Diesel Exhaust and Wood Smoke

Mechanisms, inflammation and intervention

Ala Muala
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ISBN: 978-91-7601-028-0
ISSN: 0346-6612
Cover illustration by Aziz Alhilaly & Zinah Alzaidi
Printed by Print & Media
Umeå, Sweden 2014
In the name of God, the Gracious, the Merciful

To my family, with love
and in memory of my grandmother, Hassnah Bander
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Abstract

Background Particulate matter (PM) air pollution is associated with increased respiratory and cardiovascular morbidity and mortality. Diesel engine exhaust (DE) and wood combustion are major contributors to ambient air pollution and adverse health effects. The aim of this thesis was to investigate the fate of inhaled combustion-derived PM, the subsequent effects on pulmonary inflammation and symptomatology and to explore the potential for particle filters to improve public health. Additionally, it aimed at increasing the understanding of the pathophysiological mechanisms underlying the adverse vascular effects of PM inhalation in man.

Methods In study I, lung deposition of wood smoke-derived particulates from incomplete combustion was determined in healthy and COPD subjects. In study II, airway inflammation was assessed in healthy subjects exposed to wood smoke and filtered air. In study III, vehicle cabin air inlet filters were evaluated regarding filtering capacity for DE and whether they affected the toxicological potential of the filtered PM. Healthy subjects were then exposed to filtered air and unfiltered DE, as well as DE filtered through two selected filters. In study IV, healthy subjects were exposed to filtered air and DE. Nitric oxide bioavailability was assessed by plethysmography in the presence of an NO clamp (NO synthase inhibitor NG-monomethyl locally and systemically administered) with measurements of arterial stiffness, cardiac output and blood pressure (BP).

Results Study I: The total PM number deposition fraction of the wood smoke was 0.32 and 0.35 for healthy and COPD subjects respectively. Study II: Inhalation of wood smoke caused CD3+ and mast cell infiltration in the bronchial submucosa along with CD8+ cell recruitment to the epithelium. In bronchial wash, inflammatory cells, myeloperoxidase and matrix metalloproteinase 9 levels decreased. Study III: An efficient cabin air filter with an active charcoal component was most favourable in in-vitro tests and reduced symptoms in the human exposure study. Study IV: Local NO synthase inhibition caused similar vasoconstriction after exposure to DE and filtered air, along with an increase in plasma nitrate concentrations, suggesting an increase in the basal NO release due to oxidative stress. Systemic NO synthase inhibition increased arterial stiffness and blood pressure after DE exposure along with an increase in systemic vascular resistance and reduced cardiac output, implying that the increased basal NO release could not compensate for the reduced NO bioavailability in the conduit vessels.

Conclusion Wood smoke particles from incomplete combustion tend to have a greater airway deposition than particles from better combustion. The airway inflammatory responses to the former particles differ from what have been shown for other PM pollutants, which may be of importance for subsequent health effects. The vasmotor dysfunction shown after DE exposure may largely be explained by reduced NO bioavailability. A vehicle cabin air inlet particle filter with active charcoal was effective to reduce DE exposure and subsequent symptoms. This may conceptually be of benefit when it comes to decreasing engine exhaust-related adverse health effects.
Selected abbreviations

ADMA  Asymmetric dimethylarginine
AP-1  Activated protein-1
CAC  Coronary artery calcification
CC16  Clara cell protein 16
CD  Cluster of differentiation
CIMT  Carotid intima-media thickness
CMD  Count mean diameter
CO  Carbon monoxide
DEP  Diesel exhaust particle
DF  Deposition fraction
EC  Elementary carbon
eNOS  Endothelial nitric oxide synthase
FeNO  Fraction of exhaled nitric oxide
GRO-alpha  Growth regulated oncogene-α
GSH  Reduced glutathione
GSSG  Oxidized glutathione
GSx  Total glutathione
ICAM-1  Intercellular adhesion molecule 1
IL  Interleukine
iNOS  Inducible nitric oxide synthase
L-NMMA  NG-monomethyl-L-arginine
MAPK  Mitogen-activated protein kinase
MMP-9  Matrix metallopeptidase-9
MPO  Myeloperoxidase
NF-κB  Nuclear factor-kappa B
NK  Natural killer cells
OC  Organic carbon
PAHₐ  Polycyclic aromatic hydrocarbons
RESPI  Respiratory particle deposition instrument
ROS  Reactive oxygen species
RTLF  Respiratory tract lining fluid
SDMA  Symmetric dimethylarginine
SMPS  Scanning mobility particle sizer
SNP  Sodium nitroprusside
SSA  Serum amyloid A
TC  Total carbon
TDF  Total deposition fraction
TEOM  Tapered elemental oscillating microbalance
TNF-α  Tumor necrosis factor-alpha
UFP  Ultrafine particle
VCAM-1  Vascular cell adhesion molecule 1
VT  Tidal volume
Sammanfattning

Luftföroreningar ger upphov till en rad hälsoeffekter, som drabbar såväl lungor som hjärtkärlsystem och leder till ökad sjuklighet och för tidig död. WHO har nyligen rapporterat att luftföroreningar utomhus och inomhus leder till så mycket som 7 miljoner extra dödsfall per år i världen. Mekanismerna som ligger bakom dessa stora hälsoeffekter är ej helt klargjorda. Experimentella exponeringskammarstudier med olika luftföroreningar har bidragit till ökade kunskaper inom detta område.

Syftet med denna avhandling var att ge en mer detaljerad förståelse om varför och hur luftföroreningar som vedrök och dieselavgaser påverkar hälsan, för att på sikt kunna förbättra hälsan i befolkningen. Därför har undersökningar genomförts för att klargöra hur mycket av inandade vedrökspartiklar som stannar i lungorna (s.k. deponering) och vilka efterföljande inflammatoriska effekter som uppstår i lungorna. Vidare undersöcktes om filter som renar tilluften till kupén på fordon skulle kunna minska hälsoeffekterna för personer som reser i fordonen. Dessutom studerades vilka mekanismer som ligger bakom de oönskade hjärtkärleffekternas av dieselavgaser.

I studie I fick 12 friska och 5 personer med KOL inandas utspädd vedrök från ofullständig förbränning av ved i en vedkamin. Partikeldeponeringen, dvs. hur mycket av inandade partiklar som stannade kvar i lungorna, undersöktes med en nyutvecklad teknik. Det visade sig att så mycket som 1/3 av vedrökspartiklarna fastnade i lungorna; något mer för personer med lungsjuksdomen KOL. Vedrökspartiklar från sotig vedeldning visade sig ha högre deponering än partiklar från mer effektiv pelletsförbränning.

I studie II genomgick 14 friska personer exponering för samma typ av vedrök som i studie I samt för filtrerad luft. Exponeringen genomfördes i en specialbyggd kammare på Umeå universitet. Genom bronkoskopi med provtagning från luftvägsslemhinnan samt sköljning med en liten volym koksaltlösning kunde celler och ämnen från lungorna analyseras.

Vedröksexponering orsakade inflammation i luftvägen med CD3+ och CD8+ lymfocyter samt mastceller, combinerad med en övändt minsning av celler och flera lösliga komponenter i lungskolvätskan. En förväntad ökning av neutrofila celler samt alveolmakrofager för att ta hand om och oskadliggöra inandade vedrökspartiklarna kunde inte verifieras. Den undersökta typen av sotig vedrök ger inflammation i bronkväggen samtidigt som immunförsvaret kan påverkas på ett negativt sätt. Detta kan vara kopplat till den ökade
frekvensen av luftväggssjukdom och luftvägsinfektioner som kunnat härledas till vedeldning i folkhälsostudier.

I studie III genomgick 30 friska försökspersoner exponeringar för filtrerad luft, dieselavgaser samt avgaser som filtrerats med två olika partikelfilter, som utvalts baserat på cellförsök med filtrerade diesel partiklar. Exponeringarna genomfördes liksom i tidigare studier på Svensk maskinprovnings (SMP) anläggning i Umeå. Ett speciellt partikelfilter som innehåller aktivt kol var mest effektivt, i såväl cellförsök, som att minska symptom av exponering för dieselavgaser hos friska forskningspersoner.

I studie IV undersöktes genom vilka mekanismer i blodkärlen som dieselavgaser ger negativa effekter. Biotillgängligheten för kväveoxid (NO), ett centralt ämne för blodkärlsfunktion, undersöks genom att exponera 30 försökspersoner för dieselavgaser som genereras under samma förhållanden som vid studie III. Betydelsen av blockering av kväveoxid, både lokalt i armen och systemiskt i hela kroppen, för blodkärlsfunktion i samband med dieselavgaseexponering studerades med hjälp av halvautomatiska detektorer. Systemisk blockering av produktionen av kväveoxid gav ökad artärstyp och ökat blodtryck men minskning av hjärtats slagsvolym, efter exponering för dieselavgaser. Tillgängligheten av kväveoxid i blodkärlen förefaller ha stor betydelse för hjärtkärlsfunctionen av dieselavgaser. Detta fynd kan på sikt leda till förbättrade möjligheter att terapeutiskt minska hjärtkärlsfunctionerna av luftföroreningar.

Sammantaget har denna avhandling gett ny kunskap om hur inandad sotig vedrök tenderar att fastna i lungorna och ge oväntade effekter på immunförsvaret samt hjärtkärlsen. Tillgängligheten av det viktiga ämnet kväveoxid i blodkärlen störs av dieselavgaser, och avhandlingen har gett ny information om mekanismerna bakom detta. Luftföroreningar ger mycket stora hälsoeffekter i lungor och hjärtkärlsystem, och det är av största betydelse att minska emissionerna av dessa. Denna avhandling visar även på möjligheten att minska symptom och hälsoeffekter genom att utveckla effektiva partikelfilter för fordon.
Original Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals


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Introduction

Air pollution is recognised as an increasing health problem globally. In the modern history, several disasters occurred during the first half of the 20th century (1, 2) which brought air pollution into focus for research and official recommendations for threshold.

Air pollution is a mixture of particulate matter (PM) and gaseous compounds, such as ozone and nitric oxides that have been linked to adverse health effects. The present thesis investigated air pollution particle deposition and adverse health effects of wood smoke and diesel exhaust, which contains particles with different characteristics. Therefore, particle characteristics are explained followed by a description of health effects and the research literature relevant for this thesis.

PM air pollution can be divided into; a) naturally originated particulates from volcanoes, dust storms and sea spray and b) those, which are a result of combustion for transport, heating, cooking and industrial processing.

PM may also be divided according to its size into coarse particles (PM\textsubscript{10}); with a diameter less than 10 μm such as silica-based crustal particles, break wear, road dust and volcanic ash, fine particles (PM\textsubscript{2.5}); particles with a diameter less than 2.5 μm and ultrafine particles (UFP) (PM<0.1); particles with a diameter less than 100 nm (figure 1).

Combustion is the most common source of air pollution PM, especially the ultrafine fraction that is suggested to be associated with adverse health effects following its ability to deposit in the lungs. The respiratory deposition of wood smoke combustion has previously not been well studied. Therefore, the present work tried to address the deposition probability and investigate the inflammatory effects of the deposited wood smoke particles.

Respiratory deposition

Chemical and physical characterization of wood smoke particles

The physicochemical properties of the inhaled particles are of major importance for the respiratory tract deposition and the adverse health effects. The particle size, density, shape, hygroscopicity (water solubility) and
chemical composition have been proposed to influence the toxic and inflammatory potency of the particulate matter (3-5).

