 jaar aangetoond dat er een duidelijk verband bestaat tussen blootstelling aan luchtvervuiling enerzijds en cardiovasculaire en respiratoire morbiditeit anderzijds, zowel op korte termijn (acute blootstelling) als op lange termijn (chronische blootstelling). De meest recente studie van de Wereldgezondheidsorganisatie (WGO, in 2014) rapporteert dat er jaarlijks wereldwijd 3,7 miljoen mensen overlijden ten gevolge van luchtverontreinging. Hierdoor staat dit in de top 10 van de meest voorkomende risicofactoren voor vroegtijdig overlijden.

Luchtvervuiling in de atmosfeer bestaat uit fijn stof (PM, *particulate matter*) en gassen, zoals NO_2, SO_2 en ozon. Van alle polluenten heeft fijn stof de duidelijkste invloed op de menselijke gezondheid. Fijn stof wordt gewoonlijk ingedeeld volgens deeltjesgrootte. Fijn stof waarvan de deeltjes kleiner zijn dan 10 µm (PM_{10}) wordt het vaakst gemeten en gebruikt in studies. De Europese Unie (EU) heeft twee grenswaarden voor deze PM_{10} waarden vastgelegd. In alle EU-lidstaten moet het gemiddelde jaarniveau van PM_{10} minder dan 40 µg/m³ bedragen in elk meetstation van luchtkwaliteit (en 25 µg/m³ voor $PM_{2.5}$). Bovendien mag het daggemiddelde op niet meer dan 35 dagen per jaar de 50 µg/m³ overschrijden. De WGO daarentegen adviseert dat het gemiddelde jaarniveau van PM_{10} niet meer dan 20 µg/m³ mag bedragen (10 µg/m³ voor $PM_{2.5}$) en dat het daggemiddelde niet meer dan 3 dagen per jaar de waarde van 50 µg/m³ mag overschrijden.

DOELSTELLINGEN

De algemene doelstelling van dit doctoraatsonderzoek was meer inzicht krijgen in de relatie tussen fijn stof en menselijke gezondheid in gevoelige populaties zoals zuigelingen, longtransplantatiepatiënten en ouderen. In twee van de vier studies onderzocht ik eveneens mogelijke biologische mechanismen die het verband verklaren tussen inademing van fijn stof en het optreden van ziekten. Ten slotte trachtte ik op basis van onze studieresultaten de EU-grenswaarden en de WGO-richtlijnen voor fijn stof kritisch te evalueren.

BELANGRIJKSTE ONDERZOEKSRESULTATEN

In de eerste studie (hoofdstuk 1) onderzocht ik de effecten van de dagelijkse variatie in PM_{10} concentraties op het risico op zuigelingensterfte (< 1 jaar oud) in Vlaanderen. 2382 baby's stierven tijdens de onderzoeksperiode (1998-2006). De concentratie van fijn stof (PM_{10}) tijdens deze periode was gemiddeld 31.9 µg/m³ en er waren 321 dagen (of gemiddeld 35.7 dagen per jaar) met een gemiddelde dagelijkse concentratie hoger dan 50 µg/m³. De luchtkwaliteit voldeed dus aan de EU-normen voor het jaarlijkse gemiddelde, maar de norm voor het daggemiddelde werd echter nauwelijks gehaald. Het is ook duidelijk dat de veel strengere WHO-richtwaarden ruim overschreden werden in Vlaanderen.

Met een bidirectionele tijd-gestratificeerde *case-crossover* analyse toonde ik aan dat PM_{10} gerelateerd was met kindersterfte, voornamelijk in de late neonatale fase (d.w.z.tussen een week en een maand oud N=372). Voor elke stijging van PM_{10} met 10 µg/m³ steeg het risico op sterfte met 11% (95% CI 1-22%). Op de dagen waarop het gemiddelde PM_{10}-niveau de EU-grenswaarden van 50 µg/m³ oversteeg was de kans op overlijden 74% hoger (95% CI 18-158%) dan op de dagen waarop de PM_{10}-waarden onder de grenswaarden bleven. Hieruit blijkt dat de huidige EU-waarden voor PM_{10} niet streng genoeg zijn om het risico op kindersterfte zo klein mogelijk te houden. Bovendien is het verband tussen de blootstelling aan fijn stof en overlijden lineair, wat betekent dat er geen 'veilige' drempelwaarde kan worden bepaald.