The physical and chemical properties of wood smoke particles differ substantially depending on the combustion conditions. Based on the chemical combustion and morphology, fine particles (PM$_{2.5}$) generated from wood smoke combustion have been divided into three particle classes:

**Spherical organic carbon particles**
These particles are generated from incomplete wood smoke combustion at low temperature and air deficiency and have a diameter ranging from 50 to 600 nm (6). Particles emitted form this type of combustion contain high levels of organic carbon and the ratio of elemental carbon (EC) to the total carbon (TC) EC/TC lies between 0.01 to 0.11 (7).

**Soot (Elemental carbon aggregates)**
Soot formation is a complex process. Solid carbon aggregates with a diameter range of 20-30 nm are emitted from incomplete, air-starved and high temperature combustion conditions (6). In contrast to spherical organic carbon particles, soot particles are composed of higher levels of elemental carbon and lower levels of organic compounds with EC/TC ration of 0.5-0.75 (7).

**Inorganic ash particles**
Complete combustion conditions of wood logs, wood chips and pellets with modern stove technology at high temperature and air-rich supply result in emissions of inorganic ash particles (alkali salts of potassium/ sodium sulphate, chloride and carbonate) with low levels of organic and elemental carbon which may be below 1% of the particle mass (8, 9).

The characteristics of the emitted particles are overlapping in real life as the combustion conditions may change during burn cycles (10).

**Factors affecting particle deposition**
To exert adverse health effects, inhaled particles need to deposit in the respiratory tract and interact with the respiratory tract lining fluid, inflammatory cells and lung tissue. The major factor for this is the number and distribution of particle deposited locally in the lungs and not the particle
mass concentration that can be measured in the ambient air. This is furthermore of importance as the lung deposition fraction varies considerably between different types of particles (11).

The vast majority of the epidemiological and toxicological studies relay the observed health effects to the particle mass concentration, however the probability of the particle to deposit is not only a function of mass concentration but depends also on other important parameters, such particle size, hygroscopicity, exposure duration and ventilation physiology.

The particle size is the major determinant of a particle’s deposition property, with smaller particles depositing much more that the larger particles.

There are three mechanisms by which particles of different size deposited in the respiratory tract particle deposited in the respiratory depending on particle size (12):

- Particles sized > 3 \( \mu \text{m} \) deposit mainly by impaction in the airways. Impaction increases with particle size and flow rate.
- For the particles size range 0.3-3 \( \mu \text{m} \) the deposition occurs mainly by sedimentation because of the gravitational force. The particles settle in the lower airways where the flow rate is slow and the airways are small. The deposition of the particles differs according to the direction of the particle flow and the direction of the gravitational force.
- Very small particles (< 0.3 \( \mu \text{m} \)) deposit by diffusion. These particles are constantly moving and colliding resulting in a random movement, Brownian motion, which causes diffusion when there is a difference in the particle concentration between two mediums. The diffusion increase with decreasing particle size and flow rate. The main site for diffusion is in the alveolar region because of the smaller airways and long residence time for the aerosol.

Hygroscopicity is another important factor for particle deposition as the high relative humidity within the bronchial tree may reach as much as 99.5% (13, 14). Hygroscopic particles may grow to a diameter up to six times the dry size, whereas the hydrophobic particles are water insoluble and almost keep their original dry size, though deposited more compared with hygroscopic particle of the same aerodynamic size (15).

In addition to the particle size and the hygroscopicity, the pulmonary deposition of the particles depends also on tidal volume and respiratory rate, and thereby minute volume. The total deposition fraction (DF) increases
with higher tidal volume ($V_T$). When it comes to particles below 300 nm which deposit mainly by diffusion, the relation with respiratory rate is inversed (16). This is due to the decreased residence time in the lungs during high respiratory rate (12). The minute volume depends on the $V_T$ and RR. This means that in patients with chronic obstructive pulmonary disease (COPD), who used to have high respiratory rate RR as a compensatory mechanism, the minute volume tends to be higher compared with healthy subjects with a subsequent increment in the particle deposition dose rate. Furthermore, during exercise, both the $V_T$, the RR are increased, resulting in a substantial increment of particle deposition dose rate compared with exposure at rest or under normal physical activities (17, 18).

Aerosol deposition in pulmonary diseases

Airway geometry and lung morphology affect the pulmonary deposition of inhaled particles. Bronchoconstriction, decreased lung compliance and increased mucus production, with or without impairment in ciliary function have a major impact on the total lung deposition in the lungs. Patients with obstructive lung and chronic bronchitis diseases have been reported to have higher deposition than healthy subjects (19-21). UFP deposition may be enhanced due to airway obstruction and increased alveolar volumes, which facilitates particle diffusion. In previous study carbon soot particles, which were generated from graphite electrodes by spark discharge in anhydrous argon, using a commercial generator, were used to compare the deposition in healthy and asthmatics during spontaneous breathing. It was shown that the UFP deposition dose was greater by 74% in asthmatics than healthy subjects due to higher DF and minute ventilation (22). Furthermore, TDF and normalized deposited dose rate have been reported to be 50% and 32% higher, respectively, in asthmatic children compared with nonasthmatic adults. TDF was 21% higher in asthmatic than non-asthmatics children (23). Patients with emphysema often have increased residual volume. Therefore, particle residence time is usually increased leading to enhanced particle deposition (24). Deposition in cystic fibrosis has also been demonstrated to be higher compared with deposition in healthy subjects (25).

Up to date, no study of particle deposition in asthma has been performed under more real life circumstances such as studying deposition of particles generated from fuel combustion by using flow through systems with either mouth-piece or face mask ventilation.
Air pollution and health effects

There exists today an extensive documentation on the adverse health effects of air pollution on respiratory and allergic diseases, as well as contribution to acute and chronic cardiovascular events. The Word Health Organization (WHO) had estimated the number of premature deaths attributable to urban outdoor air pollution amount to 3.2 million worldwide annually 2010 (26).

The adverse effects of air pollution have been strongly associated with combustion processes for transport and heating purposes. Traffic has been identified as a major contributor to respiratory and cardiovascular morbidity and smoke form biomass such as wood smoke has also been considered to be of considerable importance globally.

Respiratory effects

A number of studies have demonstrated that exposure to ambient air pollution is associated with cough, wheeze, asthma and respiratory tract infections (27, 28). Traffic-related air pollution has been suggested to contribute to asthma development (29-31). A recent report from an infant cohort examined the birth-year home exposure up to 7 years of age and showed an increased risk of asthma with an odds ratio (OR) of 3 for an interquartile range increase of PM$_{2.5}$ (4.1 μg /m$^3$) (32).

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory lung disorder with irreversible airways obstruction with tobacco smoke as a well-recognized causative factor. It is considered to be the 4th most common cause of death worldwide and estimated to reach no. 3 by 2020 (33). Epidemiological studies have delineated a strong relationship between exposure to PM air pollution and worsening of pre-existing COPD in elderly, associated with high hospital admission rate and increasing mortality rate (34-36). COPD has also been suggested to caused by exposure to high concentration of wood smoke and other biomass smoke from heating and cooking. This has been recognized as a major health problem globally.

Cardiovascular effects

Exposure to particulate matter (PM) air pollution, especially traffic-related PM, has been suggested to be an important factor for the development and progression of atherosclerosis and further increase the incidence of acute myocardial infarctions as well as mortality (37, 38). Particulate matter, especially the fine and ultrafine particle fractions, have been proposed to be
the causative agents for most other traffic-related cardiovascular morbidity and mortality (37). It has been well demonstrated that the risk for acute myocardial infarction increased by 2.5% with each 10 µg m⁻³ increase in exposure to particulate matter with a mean aerodynamic diameter of < 2.5 µm (39).

Several epidemiological studies have linked long-term exposure to traffic-related PM and atherosclerosis. In one cross sectional analysis, the relationship between residential proximity to major road and abdominal aorta calcification, as an indicator of systemic atherosclerosis, was investigated in five US metropolitan areas. Exposure to PM2.5 was associated with increased risk of aorta calcification (40). Another study showed that traffic proximity was positively associated with carotid intima thickness progression (CIMT) with an acceleration of 5.5 micrometer/year among those living within 100 m from a highway in the Los Angeles area (38). Furthermore, a prospective cohort of over four thousand participants from the German Heinz Nixdorf Recall study showed a clear association between residency near a major road and increase in the coronary artery calcification (CAC). A 50% shorter distance between the residence and a major road increased the CAC by 7% (41). The same group recently demonstrated both long-term exposure to PM2.5 and nighttime traffic noise to be independently associated with increase in thoracic aorta calcification as measure of subclinical atherosclerosis (42).

Stroke is a major cause of morbidity and mortality both in developed and developing countries (43). It was estimated that approximately 17 million stroke cases worldwide of which 1/3 resulted in death in 2010 (44), making stroke the second leading cause of death worldwide. Ambient air pollution has been associated with stroke mortality and subsequent hospital admissions in several studies (45-49). Deep vein thrombosis has similarly been associated with exposure to traffic and PM air pollution PM (50, 51).

**Special considerations on biomass smoke and health effects**

Approximately 3 billion people worldwide depend on solid biomass fuels as the major source of energy for heating and cooking (52, 53). The exposure to solid biomass fuel was estimated to contribute to about 3.5 million premature deaths per year in 2010 and is now considered as the third leading risk factor for global disease burden (26) and in a recent paper Smith et al estimated that household air pollution was responsible for about 3.9 million premature death reaching the highest rank among 60+
environmental risk factors (54). In a previous review, ambient wood smoke was estimated to be no less harmful than other sources of ambient particulate matter (55).

A three-fold increase in the prevalence of asthma symptoms was reported in children exposed to wood smoke (56). Likewise, elderly men and women exposed to wood smoke have been shown to have higher prevalence of asthma symptoms compared with those who were depending on cleaner fuels (57).

The association between wood smoke exposure and COPD has been evaluated by several epidemiological studies (58-60). A consistent evidence for an increasing risk, at least a doubling, for COPD and chronic bronchitis has been found to be associated with indoor air pollution, specifically wood smoke (61).

Systemic reviews and meta-analyses have concluded wood smoke exposure to increase the risk for acute and chronic lower respiratory tract infections (62, 63). Exposure to indoor wood smoke was estimated to double the risk of acute lower respiratory tract infections and pneumonia in children under 5 years age estimated to cause nearly 800,000 death in 2000 (64). Furthermore, in a cross sectional study, exposure to biomass was found to increase the risk of pulmonary tuberculosis (65).

Several studies have addressed the risk for lung cancer with wood smoke exposure. In a multivariate logistics regression analysis, a significant correlation was obtained between exposure to biomass related air pollution and lung cancer with an OR of 3.6, after adjustment to smoking and passive smoking (66). Moreover, increased DNA damage in buccal epithelial cells was observed in non-smoking women exposed to wood smoke compared with non-biomass exposed controls (67).

Low birth weight has also been suggested to be associated with prenatal exposure to biomass fuel exposure (68, 69). Carbon monoxide (CO) concentrations, when using biomass for cooking, is often 2-50 ppm, and may reach up to peaks of 500 ppm. This may reduce the free Hb available in the metabolic-and maturation process in utero and contribute to low birth weight and potentially reduce lung growth and increase mortality (69, 70). Low birth weight has a prognostic importance in which future decline in lung function, mainly forced expiratory volume in one second (FEV1), is related to low birth weight with subsequent increase in mortality rate for COPD.
Experimental studies of air pollution effects

A vast number of experimental studies have investigated the effects of various air pollutants using in-vitro models, animal studies and experimental exposures in human subjects. In this short review wood smoke and diesel exhaust exposure studies relevant to the studies in this thesis are given.

Wood smoke

Human exposure studies

The mechanisms by which wood smoke exert its effects have not been well studied compared with the huge amount of experimental studies that have focused on DE exposure. The interest has been increasing to perform human wood smoke exposure studies during the last decade.

The first controlled wood smoke exposure study in humans was, to our knowledge, performed by Barregard and co workers and published in 2006. Thirteen subjects were exposed to filtered air and wood smoke by mass concentration 240-280 μg/m³ inside the exposure chamber for 4 hours. The wood smoke was generated in a small cast-iron wood stove and a partial flow was mixed with indoor air. Wood smoke exposure resulted in increased levels of serum amyloid A (SAA) suggested to be a marker for inflammation and cardiovascular risk together with increase in factor VIII in plasma and factor VIII/von Willebrand factor ratio. Furthermore, the authors reported increase in urinary free 8-iso-prostaglandin 2-α, suggested to reflect an increased free radical-mediated lipid peroxidation (71, 72). More published data have originated from the same study and showed increase in exhaled nitric oxide and malondialdehyde levels in breath condensate post wood smoke exposure (73). To investigate the genotoxicity related to wood smoke exposure, analyses were done for the damaged DNA and repair capacity in the peripheral blood mononuclear cells. There was significant decrease of mononuclear cells levels of DNA strand breaks reported while the mRNA level of oxoguanine glycosylase 1 was significantly increased.

Pulmonary and systemic inflammation has been suggested to be the underlying mechanism of most adverse health effect of exposure to wood smoke. This was the primary hypothesis behind the first bronchoscopy study published in this field. The study included nineteen healthy volunteers exposed to wood smoke at a PM mass concentration of 224 μg/m³ for 3 hours, generated by a wood pellet burner under incomplete combustion.
Mucosal symptoms like nasal- and throat irritation and unpleasant smell were reported after wood smoke exposure. An increase in glutathione concentrations in bronchoalveolar lavage (BAL) may have had a protective role as a response to the oxidative challenge as no increase in proinflammatory components was seen (74).

In a study by Ghio et al, ten healthy subjects were exposed to wood smoke at a PM mass concentration of 485 µg/m³ for 2 hours alternating with air, and 3 weeks apart. The authors reported an increase in the percentage of neutrophils in the peripheral blood parallel with a neutrophilic influx in bronchial wash (BW) and in BAL whereas there were no significant changes in pulmonary function test and symptoms (75).

The scope of experimental human exposure studies with wood smoke has also expanded to cover allergic individuals. In a controlled cross over wood smoke exposure study in Denmark, 20 atopic volunteers were exposed on three different occasions to air and to wood smoke generated from complete wood stove combustion at a PM mass concentration of 220 and 354 µg/m³. The endpoints were changes in microvascular function measured by peripheral arterial tonometry during reactive hyperaemia, measurements of oxidatively damage DNA, levels of inflammatory markers and soluble adhesion molecules in the blood before, 6 and 20 hours after each exposure. There were no significant changes reported for these parameters in the atopic subjects examined (76).

The emerging knowledge of the importance of the combustion conditions and the chemical characterization of the wood smoke has given the human experimental studies a new horizon to investigate different types of wood smoke combustion to understand the relationship with adverse health effects.

In a recent study by the Gothenburg group of Barregård and colleagues, thirteen healthy subjects were exposed to two types of wood smoke combustion; one from the start-up phase (incomplete combustion with more PM mass and higher polycyclic aromatic hydrocarbons, PAHs) and another from burn-out phase (complete combustion with higher alkali salts) at a PM mass of 295 µg/m³ and 146 µg/m³, respectively. After exposure to smoke from the burn-out phase of wood smoke, clara cell protein 16 (CC16), secreted by clara cells into the epithelial lining fluid and supposed to have a protective role against inflammation and oxidative stress, was elevated in serum after 4 hours and in urine the next morning. The fraction of exhaled nitric oxide (FeNO) increased after exposure to the burn-out phase smoke (Stockfelt Inh Tox 2012). In the same study, by further investigation of
possible systemic inflammation, there were unaltered levels of inflammatory markers interleukine-6 (IL-6), Tumor necrosis factor-alpha (TNF-α) and SAA in the peripheral blood, white blood cell counts, adhesion molecules, coagulation factors and the lipid peroxidation degradation product 8-iso-prostaglandin F2α (8-iso-PGF2α) (77).

**In-vivo animal toxicological studies**

Animal PM instillation studies have been performed to study mechanisms behind the adverse effects of wood smoke exposure. In an extensive review by Naeher et al, short-term wood smoke exposure of mice induced oxidative stress responses with altered antioxidant status, increased cytokine and inflammatory cell levels in the BAL along with down regulation of immune defence functions including reduction of macrophages phagocytic ability. Moreover, The reviewed animal studies suggested wood smoke to be highly mutagenic and may also be carcinogenic (78).

**In-vitro exposure studies**

Experimental exposure to liquefied wood smoke on cultured mouse macrophages has been showed to generate free radicals, DNA damage and TNF-α release (79). Similar findings were demonstrated when a contact co-culture of monocytes and penumocytes was exposed to wood smoke particles with proinflammatory cytokines release combined with cell toxic effects and reduction in cell number (80). Furthermore, it has been demonstrated that PM related to wood smoke created more DNA damage than traffic-related PM (81). Similar data were reported form Finland in a series of studies using macrophage cultures (82-84).

**Diesel Exhaust**

**Human exposure studies**

A series of experimental human diesel exposure (DE) studies mimicking traffic exposure have shown a wide range of proinflammatory responses and adverse cardiovascular effects The addition of bronchial mucosal biopsy sampling added new information to the early experimental DE studies that relied on BAL analysis (85). In the Salvi et al 1999 paper, bronchoscopy and blood sampling were done 6 hours after 1 hour of exposure to diluted DE and filtered air in healthy subjects. There were significant increases in neutrophils and B lymphocytes in bronchial wash (BW) and bronchoalveolar lavage (BAL) respectively, together with a significant increase in neutrophils,
mast cells, CD4+ and CD8+ T lymphocytes combined with upregulation of the endothelial adhesion molecules ICAM-1 and VCAM-1 in the bronchial biopsies, along with increased numbers of neutrophils and platelets in the peripheral blood (86). Furthermore, from the same study, further analyses showed interleukine-8 (IL-8) in the BW and bronchial mucosa along with growth regulated oncogene-alpha (GRO-α), IL-8 and IL-13 in the bronchial epithelium to be significantly increased after exposure to DE compared to air (87, 88). Moreover, DE caused activation of redox sensitive transcription factors and kinases along with induction of oxidative stress with subsequent cytokines production after exposure DE (89). Additional studies extended the focus to responses in asthmatics subjects as well as complementary time points for bronchoscopy, concentrations and engine running cycle.

Cardiovascular endpoints have more recently been studied to address the background mechanisms by which DE exerts its effects. Controlled DE exposures have been shown to cause impairment in vasomotor function and endogenous fibrinolysis combined with increased arterial stiffness and enhanced ex vivo thrombus formation (90-92). In patients with stable coronary heart disease, ST-T segment depression on ECG was shown after DE exposure despite full preventative treatment (93).

**In-vitro and in-vivo animal studies**

The effects of DE particles have been studied thoroughly by many *in-vitro* and animal models over the years as recently reviewed by Schwarze et al (94). DE particles have been shown to induce release of wide range proinflammatory markers including cytokines, chemokines in line with *in-vivo* studies findings in animals and humans.

**Oxidative stress**

Oxidative stress is defined as an imbalance between free radicals or reactive oxygen species (ROS) and the amplitude of the protecting antioxidant defences. In the absence of antioxidants, oxygen-derived free radicals such as superoxide (O²⁻) and hydroxyl free radicals triggers a cascade of inflammatory processes resulting in activation of inflammatory cells, mitochondrial reactions and cell damage, as well as oxidization of lipids, proteins and DNA. The respiratory system is affected by the oxidative stress by diesel exhaust particles (DEP) resulting in reduced antioxidant levels of glutathione and ascorbate in a synthetic respiratory tract lining fluid (RTLF), along with increased flux of reduced glutathione and urate in the lower airways and activation of redox sensitive elements and transcription factors.
Oxidative stress may also be involved in the reduction of nitric oxide bioavailability in the vascular wall associated with reduction in the vasomotor responses and impaired fibrinolytic functions (90, 97, 98). The degree of oxidative stress by DE exposures in human in-vivo is far less extensive than for strong oxidants such as ozone, which give more pronounced local and systemic effects.

Figure 1. Particle classification according to its size.
Aims

The overall aim of this thesis was:

The aim of this thesis was to investigate the fate of inhaled combustion-derived PM, the subsequent effects on pulmonary inflammation and symptomatology and to explore the potential for particle filters to improve public health. Additionally, it aimed to further understanding of the pathophysiological mechanisms underlying the adverse vascular effects of PM inhalation in man.

Specific aims were:

- To evaluate the airway deposition of wood smoke particles emitted during incomplete combustion in healthy volunteers and subjects with chronic obstructive pulmonary disease.

- To study whether wood smoke generated during incomplete combustion would induce airway- and systemic inflammation.

- To evaluate the efficacy of different vehicle cabin air inlet filters to reduce the pro-inflammatory and oxidative potential of diesel exhaust and to reduce diesel exhaust-induced symptoms in healthy human subjects.

- To test the hypothesis that changes in NO bioavailability contributes to the cardiovascular dysfunction induced by diesel exhaust inhalation.
Subjects and methods

Subjects

Subject’s characteristics are summarized in table 1. Healthy subjects were included in all studies. In study I, 5 subjects with COPD were included. COPD severity was ranging between stage I-III according to GOLD standard (99).

Significant exposures to air pollution or those with concomitant illness were excluded. All subjects were free of symptoms of respiratory tract infection for at least 6 weeks leading up to the study. All subjects underwent a physical examination, baseline blood count and renal function assessment, spirometry (FEV₁, VC, FEV₁/VC) and 12-lead electrocardiogram prior to participation. All subjects gave their written informed consent and the study was approved by the local ethical review board, and carried out in accordance with the Declaration of Helsinki.