In hoofdstuk 2 bestudeerden we het verband tussen de acute afstoting van een donorlong na longtransplantatie (LTx) en recente blootstelling aan fijn stof, met aandacht voor de onderliggende mechanismen die hier mogelijk bij betrokken zijn. In het UZ Leuven werden tussen 2001 en 2011 bij 397 LTx-patiënten herhaaldelijk transbronchiale biopten genomen. Ik bracht voor deze patiënten de geschatte PM_{10}-waarden op hun thuisadres in verband met symptomen van afstotingsverschijnselen en de daarbij horende fysiologische parameters.

Uit het onderzoek bleek dat een stijging van de PM_{10}-waarden met 10 µg/m³, gemeten drie dagen voor de biopsie, het risico op lymfocytische luchtwegaandoeningen (*lymphocytic airway disease*, LAD) deed stijgen met 12% (95% CI 1-25%). LAD is de pathologische uitingsvorm van acute afstoting na LTx. Daarnaast vonden we een positief verband tussen blootstelling aan PM_{10} en BAL tellingen van neutrofielen en lymfocyten, wat betekent dat ontstekingsreacties mogelijk een rol spelen in het fysiologisch proces. Een preventieve behandeling met het antibioticum Azithromycine bleek het effect van PM_{10} op de acute afstoting tegen te gaan.

In een derde studie (hoofdstuk 3) onderzocht ik of een toe- of afname van luchtverontreiniging gedurende verschillende opeenvolgende dagen (vergeleken met het 'normale' blootstellingsniveau van een persoon) kan resulteren in veranderingen in cardiovasculaire biologische merkers. Een jaar lang onderzochten we de luchtvervuiling, stijfheid van de halsslagader (een gekende merker van hart- en vaatziekten) en andere biomerkers in een testgroep van 20 gepensioneerde, gezonde mannen en vrouwen op verschillende locaties: tijdens een verblijf van 10 dagen in Milaan (Italië , PM_{10} > 50 µg/m³),een gelijkaardig verblijf in Vindeln (een landelijk gebied in het noorden van Zweden, PM_{10} < 10 µg/m³) en op verschillende tijdstippen in Leuven (als referentieplaats, $PM_{10} \approx 30$ µg/m³).

In vergelijking met Leuven was de luchtvervuiling groter in Milaan en kleiner in Vindeln. Het grootste verschil vonden we in de waarden van NO_2, met een gemiddelde van 63.7 µg/m³ in Milaan en 4.4 µg/m³ in Vindeln. Uit het onderzoek kwam een duidelijk verband naar boven tussen blootstelling aan luchtvervuiling gedurende 7 dagen en de stijfheid van de halsslagader. Zo vonden we bijvoorbeeld een 4.7% daling van de compliantie (dus een stijging van de stijfheid van de wand) bij een stijging van 10 µg/m³ van PM_{10}. We vonden echter geen directe inflammatoire effecten, zoals een stijging van de concentratie leucocyten of plasma CRP.

Beroerte of cerebrovasculair accident is wereldwijd een van de meest voorkomende doodsoorzaken en wordt bovendien in verband gebracht met blootstelling aan luchtvervuiling. Ik voerde een meta-

analyse uit van de huidige vakliteratuur om het verband tussen beroerte en langdurige blootstelling aan PM_{10} of $PM_{2.5}$ te kwantificeren (hoofdstuk 4).

Ik vond 20 studies over het verband tussen langdurige blootstelling aan fijn stof en het voorkomen van een beroerte. Ik vond een positief verband voor Noord-Amerika (N=7 studies), Europa (N=8) en China (N=3, met extreem hoge blootstellingen) en een negatief verband in Japan (N=2). Het geschatte effect van $PM_{2.5}$ [een stijging van 6% (95% CI 2-11%) van het risico op beroerte bij een toename van 5 $\mu g/m^3$ in $PM_{2.5}$] was groter dan het effect van PM_{10} [een stijging van 2% (95% CI -2 to 7%) van het risico op beroerte bij een toename van 10 $\mu g/m^3$ in PM_{10}]. Deze bevindingen duiden op het belang van directe metingen van $PM_{2.5}$ en bevestigen de hypothese dat de kleine partikels in fijn stof ($PM_{2.5}$) schadelijker zijn voor de gezondheid dan de grovere partikels ($PM_{2.5-10}$).