Table 1: Subject characteristics

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Study I Healthy</th>
<th>Study II Healthy</th>
<th>Study III Healthy</th>
<th>Study IV Healthy</th>
<th>Study IVa Healthy</th>
<th>Study IVb Healthy</th>
</tr>
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<tr>
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<td>66 62-69</td>
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<td>26 18-33</td>
<td>23 21-27</td>
<td>26 23-34</td>
</tr>
<tr>
<td>Male/Female</td>
<td>7/5</td>
<td>4/1</td>
<td>8/6</td>
<td>17/13</td>
<td>9/7</td>
<td>8/6</td>
</tr>
</tbody>
</table>

Wood smoke exposures in study I and study II

Wood smoke was generated using a common Nordic wood stove (chimney stove) applying a firing procedure that aimed for incomplete combustion conditions with soot-dominated particle emissions. Birch wood logs were inserted every 5-15 minutes to obtain a high burn rate. In addition, the fire was disturbed 2-3 times during a batch either by adding some extra fuel (i.e. a small wood log) or by reducing the air supply momentarily. This procedure caused occasions with high burn-rate air-starved combustion conditions, presumable generating a soot-rich aerosol emission. Excess oxygen in the flue gases varied from 3 to 17%. Due to the varying combustion conditions, CO concentrations in the flue gases varied typically in the range 1000-5000
ppm, with peaks up to 12,000 ppm. Previous studies have shown that this kind of combustion, with a high burn rate of wood, is associated with high elemental carbon (EC), soot and polycyclic aromatic hydrocarbon (PAH) emissions (100). The wood smoke was diluted with HEPA and activated carbon filtered air in three steps and continuously fed into specially designed exposure chamber (15.3 m³) of stainless steel at the Thermochemical Energy Conversion Laboratory (TEC), Umeå University, with an air exchange rate of around 3 times per hour. Continuous measurement of nitrogen oxides (NOₓ) (chemiluminescence, CLD 700 Ecophysics, >0.001 ppm) and carbon monoxide (CO) (IR, UNOR6N Maihak) was done to monitor the gaseous pollutants in the exposure chamber. PM₁ (particulate matter with an aerodynamic diameter of <1 µm) mass concentration was measured on-line using a tapered element oscillating microbalance (TEOM 1400 by Thermo Scientific) equipped with a PM₁ pre-cyclone. Integrated with the TEOM, a Teflon filter sampler was used to determine the particle mass concentration gravimetrically. The mobility diameter of the wood smoke particles was measured in the chamber using a scanning mobility particle system (SMPS, including a DMA model 3071A and a CPC model 3010, TSI Inc, USA). Organic (OC) and elemental carbon (EC) was determined using a thermal-optical carbon analysis (Method NIOSH 5040) (101). Polycyclic aromatic hydrocarbons (PAH) were sampled in the chamber by glass fibre filters (Ø 47 mm) for the particulate fraction followed by a polyurethane foam (PUF) plug (Ø 75 mm × 50 mm) for the semi-volatile fraction. PAH compounds (3-6 rings) were analysed by high-pressure-liquid-chromatography coupled with a gas chromatography-mass spectrometry system (HPLC-GC-MS) as described previously (102).

Study I

The subjects inhaled wood stove smoke from the exposure chamber through a mouthpiece. A pre study test measurement was carried out in order to allow all subjects to adjust to the equipment and be comfortable. Deposition of wood smoke particles was measured during three 15 minutes exposure sessions for each subject using the RESPI technique (figure 2).
Study II
A randomized, double blind, cross over study was carried in order to examine the respiratory effects of wood smoke using bronchoscopy and complementary methods. Each subject was exposed, over two different occasions at least 3 weeks apart, to air and wood smoke in the exposure chamber (figure 3). The PM, concentration of wood smoke was 350 µg/m³. The exposure lasted for 3 hours, during which, each subject performed an exercise on a bicycle ergometer over 15 min alternate with rest for 15 min, under a predetermined load to keep the minute lung volume at 20 L/min/m² body surface area.
Diesel exhaust exposures

Diesel exhaust was generated by an idling Volvo diesel engine (Volvo TD40 GJE, 4.0 L, four cylinders, 1996) running on a well-characterized diesel fuel (Preem, UN 1202, VSD 10). Over 90% of the exhaust was shunted away, the remaining part diluted with high-efficiency particulate air (HEPA) filtered air and fed into the exposure chamber as previously described (103). The exposures were standardized based on particulate matter (particles with a mean aerodynamic diameter ≤10 µm (PM_{10}) concentrations and a mean airborne particle concentration of 350 ± 56 µg/m³ and 333 ±11 µg/m³ for study III study IV respectively.
The exposures were separated by at least one week. Each exposure lasted for one hour during which the subjects performed moderate physical exercise (minute ventilation 20 L/min/m² body surface area) on a bicycle ergometer for 15 min followed by 15 min rest (104).

**Study III**

This study was designed to test the efficiency of two ultrafine particle filters (with and without active charcoal component) selected after performing *in-vitro* proinflammatory and antioxidant tests, see below in additional methods. Subjects were exposed on four separated occasion, at lease one week apart, to filtered air, unfiltered and filtered diesel with ultrafine particle filter A and filter B (with active charcoal). The tested filters were located prior to the airflow entering the chamber (figure 4). The tubing immediately before and after the filter, which was located immediately before the air entered the chamber, allowed for continuous control of pressure changes across the filters as well as SMPS measurements. For the two filters employed for the human exposure studies, the particle number size distributions in the chamber determined by a scanning mobility particle sizer (SMPS) ranged from 0.014 to 0.660 µm.

![Figure 4. Diesel exhaust exposure setup with filter location.](image-url)
Subjects were exposed separately to filtered air or diesel exhaust, filtered and unfiltered, on four separate occasions in a randomized double-blind controlled crossover manner. Filtration of the diesel exhaust was performed using two separate filters; Filter A, which was a particle filter and Filter B, which was the same particle filter with an active charcoal filter medium.

**Study IV**

This study was designed to investigate the effects of nitric oxide (NO) inhibition, locally and systemically, on endothelial vasomotor function. In 2 randomized double blind cross over studies, subjects were exposed on two occasions to either filtered air or diluted diesel exhaust at a target of 300 μg/m³ in a diesel exposure chamber as previously described (104).

**Effect measurements**

**Study I**

*Respiratory tract deposition measurement*

For the deposition measurements, the research subjects were wearing a nose clip were breathing wood smoke through a mouthpiece while sitting in a relaxed position. Wood smoke was drawn from the exposure chamber for inhalation, while exhaled smoke was directed into a 2 dm³ stainless steel container open to atmosphere. The container for exhaled air was heated to approximately 37°C to avoid condensation of water vapour.

The particle concentrations in inhaled and exhaled air respectively were measured with a scanning mobility particle sizer (SMPS, design: Lund University) consisting of a differential mobility analyser (DMA, 28 cm long Vienna-type) and a condensation particle counter (TSI Inc., model 3010). The SMPS measures the particle number size distribution in the range of 10-500 nm, classified according to the particle mobility diameter. This is also the appropriate equivalent diameter for this application, since diffusion is the dominating deposition mechanism in the respiratory tract for particles below 500 nm (16). Scan time was set to 90 s (both up and down scan). The first 90 s of data from each exposure session were wasted to ensure complete mixing of the aerosol in the lungs. The aerosol was dried to below 20% relative humidity before measurement.

Fresh wood smoke particles are generally a mixture of agglomerated soot particles and hygroscopic salt particles that may change in size when
exposed to high relative humidity (105, 106). A particle size shift between inhaled and exhaled sample due to the elevated humidity in the lungs may cause errors in the measured respiratory tract deposition (16, 107). To minimize such errors, the aerosol sampled from the inhaled air was humidified to 90% relative humidity and subsequently dried. Thereby, the inhaled and exhaled size distributions were comparable.

**Deposition fraction and dose rate**

The size-dependent deposition fraction, $DF (d_{me})$, was assessed from the general equation

$$DF(d_{me}) = 1 - \frac{C_{in}(d_{me})}{C_{ex}(d_{me})}$$

where $C_{in}$ and $C_{ex}$ are the measured inhaled and exhaled particle concentrations, respectively. The mobility equivalent diameter, $d_{me}$, is used for particle size determinations in this work. As discussed elsewhere, this is the most relevant diameter measure in the diffusion-dominated size regime (approximately < 700 nm for particles with effective density similar to wood smoke) (16). The calculated DF was corrected for particle losses in the instrument between inhaled and exhaled sampling points and for mouthpiece dead space (108, 109).

**Study II**

**Bronchoscopy**

Bronchoscopy was done 24 hr after each exposure using a flexible video bronchoscope (Olympus BF IT200, Tokyo, Japan), as previously described (85). The subject received local anaesthetics in pharynx, epipharynx, over the vocal cords and along the entire of the bronchial tree. Bronchial wash (BW) was performed twice by instillation of 2 x 20 ml of normal saline (pH 7.4) at 37 °C. The first BW was used for total differential cell counts and analysis of soluble mediators whereas the second BW was used for antioxidant determinations. Bronchoalveolar lavage (BAL) was taken by instillation of 3 x 60 ml normal saline. The obtained aspirates from the BW and BAL were collected in separate containers and placed on iced water and filtered with a nylon filter having a pore diameter of 100 µm (Syntab Product AB, Malmö, Sweden) and centrifuged at 400 g for 15 min. It was then kept frozen at -80°C for further testing for differential cell counts and for inflammatory markers testing. The bronchial biopsies were taken, in a randomized order, either from the anterior aspect of the main carina and the
subcarinae down to the fourth generation bronchi of the right side, or from the posterior aspect of the carina and the corresponding bronchi in left side.

**Immunohistochemical analysis**

Endobronchial mucosal biopsies obtained during bronchoscopy were processed and embedded in glycol methacrylate resin (Polyscience; Northampton, England), as previously described (86). 2 µm thick sections were cut and immunostained with monoclonal mouse antibodies for the following markers: Neutrophil elastase; mast cell tryptase (Dako); CD3 (Biolegend); ICAM-1 (Biosource); p-selectin (AbD Serotech); and for EN4 (Monosan). The immunostaining procedure followed has been described previously (86). Briefly, endogenous peroxidases were inhibited using a sodium azide and hydrogen peroxide solution and nonspecific antibody binding was blocked using undiluted culture medium (Sigma; St Louis, Missouri). Mouse anti-human antibodies were applied and incubated overnight at room temperature. After washing with Tris-buffered saline, the biotinylated rabbit anti-mouse secondary antibody (IgG F[ab']2; Dako) was applied and incubated for 2 hours. After further washing a streptavidin-biotin horseradish peroxidase complex (Vector Laboratories) was added and incubated for 2 hours. The sections were then visualized using 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) and counterstained with Mayer's haematoxylin. Positively stained nucleated cells were counted within the submucosa, excluding smooth muscle and glands, and in intact epithelium. Counts were corrected for submucosal area and epithelial length using the program LeicaQWin V3 (Leica Q500IW; Leica, Cambridge, UK). Activated blood vessels were expressed as the ratio of adhesion molecule-positive vessels to the pan-endothelial marker EN4-positive vessels and corrected for submucosal area.

**Flow cytometry measurements**

Flow cytometry is a technology that measures the physical characteristics of a single particle, commonly cells in medical research, when they flow in a fluid stream through a beam of light. Cells size, granularity and relative fluorescence intensity are examples of the properties that are included in the measurements by using optical-to-electronic coupling systems to record how the cells receive laser light and emit fluorescence. Basically, the flow cytometer consists of three parts; a) the transport system by which the cells become aligned in single cell line, b) the optics system with lasers to illuminate the particles and optical filter to detect the resulting light signals using special detectors and c) the electronics system that converts the detected light signals into electronic signals that will be processed by a
computer. Scatter light occurs when the cell deflects laser light. The scatter light is divided into forward-scatter light (FSC) which correlates with cell size, and slide-scatter light (SSC) which relays to inner content of the cells i.e. cell complexity. By adding fluorescent labelling, the flow cytometry can determine functional characteristics of the examined cells through monoclonal antibodies coupled with fluorochromes, which bind to specific cell surface receptors.

A flow cytometer was used in study II to analyse lymphocyte subsets in BAL and peripheral blood using FACSCalibur™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). BAL cells were centrifuged and diluted with PBS to a final concentration of $10^6$ cells/ml and 10 µl of antibody solution was added to 200 µl of cell suspension and allowed to bind for 30 minutes at 4°C. Red blood cells were lysed with FACS™ lysing solution. The remaining cells were washed by adding PBS to the tubes and centrifuged. Cells were then fixed with 500 µl CellFIX™ (Becton Dickinson).

Up to 3,000 total events were collected in the lymphocytes gate per sample and monoclonal antibodies were added before processing the scatter readings as previously described (110).

Figure 5. Example of flowcytometry of lymphocyte subsets with gatings.
Antioxidant determination

The second BW and the BAL samples were analysed for antioxidants, including the reduced glutathione (GSH), glutathione disulphide (GSSG) and total glutathione (GSx). GSH and GSSG were measured according to the method of Baker et al (111). Briefly, standards containing 0–165 pmol/50 µl GSSG, equivalent to 0–330 pmol/50 µl GSx were prepared in 150 mM NaCl, 1 mM EDTA, pH 7.5. Fifty microliters of standard and sample were then transferred to microtitre plate wells and 100 µl of a reaction mixture was added to each well to give a final concentration in each well of 0.15 mM DTNB, 0.2 mM NADPH, and 1 U of glutathione reductase. Immediately after the addition of the reaction mixture, the microtitre plate was transferred to a plate reader (EL 340, Biokinetics Reader) for analysis. The rate of TMB formation was then followed by the rate of change of absorbance at 405 nm, over a 2 min period at 30% °C. To determine the concentration of GSSG, 5 µl of undiluted 2-vinyl pyridine (Aldrich Chemical Co, Poole, Dorset, UK) was added to 130 µl of the samples and standards, vortexed for 5 sec, and then incubated at room temperature for 1 h. The GSH pool was determined by the subtraction of the GSSG (x 2) from the total GSx concentration.

Blood samples

Blood samples were obtained at baseline, 24 and 44 hours after exposure. The samples were centrifuged at 3,000 g for 30 min at 4°C before plasma was removed and frozen at -80°C for further analysis. Plasma samples were analysed for markers of acute inflammation: Interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), soluble Intercellular Adhesion Molecule-1 (sICAM-1) and Clara cell secretory protein 16 (CC16) using DuoSet ELISA kits (R&D Systems, Abingdon, UK), according to the manufacturer’s instructions.

Lung function test

Lung function tests were done at inclusions, before, after each exposure and 24 hours after exposure (pre-bronchoscopy) using a computerized spirometer (Jaeger MasterScreen™ Pneumo, Germany). The tests were performed according to the guidelines of the American Thoracic Society (112).
**Fraction of exhaled nitric oxide**

The fractions of exhaled nitric oxide at the flow rates of 50ml/ s and 10ml/ s were tested before, after each exposure and after 24 hr by using a chemiluminescence analyser (NiOX; Aerocrine AB, Stockholm, Sweden). The procedure was done according to ATS/ERS recommendations (113).

**Study III**

**Symptoms**

The subjective symptoms headache, eye irritation, nasal irritation, unpleasant smell, throat irritation, bad taste, nausea, cough and difficulty in breathing were assessed by questionnaire before and every 15 minute throughout the duration of the exposure, with scores based on a modified Borg scale, ranging from no symptoms (ranked 0) to maximal symptoms (ranked 11) (104).

**Lung function test**

Spirometry with determination of FEV, and FVC was performed before and one hour after each exposure using a Jaeger MasterScreen™ Pneumo, Germany.

**Systemic inflammatory markers**

Blood samples were obtained at baseline and 5 hours after exposure and handled as described above. Plasma samples were analysed for markers of acute inflammation: Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), P-selectin, soluble Intercellular Adhesion Molecule-1 (sICAM-1), and cluster of differentiation 40 ligand (CD40L) using DuoSet ELISA kits (R&D Systems, Abingdon, UK).

**Blood pressure and pulse rate**

Blood pressure and pulse rate were measured before exposure, 30 min and 60 min of exposure using an automated blood pressure device.
**In-vitro testing of PM**

Four filters, including the two selected for the human exposure campaign, were analysed regarding influence on oxidative and inflammatory potential. PM oxidative potential was assessed in a synthetic respiratory tract lining fluid (RTLF) model at a particle concentration of 50 µg/ml. PM oxidative potential was expressed as the percentage loss of ascorbate and glutathione from this model over a 4 hour incubation (pH 7.4, 37 °C) as previously described (114).

The human type II alveolar epithelial cell line A549 (ATCC CCL-185) was cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS, HyClone, Perbio Science, Aalst, Belgium) and 50 µg/mL gentamicin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. For experiments, cells were seeded in 24-well culture plates at 5 × 10⁴ cells per well and allowed to attach overnight before particle stimulation. Stock solutions of particles were generated at a concentration of 5 mg/mL in a solution of 0.0004% dipalmitoyl lecithin (DPL; Sigma) in distilled water and sonicated for 4 × 1 min, with vortexing in between. Particles were diluted in cell culture medium without FCS or supplements and used at 10, 30, 50 or 100 µg/cm². After a 24-hour incubation the cell free supernatants were harvested and the concentrations of IL-8 were measured using DuoSet ELISA kits (R&D Systems, Abingdon, UK). After removal of supernatants, the cells were washed with PBS and visually given a viability score, assessing cells as either normal, slightly inhibited, growth inhibited or dead.

Filter B, which was a particle filter with active charcoal and high filtrating capacity, was selected as most promising. Filter A, which was the same filter but without charcoal, was included as reference.

**Study IV**

**Study IVa: Local NO Synthase Inhibition**

Eighteen subjects were recruited into this study. Forearm blood flow was determined using forearm venous occlusion plethysmography and detected using mercury-in-silastic strain gauges placed around each forearm as described previously (115). All subjects underwent unilateral brachial artery cannulation using a 27-gauge steel needle (Coopers Needle Works Ltd) under controlled conditions. After a 30-min baseline infusion of 0.9% saline, subjects received an infusion of the NO synthase (NOS) inhibitor NG-
monomethyl-L-arginine (L-NMMA; Clinalfa® basic, Bachem) at incremental doses of 2, 4, and 8 µmol/min. The infusion of L-NMMA was continued at 8 µmol/min for the remainder of the study to fully inhibit all basal NOS activity (116, 117).

Sodium nitroprusside (SNP), an NO donor, was then coinfused with L-NMMA at doses of 90 to 540 ng/min and titrated to restore basal blood flow. Once basal blood flow was restored, the SNP infusion was continued for the remainder of the study to produce an “NO clamp” as described previously (116). This permits the assessment of vascular function in the absence of endogenous NO generation but without the potential confounding effects of basal vasoconstriction induced by isolated NO synthase inhibition. The dose-response relationship of the vasodilators acetylcholine (5, 10, and 20 µg/min; endothelium-dependent NO donor) and SNP (2, 4, and 8 µg/min; endothelium-independent NO donor) were assessed in the presence of the clamp. The vasodilators were given in a random order and were separated by an infusion of 0.9% saline for 20 minutes.

**Study IVb: Systemic Nitric Oxide Synthase Inhibition**

Fifteen subjects were recruited into this study. Subjects had a 20-gauge arterial line inserted to the radial artery of the non-dominant arm (LeaderCath, Vygon SA). The arterial catheter was attached to a validated pressure transducer (Deltran® II, Utah Medical Products) and data were recorded in real-time using Powerlab instruments and LabChart software (ADInstruments Pty Ltd). A validated semi-automatic oscillometric sphygmomanometer was placed around the upper arm of the dominant arm. Cardiac output, peripheral resistance, and stroke index were recorded using thoracic bioimpedance (Hotman®, Hemo Sapiens, Inc) and central arterial stiffness (pulse-wave velocity and pulse-wave analysis) recorded using the Vicorder (Skidmore Medical) and SphygmoCor™ devices (AtCor Medical).

A 17-gauge intravenous cannula inserted into a large antecubital vein of the dominant arm. After lying supine at rest for at least 20 minutes, L-NMMA was infused intravenously over 5 minutes (total dose 3 mg/ kg), and hemodynamic measurements were recorded for the following 45 minutes (118).
**Biochemical Analyses**

Blood samples were analysed for total and differential cell counts by an auto analyser (Sysmex XE2100, Sysmex Europe GmbH). Plasma samples were collected into ethylene diamine tetra-acetic acid and kept on ice until centrifuged at 2000g for 30 minutes at 4°C. Serum samples were collected and left to clot on melting ice for 60 minutes before being centrifuged at 2000g for 10 minutes at 4°C. Samples were immediately frozen and stored at -80°C prior to subsequent analysis.

Plasma was analysed for the endogenous NOS inhibitors asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) as well as the NO precursors L-arginine and L-homoarginine using a high-performance liquid chromatography (HPLC) method as described previously (119).

**Lung function test**

Lung function was determined by spirometry at baseline and 2 hours following the exposure (Jaeger MasterScreen™ Pneumo, Germany).
Statistics

Throughout the studies, Shapiro-Wilk test was performed for normality testing. Parametric data were presented as mean ± standard deviation (SD) and student’s T test was used to compare the mean differences across the exposures. Non-parametric data were presented as medians with interquartile and Wilcoxon Signed-Rank test was used to compare data when appropriate. The specific statistical analysis for the respective study is given below.

Study I

Mann-Whitney U test was used to compare the DF and dose rate between the healthy group and the subjects with COPD. Pearson correlation test was performed for associations between DF and lung function parameters and breathing pattern. All data were analysed using SPSS, version 21 for Windows (IBM® SPSS® Statistics 21, Chicago, IL, USA).

Study II

Wilcoxon signed rank test was used for comparison of BW, BAL, flow cytometry and Immunohistochemical data. Paired sample T-test was used for lung function and FENO data. Correlations between wood smoke induced effects in BW were analysed using the Spearman rank order correlation test.

A linear mixed-effect model was used to analyse the impact of wood smoke on systemic inflammatory markers. Subjects were selected as random effect, and exposure, time, order of exposures and interaction between exposure and time as fixed effects.

Data were analysed using SPSS, version 20 for Macintosh (IBM® SPSS® Statistics 20, Chicago, IL, USA) and GraphPad Prism (GraphPad software version 6 for Macintosh, San Diego, CA, USA).

Study III

The Wilcoxon Signed-Rank test was used for calculations of differences between the delta changes (Maximum minus pre-exposure symptom score). McNemar’s Chi-square test was performed to analyse the difference between the number of subjects reporting symptoms after exposure to unfiltered and filtered DE. The Wilcoxon Signed-Rank test was used to
compare the delta change in inflammatory markers in the peripheral blood across the exposures. Paired-sample Student’s $t$ Test was used to compare the delta changes in lung function across exposures (one hour after exposure minus before exposure).

Oxidative capacity of unfiltered and filtered diesel exhaust particles was analysed using the Kruskal-Wallis One-way-analysis of variance (ANOVA) with post hoc analysis with the Games-Howell test for groups of unequal size and variances. Pro-inflammatory effects of unfiltered and filtered diesel exhaust particles were analysed using One-way analysis of variance (ANOVA) with Dunnett’s Multiple Comparison Test. A two-way ANOVA with Tukey’s multiple comparison test was performed to compare the efficacy of filter A-D with unfiltered DEP. Data were analysed using SPSS (SPSS Inc. Chicago, IL, USA, version 17) and GraphPad Prism (GraphPad Software, version 5 for PC).