CONCLUSIES

In deze thesis vond ik nadelige effecten van luchtvervuiling op de gezondheid in verschillende kwetsbare bevolkingsgroepen en voor verschillende tijdsduren van blootstelling. Zowel kortetermijn-variatie (1 tot 3 dagen) als langetermijnblootstelling (meerder maanden tot jaren) kan acute aandoeningen uitlokken, zoals een beroerte, het afstoten van getransplanteerde longen of zuigelingensterfte. Op lange termijn kan blootstelling aan luchtvervuiling bovendien de ontwikkeling van chronische cardiovasculaire en respiratoire aandoeningen versnellen, zoals atherosclerose of longkanker. Uit de twee panelstudies (LTx-patiënten en gepensioneerden) verkregen we gemengde resultaten aangaande de rol van ontstekingen in dit proces, gemeten als concentraties van leukocyten en plasma CRP.

Op individueel niveau heeft blootstelling aan luchtverontreiniging een kleiner effect op de gezondheid dan andere omgevingsfactoren (zoals roken, voeding en lichamelijke activiteit). Blootstelling aan luchtvervuiling gebeurt echter onvrijwillig en is alomtegenwoordig. Aangezien de hele bevolking wordt blootgesteld tonen onze studies aan dat luchtverontreiniging wel degelijk een belangrijk probleem is voor de volksgezondheid.

Gedurende de afgelopen 10 jaar zijn in België (en in West-Europa in het algemeen) de PM_{10}-concentraties gedaald tot waarden duidelijk onder de EU-grenswaarde van 40 µg/m³. De waarden overschrijden echter nog steeds de richtlijnen van de WHO van 20 µg/m³,voornamelijk in stedelijke gebieden, waar het grootste deel van de bevolking woont. Zoals ook uit eerdere studies bleek, vonden we geen aanwijzing voor een 'veilige' grens voor luchtvervuiling. Dit betekent dat de volksgezondheid gebaat is bij elke µg/m³ daling in de concentratie van fijn stof. Dit is een gedeelde verantwoordelijkheid van de overheid, de industrie en ook de algemene bevolking: het toenemende wegverkeer en huishoudelijke houtverbranding zijn immers een steeds belangrijkere vorm van luchtvervuiling.

SHORT CURRICULUM VITAE and

LIST OF PUBLICATIONS

SHORT CURRICULUM VITAE

Hans Scheers was born on August 31st 1979 in Wilrijk (Antwerp). He attended the Sint-Ursula-Instituut in Onze-Lieve-Vrouw-Waver from 1991 to 1997 (Latin & Mathematics). In 2001, he obtained a Master's degree (officially 'licentiate', as it was called in the days of yore) in Biology, with the Highest Distinction, and a teacher's certificate, with Distinction, both at the University of Antwerp (UA). He then worked for 4 years at the UA's lab of Functional Morphology, and for 5 months as a teacher in Chemistry at a secondary school. In 2007, he obtained a Master's degree in Applied Statistics, with Distinction, at the University of Hasselt.

After a 9 months stay at the INBO in Brussels and a very fortunate meeting in a train, Hans continued his tour around the Flemish universities and started working in the laboratory of Pneumology at the KU Leuven in 2008. After 3 years of working as a scientific employee, he started a PhD on the epidemiology of air pollution exposure in 2011, under supervision of Ben Nemery and Tim Nawrot. His stay at the lab came to an end in December 2015, and in the past 11 months, he has been struggling (but eventually managed) to combine a job as a Biology teacher at secondary schools with finalizing his PhD trajectory.

In 2009, Hans married Marieke De Haes. Together, they have three wonderful children: Kasper (°2010), Oskar (°2012), and Leonie (°2016).

LIST OF PUBLICATIONS

International Peer-Reviewed Publications

- **Scheers H,** and Van Damme R. (2002) Micro-scale differences in thermal habitat quality and a possible case of evolutionary flexibility in the thermal ecology of lacertid lizards. Oecologia (Berlin) 132: 323-331.
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Het allerlaatste woord geef ik aan de twee grootste filosofen van de 21ste eeuw.

O: Maar maar, ik wil niet dat papa naar de werk is!

K: Maar dat moet van zijn baas.

O: Maar papa is toch de baas van de werk!

K: Nee nee, die heeft een baas die boos wordt als papa niet komt werken.

O: Ah... (*lange pauze*) Papa is de baas van de fietsers dan.

Slaapwel jongens!