**Study IV**

Statistical analyses were performed using Student’s paired $t$-tests and 2-way analysis of variance (ANOVA) with repeated measures where appropriate. The extensive Plethysmography data management and analysis were done as described in details previously (120, 121). All analyses were performed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software).
Results

Study I

Wood smoke particles

The total particle concentrations inhaled by the subjects, derived from the measured size distribution and effective density, were $78700 \pm 78000 \text{ cm}^{-3}$ by number, $3700 \pm 1800 \text{ mm}^3/\text{m}^2$ by surface area and $81 \pm 35 \mu\text{g/m}^3$ by mass (Figure 6). If assuming agglomerated particles, the surface area was $8200 \pm 3500 \text{ mm}^3/\text{m}^2$. For reasons previously explained, the surface area calculated for spherical particles was used in the proceeding analysis. The mass concentration measured by the TEOM was $132 \pm 26 \mu\text{g/m}^3$. The higher mass measured by the TEOM, compared to the value calculated from the size distribution measurement, is explained by a larger size range for this instrument ($< 1 \mu\text{m}$, see also Figure 6) and possibly a different instrumental response to peaks in the wood smoke emissions.

The size distribution was clearly bimodal. Fitting two log normal functions to the distribution results in count mean diameters (CMD) of 45 and 149 nm and geometric standard deviations ($\sigma_g$) of 1.88 and 1.61 and a relative number concentrations of 64% and 36% for each mode respectively.

The variation in particle concentrations was due to fluctuations between efficient and soot-rich combustion, as is typical for this type of wood smoke. The chemical characterization of the PM in the chamber during similar combustion conditions using the same stove and fuel, showed that the carbonaceous matter was dominated by soot with an elemental carbon (EC) and total carbon (TC) ratio of $0.72 \pm 0.08$ as given in Unosson et al (122). Further, from analysis of the components in the wood smoke, it was estimated that the total PM consisted of 38% soot, 24% organics and the remainder presumably alkali salts (a correction factor was used to convert the organic carbon (OC) content to total organic PM and EC to soot PM mass concentration).
Respiratory tract deposition

The deposition fraction (DF) of wood smoke particles was decreasing with increasing particle size in the studied interval (Figure 7). The total deposition fraction (TDF) of wood smoke particles for the healthy subjects was $0.34 \pm 0.08$, $0.22 \pm 0.06$ and $0.22 \pm 0.06$ by number, surface area and mass respectively. The surface area and mass was dominated by the larger particles for which DF was low and hence also TDF. A significant part of the mass and surface area of the wood smoke particles was found at larger sizes than the measured range of 10-500 nm. For the healthy group, TDF by number correlated positively with tidal volume ($r=0.65$, $p=0.02$) and negatively with respiratory rate ($r=-0.84$, $p=0.0006$).

For the healthy subjects, tidal volume was positively correlated with dose rate by number ($r=0.88$, $p=0.0001$), surface area ($r=0.74$, $p=0.005$) and mass concentration ($r=0.71$, $p=0.009$). Furthermore, there was also a significant correlation between minute ventilation and dose rate by number ($r=0.67$, $p=0.018$), surface area ($r=0.62$, $p=0.03$) and mass concentration ($r=0.59$, $p=0.04$).
The particles emitted from the sooty wood smoke combustion that were 50-300 nm had a tendency to higher DF in subjects with COPD. Particles of a smaller size, on the other hand, had a tendency to be lower in COPD subjects. For comparison, figure 7 also shows the experimentally determined DF for diesel exhaust particles (123).

The total deposition fraction (TDF) and dose rate by surface area and mass tended to be higher in COPD, still non-significantly (p=0.25 for TDF and p=0.21 for dose rate; Mann-Whitney U test).

There was a wide distribution of tidal volumes, respiratory rates and minute ventilation in the subjects with COPD and no additional significances were found.

Figure 7. Deposition fractions of inhaled wood smoke particles in healthy and COPD subjects. Diesel exhaust data are included for comparison.
Study II

Wood smoke characterization

The average total PM$_1$ concentration in the chamber during the exposures, measured by the TEOM, was of 314 $\mu$g/m$^3$ (range 232–356 $\mu$g/m$^3$), which was associated with average NO$_x$ and CO concentrations of 0.41 ppm and 25 ppm, respectively. Due to the combustion procedure, as described above, the concentration of PM and gases in the chamber did vary, although the aerosol residence time in the chamber (~20 min) compensated for this. During a typical exposure period the total particle number concentration in the chamber varied between 1–2.5 × 10$^5$ part/cm$^3$. The particle number size distribution was clearly bimodal with one peak at 60–70 nm and one peak at 150–200 nm. Previous studies show that the 60–70 nm peak consist of alkali salt particles (e.g. K$_2$SO$_4$ and KCl) and the 150–200 nm peak consist of a soot mode with more or less condensed organic material (9, 124).

The total carbonaceous (TC) PM of the wood smoke was dominated by EC with a EC/TC ratio of 0.72 ± 0.08. Based on estimation of the components of the total PM in the wood smoke, the PM consisted of 38% soot, 24% organics and the remainder presumably alkali salts. A factor of 1.8 was used to convert the OC content to total organic PM and a factor of 1.1 was used to convert EC to soot PM mass concentration. The total PAH concentration in the chamber was 1.1 ± 0.7 $\mu$g/m$^3$, of which 74% (0.78 $\mu$g/m$^3$) was in the particulate phase (table 2). The 12 dominating PAH compounds in the PM fraction, accounting for 86±2% of the total analysed PAH, were (in descending order); Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(ghi)perylene, Benzo(e) pyrene, Benz(a)anthracene, Indeno(1,2,3-cd)pyrene, Benzo (k)fluoranthene, Benzo(ghi)fluoranthene, Coronene, Pyrene, Perylene and Fluoranthene.
Table 2: PM characteristics of wood smoke.

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM, mass conc. (TEOM)</td>
<td>µg/m³</td>
<td>314 ± 38</td>
</tr>
<tr>
<td>PM, mass conc. (filter)</td>
<td>µg/m³</td>
<td>294 ± 36</td>
</tr>
<tr>
<td>CO</td>
<td>ppm</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>NOx</td>
<td>ppm</td>
<td>0.41 ± 0.12</td>
</tr>
<tr>
<td>EC/TC (elemental/total carbon)</td>
<td></td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td>Organic fraction of total PM</td>
<td>%</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>Soot fraction of total PM</td>
<td>%</td>
<td>38 ± 9.9</td>
</tr>
<tr>
<td>PAH-PM associated</td>
<td>µg/m³</td>
<td>0.78 ± 0.56</td>
</tr>
<tr>
<td>PAH-semi-volatile</td>
<td>µg/m³</td>
<td>0.28 ± 0.12</td>
</tr>
</tbody>
</table>

**Immunohistochemistry of the bronchial biopsies**

Representative bronchial biopsies had been obtained from all 14 subjects after both air and wood smoke exposure. There was a significant increase in submucosal and epithelial CD3+ T-cell lymphocytes (p<0.01 and <0.05 respectively), together with CD8+ T-cells in the epithelium (p<0.05) after exposure to wood smoke vs. filtered air (Figure 8). CD4+ cells were unaffected by exposures. Mast cells were significantly increased in the submucosa (p<0.01) after wood smoke exposure. There were no significant changes in other cell types nor adhesion molecule expressions after exposure to wood smoke.
Figure 8. Microscopy photographs (x40) of cell stainings in bronchial biopsy mucosa together with scatterplots of study data.

**Bronchial wash and bronchoalveolar lavage**

Representative paired samples from both air and wood smoke exposure were obtained from thirteen subjects. After exposure to wood smoke vs. filtered air there were statistically significant reductions of macrophages, neutrophils and lymphocytes in the bronchial wash (BW) (p<0.05 - <0.01, figure 9). Moreover, wood smoke exposure significantly reduced the BW levels of soluble Intercellular Adhesion Molecule-1 (sICAM-1), myeloperoxidase (MPO) and matrix metallopeptidase 9 (MMP-9) (p<0.05- <0.01).
There were significant positive correlations between the reduced number of neutrophils and decreased amounts of MPO ($p<0.01$, $r=0.791$) and MMP-9 ($p<0.01$, $r=0.70$) after wood smoke exposure (figure 10).

Figure 9. Bronchial wash cell scatterplots.

Figure 10. Spearman’s rank correlations between the number concentration of neutrophils in BW with the amount of MPO and MMP-9 obtained after exposure to wood smoke.
In bronchoalveolar lavage (BAL), there was a significant decrease in the total lymphocyte numbers as well as the total number of CD3+, CD4+, CD8+, CD4HLADR+, CD8+CD314+, CD4+CD25+ cells after exposure to wood smoke ($p<0.05$-$0.01$). The BAL levels of IL-6, sICAM-1, CC16, GrzA, MPO and MMP-9 were unaffected by the exposures.

**Antioxidant levels**

Compare with exposure filtered air, there was significant increase in the total glutathione (GSx) in BAL after wood smoke exposure ($p=0.013$), along with a tendency for increase in reduced glutathione (GSH). No statistical differences regarding the oxidized glutathione (GSSG) were found.

**Peripheral blood**

Flow cytometry analyses on peripheral blood demonstrated significant increases in CD16+56+ cells as well as CD4HLADR+ and CD8HLADR+ cells ($p<0.05$-$0.01$,) by wood smoke exposures (figure 5), other cell types were unchanged. No significant changes were seen for IL-6, TNF-α, sICAM-1 and CC16 over exposures.

**Lung function test**

Lung function parameters were not significantly changed by wood smoke of filtered air exposures.

**FEnO**

There were no significant differences between changes in FENO after exposure to wood smoke vs. air.
Study III

Exposure details
During the unfiltered DE exposure the mean PM concentrations within the chamber was 350 µg/m³, with NO and NO₂ concentrations of 2.49 and 0.68 ppm, respectively. Filter A reduced the average PM₁₀ concentration by 47% (p<0.001) and the particle number concentration by 36% (p=0.01). Filter B, which contained an active charcoal component, was even more effective, reducing PM₁₀ concentrations by 74% (p<0.001), with an associated reduction in particle number of 75% (p=0.001). The combination filter also reduced the concentrations of NO₂ by 85% (p<0.001), whilst Filter A showed no significant effect on NO₂. Hydrocarbon concentrations were not significantly reduced with filter A, but filter B reduced concentrations by an average of 58% (p<0.001).

Symptoms
All of the subjective symptoms examined, eye and nasal irritation, bad smell and taste, plus headache, were experienced in an increased proportion of the subjects following the unfiltered DE challenge compared with filtered air.

Figure 11 displays the number of subjects reporting symptoms after each exposure. Filter B significantly reduced the number of subjects reporting eye irritation, nasal irritation and bad taste (p<0.05-0.01).

Eye irritation, nasal irritation and bad taste were significantly reduced after exposure with diesel exhaust filtered with filter B (combined particle and active charcoal filter) compared with unfiltered exhaust (p<0.01-0.001). Only a few subjects experienced eye irritation before and after the filtered air exposure while the number increased to 18 out of 28 following the unfiltered diesel exhaust challenge. Filter B was associated with a significant (p<0.001) reduction in eye irritation by 80%. Unfiltered diesel exhaust elicited nasal irritation, with in 20 out of 28 subjects reducing significantly (p<0.001) to only 6/28 after inclusion of filter B in the exhaust outlet. Filter A did not affect the individual symptom perception.
Figure 11. Subjects reporting symptoms.

**Lung function test**

A statistical reduction ($p=0.035$) in the FEV$_1$ response with DE, plus filter B, versus unfiltered DE, but the difference was small with no clinical significance.

**Systemic markers of inflammation**

No changes in the concentration of any of the markers of systemic inflammation were observed when responses across the filtered air and unfiltered DE exposure. Meanwhile, a significant reduction in the sICAM-1 responses with both DE passed through filter A ($p=0.040$) and B ($p=0.026$) compared with the unfiltered DE exposure, the response though was minimal and difficult to interpret in the absence of a clear systemic response with the unfiltered DE.

**In-vitro measurements**

The oxidative potential, assessed by the capacity of the sampled PM to drive the oxidation of ascorbate and glutathione from a simple synthetic respiratory tract lining fluid, was low for all PM samples and was not affected by passing the PM through either of the filters tested when examined on a per unit mass (per $\mu$g) basis. In contrast, the capacity of the collected PM to induce A549 cells to release IL-8 appeared significantly diminished after the samples were passed through the filtered containing activated charcoal, though the difference was only apparent at the higher concentrations: 30, 50 and 100 $\mu$g/cm$^2$ ($p<0.05$-$0.0001$).
Study IV

Thirty subjects completed the study protocols. There were no changes in any indices of lung function (FEV1, FVC and VC) or in hemoglobin concentration, white cell count or platelet count throughout all studies.

Plasma nitrite concentrations were higher as compared to the filtered air exposure (68 ±48 versus 41 ±32 nmol/L; \( p=0.006 \)) two hours following exposure to diesel exhaust. In contrast, there were no differences in plasma concentrations of the NO precursor L-homoarginine, or in the endogenous NOS inhibitors ADMA and SDMA at 2 or 6 hour following exposures, whereas plasma concentrations of the NO precursor L-arginine were lower after exposure to diesel exhaust as compared to filtered air (\( p=0.043 \)).

Study IVa: Local Nitric Oxide Synthase Inhibition

Sixteen subjects, median age 23 years, completed the study protocol: one subject failed to attend scheduled visits and one was withdrawn for technical reasons (failed cannulation). Blood pressure, heart rate, and baseline forearm blood flow were not different following either exposure (\( p>0.05 \) for all; data not shown). Infusion of the NOS inhibitor, L-NMMA, resulted in a similar dose-dependent vasoconstriction following both exposures (\( p=0.559 \); Figure 12A). After establishing the NO clamp by restoring basal blood flow with SNP coinfusion (208 ±33 and 236 ±26 ng/min following air and diesel exhaust exposure respectively; \( p=0.453 \)), both acetylcholine and SNP caused dose-dependent vasodilatation (\( p<0.01 \) for both) that was attenuated in comparison to previous studies (90). This vasodilatation, however, was similar following both exposures (dilute diesel exhaust versus filter air: \( P=0.209 \) for acetylcholine and \( p=0.613 \) for sodium nitroprusside; Figure 12B).

Study IVb: Systemic Nitric Oxide Synthase Inhibition

Fourteen subjects completed the study protocol. L-NMMA caused a greater increase in blood pressure (\( p=0.048 \)) and central arterial stiffness (\( p=0.007 \)) after exposure to diesel exhaust whereas there were similar reductions in cardiac output and increases in systemic vascular resistance (\( p>0.05 \) for both) as compared to filtered air (figure 13).
Figure 12 A. Forearm blood flow response during infusion of L-NMMA (2-way repeated measures ANOVA: p=0.559 for exposure). B: Forearm blood flow during infusion of the vasodilators acetylcholine and sodium nitroprusside.

Figure 13. Changes in invasive mean arterial blood pressure, central arterial stiffness (PWV), cardiac index, and stroke volume following systemic infusion of L-NMMA.
Discussion

Study I

Methods discussion

There are several types of experimental setups for measurements of respiratory deposition. In general, they all share the same principles, in which the setup is composed of an aerosol generation source, aerosol conditioning and inhalation system, and a particle detection method. The aerosol source in the current study was a common Nordic wood stove by which sooty wood smoke was generated under incomplete high temperature combustion. The generated aerosol was bimodal with particle count mean diameter (CMD) of 45 and 149 nm.

The RESPI technique is an example of flow-through-system which is suitable to use when there are varying in particles concentrations. In addition, the estimated loss of particles due to electric deposition is very low. The collection tanks have a connection to the atmosphere and thereby no pressure differences inside the instruments. The pneumotachograph connected to the exhaled tank provides data on actual breathing parameters.

SMPS is a useful method to measure particle concentration of the inhaled and exhaled air because of the wide particle size detection range (10-500 nm) and its superiority in size resolution. However, using SMPS has a disadvantage due to its low time resolution leading to limitation in the number of size scans in the inhaled and exhaled air during an inhalation setting. Therefore, the wood smoke generation process should be highly stable during the combustion process, which is rather difficult reality with the type of wood stove combustion. The generated particles in this study simulated well the particle characterization emitted form the most common way of wood burning worldwide, which as stated in study II, caused significant effects on inflammatory cell responses and induced inflammatory processes in the bronchial wall. No similar study had investigated the deposition probability of this type of experimental incomplete wood smoke combustion previously, however the RESPI technique had been used to investigate the deposition of wood smoke generated from pellet, under complete and incomplete combustion (9), DE in healthy and CODP subjects (123, 125), and ambient air UFP at a busy street (126). UFP deposition in the respiratory tract has been investigated previously by flow-through inhalation systems but the models differed in source generation, which was spark discharge carbon soot with the breathing pattern was predetermined, and the model had no contact with atmosphere (17).
Results discussion

Deposition in healthy

The combustion condition was varying during the study sessions which led to two size distributions representing predominantly smaller sized particles of alkali salt from the more efficient combustion phase and larger soot particles emitted during incomplete combustion (100). Compared with the previous particle deposition study of pellet combustions, the dry mobility size distribution peak for the emitted particles generated from the incomplete wood stove combustion was smaller than particles generated from both complete and incomplete pellet combustion, which resulted in a higher total deposition fraction by number and deposited dose whereas mass and surface area were relatively similar (9). In the other hand, DE and traffic-related particles have had higher total deposition fraction for number, surface area and mass compared with the current model (table 3) (125). The explanation is that the particles generated from the incomplete wood stove combustion were hygroscopic. They grow inside the respiratory tract under relative humidity of 99.5% and may reach up to three times the dry diameter and thereby the deposition decreases as the particle size increase. In contrast, the majority of the DE particles were hydrophobic, due to high content of organic hydrocarbons, and they grew less inside the respiratory airways. An interesting finding is that the larger sized wood smoke particles (>300 nm) showed somewhat higher DF compared with the hydrophobic DE particles which may explained by that the fact that the larger size mode of wood smoke combustion in this model was hydrophobic, with hygroscopic material on its surface and thereby grew in size and deposited by sedimentation and impaction.

<table>
<thead>
<tr>
<th>Aerosol Type</th>
<th>Number (± standard error)</th>
<th>Surface area (± standard error)</th>
<th>Mass (± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood stove smoke</td>
<td>0.34 ± 0.08</td>
<td>0.22 ± 0.06</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>Pellets, efficient combustion (9)</td>
<td>0.21 ± 0.08</td>
<td>0.23 ± 0.07</td>
<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>Pellets low-temperature combustion (9)</td>
<td>0.23 ± 0.06</td>
<td>0.23 ± 0.06</td>
<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>Diesel exhaust, idle engine (125)</td>
<td>0.64 ± 0.06</td>
<td>0.30 ± 0.06</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>Diesel exhaust, transient driving (125)</td>
<td>0.47 ± 0.08</td>
<td>0.27 ± 0.07</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>Traffic at busy street (126)</td>
<td>0.68 ± 0.08</td>
<td>0.35 ± 0.03</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Curbside (126)</td>
<td>0.60 ± 0.04</td>
<td>0.29 ± 0.04</td>
<td>0.23 ± 0.05</td>
</tr>
</tbody>
</table>
Deposition in COPD

COPD is a chronic disease characterized by a progressive inflammation in the airways, lung parenchyma and pulmonary vessels with irreversible chronic airway obstruction. Pulmonary deposition of inhaled airborne PM in COPD patients has been attractive to investigate not only because of COPD is a major importance for global public health (33), or the strong relationship between air pollution and worsening of pre existing COPD with increase morbidity and hospital admission (35, 127), but also because the data on biomass deposition in COPD is scarce and need to be explored.

The deposition fraction curve indicates subjects with COPD to have a higher DF of wood smoke particles in a range of 50-300 nm (figure 7). Subject with COPD often have high minute ventilation volume because of the high RR. As a consequence, deposited dos rate is expected to be high. This was the case in the preceding study when DE deposition in COPD had been studied using same RESPI instrument (123). Similar data have been reported from a study with an experimental deposition of ultrafine technetium-99m-labeled particles when subjects with chronic bronchitis had higher DF and deposition dose rate than a healthy group (128). A few other studies (129-131) have also showed higher DF in COPD. It is worth to mention that these studies were done with a predetermined breathing pattern and why direct comparison may not fit well as the current study, which was done with spontaneous breathing.

Differences in DF and deposited dose rate by exercise

Particle deposition dose depends on many factors, of which some are the tidal volume and respiratory rate. During exercise, both tidal volume and respiratory rate increases. Since the DF correlated positively with the tidal volume and inversely with the respiratory rate, though the net result for DF, at least theoretically, will be neutral (18). However, as the deposited dose rate depends on minute volume (see eq. of dose rate in methods section), the deposited dose rate increased by 4-4.5 folds during exercise (17, 18). Given that the COPD-and subjects with long standing asthma has higher minute ventilation; this will cause much higher deposited dose compared with healthy subjects doing the same physical exercise, supposing that the DF is the same.
Limitations

While there are many advantages to use the RESPI technique such as; high size resolution; rapid measurements; minimal size shift of the particles and negligible pressure variation related to breathing cycle, there was some limitation in the present study to fully interpret the result of the COPD deposition dose because of the minute volume inter-subject variability.

In addition, there was a difficulty in recruitment of COPD patients fitting the study inclusions criteria, hence the COPD part of the study might be underpowered. Noteworthy, some studies had investigated UFP deposition probability in the lungs using approximately the same power as in the current study (16, 129).

The hygroscopicity was not measured in the current study, which has been shown previously to have a great influence in deposition probability. However the particle characteristic were known, which make the interpretation rely on previous experience of particle hygroscopicity (9, 18, 125, 126).

One of the most challenging targets in studying particle deposition is to determine the deposition of specific particle types in specific regions of the lung, i.e. regional deposition. Use of combined particle labelling with emission tomography could potentially increase the understanding of the of respiratory deposition of specific particles.

Conclusions

The particles emitted from incomplete wood combustion in a traditional wood stove had a higher deposition probability than the particles generated from wood pellet burner, and lower than for diesel exhaust. The chemical and physical properties of the inhaled particles, mainly the size and hygroscopicity, determine the particle deposition in the lungs. Compared with healthy, subjects with COPD appear to have a tendency for higher deposition probability for particle sized 50-300 nm where the majority of the particles that originated from incomplete combustion can be found. Further studies with larger numbers of COPD subjects with different characteristics, are warranted to better understand PM deposition in this sensitive population.
Study II

Methods discussion

Although the adverse health effects of wood smoke exposure have been observed and studied in many epidemiological, cross sectional and in-vivo animal studies, the experimental human wood smoke exposure studies still sparse. One of the most challenging issues is the heterogeneity of wood smoke combustion. The adverse health effects of wood smoke are related to the chemical and physical characterizations of wood smoke which in turn are determined by source, appliance design, temperature and air supply (4, 132). The novelty of the present study is the investigation of wood smoke emitted from incomplete sooty combustion by a conventional wood log stove. The motivation of choosing such combustion was that it is a very common type of wood smoke combustion worldwide and many epidemiological and cross sectional studies have demonstrated a wide range of adverse health effects associated with indoor biomass exposure due its PAH and soot rich content (52, 78, 133). Moreover, in-vitro studies have demonstrated that wood smoke that contained high PAH elicited a broad spectrum of inflammatory and oxidative stress responses including TNF-α, IL-6 and IL-8 and free radical releases along with DNA damage and cell growth arrest (82, 83, 134, 135).

Bronchial biopsy findings

The expected findings regarding bronchial biopsies were infiltration of acute inflammatory cells following the experimental exposure to wood smoke generated under incomplete combustion. The obtained inflammatory pattern in the bronchial mucosa was unexpected due to absence of neutrophilic infiltration, which has been shown previously as a predominantly finding following experimental exposures to other types of air pollutants such as diesel exhaust and ozone (85, 86, 136, 137). Furthermore, increase in neutrophils have been observed both in blood and in sputum along with enhanced expression of cellular surface markers on neutrophils in peripheral blood taken from women chronically exposed to indoor biomass smoke generated mainly under incomplete air starved combustion (138). Along with this finding, increase in neutrophils percentages in BW and BAL was obtained after 2 hour exposure of healthy subjects to wood smoke emitted from smouldering oak wood with a particle mass concentration of 500 µg/m³. However, there were no data given about the absolute number of neutrophils and whether there was a shifts in the percentages of other inflammatory cell types (75).
In *in-vitro* studies using human cell cultures exposed to wood smoke PM have demonstrated an increase in proinflammatory cytokines such as TNF-α and IL-8 (135). The later is a known potent chemotactic factor for neutrophils. Theoretically, this meant that a neutrophilic infiltration was expected to occur as an acute response but could not be demonstrated at the time of bronchoscopy 24-hour post wood smoke exposure. An earlier neutrophilic infiltration cannot be excluded, although this is unlikely according to exposure studies with other air pollutants, especially as no increase in neutrophils associated soluble component was found.

A novel finding was the presence of a lymphocytic inflammation in the submucosal and epithelial regions, which was dominated by T lymphocyte infiltration with significant presence of epithelial CD8+ cells, in the absence of neutrophilia in the examined biopsies. The present study is the first study to demonstrate an isolated increase in airway epithelial CD8+ lymphocytes without concomitant CD4+ lymphocyte recruitment. This finding combined with the fact that the presence of CD8+ lymphocytes increased in the bronchial epithelium of smokers who developed COPD (139, 140) may indicate a relationship between wood smoke exposure and development and worsening of COPD. In addition, chronic exposure to biomass fuel has been suggested to have a causal relationship with COPD and chronic bronchitis (61).

Along with T lymphocyte infiltration in the bronchial submucosa, mast cells were also increased. The recruitment of mast cells has been demonstrated in many diesel as well as Ozone (O₃) exposure studies. Mast cells may have a central role to orchestrate the immunity responses to pathogens (141). It has been demonstrated that, upon activation, mast cells may deliver peripheral signals for lymphocyte recruitment (142), which may explain the presence of both mast and lymphocytes in the bronchial biopsies. The challenging question still, though, about the plausible pathways that causes mast cell influx in the bronchial mucosa after exposure to air pollutants.

**Bronchial wash and bronchoalveolar lavage**

The decrease in the inflammatory cells and soluble compounds in BW and BAL after wood smoke exposure were unpredicted results. The MPO and MMP-9 reductions correlated well with the decreasing numbers of neutrophils after wood smoke. The presented findings are in line with previous study where no detectable increase in inflammatory cells in BW and BAL had been observed after exposure to complete pellet combustion (74). However, it is worth to mention that the combustion conditions differed between these two studies, mainly regarding the chemical composition of the
emitted wood smoke where there were lower PAH and soot contents in the pellet study.

In previous in-vitro studies, pro inflammatory responses to wood smoke particle samples were investigated by analysing the release of TNF-α, IL-6 an IL-8 from co-culture of monocytes and penumocytes exposed to particles from wood smoke and traffic. Wood smoke particles enhanced lower levels of these inflammatory markers together with decrease in the number of viable cells compared with DEP and road tunnel particles (80). Similar findings were noticed by investigation of the release of IL-6, IL-8 and TNF-α from human macrophages together with studying the genotoxicity after exposure of the human lung cell line A549. Particles from wood smoke combustion causes DNA damage, whereas the emitted particles from wood smoke combustions elicited less inflammation. The inflammatory responses were lower compared with the particles collected from tire wear, urban streets and a subway station (143). Furthermore, wood smoke PM has been shown to be more cell toxic than ambient air devoid from wood smoke PM (135).

In addition, in an in-vitro study using mouse RAW264.7 macrophages exposed to wood smoke particles demonstrated low level of proinflammatory cytokine TNF-α and the chemokine MIP-2 parallel with signs of DNA damage and apoptosis responses. This resulted in a reduction in the cell numbers in normal resting phase (G1) and accumulation of cells to G2/M phase of cell cycle (82, 83).

Thus, there are indications from these in-vitro studies that particles emitted from wood smoke combustion may exert genotoxic effects. This may explain the decreased number of neutrophils, macrophages and lymphocytes in BW and BAL after exposure to wood smoke in the present study with subsequent down regulation of immune responses.

**Wood smoke and infections**

Several epidemiological and cross sectional studies have showed a relation between wood smoke exposure and increased susceptibility for both acute and chronic respiratory infections (62, 144-146). A meta-analysis of 24 studies indicated a two-fold increased risk of pneumonia (63). Moreover, exposure to wood smoke is associated with death caused by respiratory infection in children (147). In addition, exposure to biomass smoke had been showed to associate with increased risk for tuberculosis infection (65). Reduction of wood smoke exposure by 50% has been associated with
By taking in consideration that the macrophages is the first defence mechanism against many bacteria including mycobacterium tuberculosis, the increased risk of infection after exposure to Biomass fuel could be due to down regulation of the innate and acquired immunity. This could be due to adverse effects on cell growth and maturation, specifically for macrophages and neutrophils, with subsequent decrease in important chemotactic substances needed for intact immunity like MPO and MMP-9, which were reduced in present study. In a recent in-vitro study, MMP-9 was showed to have a critical role in phagocytosis and early host immune response against S.pneumoniae in wild-type mice (149). Furthermore, MPO plays an important role in both binding and killing bacteria (150, 151). Combined with this, the flow cytometry study of BAL showed decrease in lymphocyte subsets, which might give rise to increased risk of infections. The adverse cell effects can be also explain the absence of proinflammatory markers release both in bronchoalveolar lavage and in systemic circulation. This is in line with a recent study when healthy subjects exposed to two kinds of wood smoke, emitted from start-up and burn-out phase and exhibited no release of markers for systemic inflammation (77).

**Systemic effects**

Flow cytometry of peripheral blood showed 1.5-fold increase in the CD16/CD56 NK cells combined with increased expression of the activation marker HLA-DR on CD4+ and CD8+ cells. This finding revealed that there is an immune response after acute wood smoke exposure without a fulminant effect on other cell population in peripheral blood. In line with this, Dutta and co workers has reported also increase in CD8+ and CD19+ and Treg cells with suppression of CD4+ in rural women in India. The difference in magnitude of cellular finding may explained by the exposure duration, which might be enough to modulate the immunity (152).

As a sign of systemic effect, the investigated wood smoke has been associated with an immediate increase in central arterial stiffness with reduced heart rate variability (122), such cardiovascular responses has been suggested to associated with increase morbidity and mortality (153-155).
Oxidative burden of wood smoke

It is common to find changes in antioxidant levels after exposure to PM air pollution due to increased oxidative stress leading to associated with upregulation of redox sensitive signaling cascades (86, 87, 89, 95, 96, 156).

In the present study GSH levels tended, albeit non-significantly to be increased after wood smoke exposure. This could be due to a type II error, as the results are in line with a preceding exposure study with bronchoscopy sampling, which investigated effects of wood smoke generated from a pellets burner (74). It is also in line with in-vitro studies which have indicated wood smoke to cause an oxidative burden, although this has often appeared to be less extensive than for other PM pollutants, such as metal rich PM and DEP (143). Potentially, the tendency to increased GSH could also result from an efflux from apoptotic cells, mainly alveolar macrophages (157, 158). If so, we would have expected to detect a decrease in alveolar macrophages in BAL fluid, in similarity with the cell reductions in BW. BW antioxidants were not changed, but it should be noted that the decreased cell counts were found in the recovered fluid from BW portion I, and antioxidant measurements were done on the fluid recovered from the second BW portion. There may therefore be a mismatch between cells and antioxidant levels, which could be misleading.

Limitations

It is of importance to consider that wood smoke is very heterogeneous, why conclusions from one type of exposure may not be generally adapted for all kinds of wood smoke. The present type of sooty and PAH rich smoke was selected to be relatively representative for wood smoke pollution for cooking and heating using wood stoves with inefficient combustion.

Acute wood smoke exposure over three hours for one time may of course not be representative for long-term exposures, but may still give valuable information. The time points for sampling were based on previous studies and the 24 hours bronchoscopy time point was, based on DE and ozone exposure studies, expected to result in a fully established inflammatory state. This may not necessarily have been the case. It could be that relevant effects were occurring later, and that other important effects had already passed. Complementary time points would therefore be valuable to investigate. As regards blood samples, to our knowledge, we actually covered a longer period of time, up to 44 hours, than any other wood smoke exposure study. Despite this, we did not find any strong indications of clear pro-inflammatory effects. Still, we cannot exclude that events could have been transient between the time points of our three blood samples per exposure.
The present data were obtained from healthy young adults, and other more vulnerable groups of subjects with respiratory and cardiovascular conditions would be of importance to study.

**Conclusions**

Exposure to wood smoke generated from incomplete high temperature wood smoke combustion was associated with inflammation in the bronchial mucosal dominated by lymphocyte and mast cell infiltration. The reduction of cell sin bronchoalveolar lavage is not well understood and could be due to increased cell adherence, cytotoxicity or other effects. New studies with an earlier bronchoscopy and additional measurements including apoptosis markers are recommended to fill the gap of current knowledge about the health effects of wood smoke.
Study III

Methods discussion

Individuals travelling in cars, buses and other vehicles in traffic are exposed to high concentrations of air pollution, which may cause acute as well as chronic cardiovascular and respiratory effects (29, 37, 38, 159-161). In an earlier study, the concept of efficient vehicle cabin air inlet filters to reduce acute symptoms was proven, using diesel engine exhaust as a model for traffic-related air pollution associated with acute effects (103, 162). The present study was therefore designed to identify a new vehicle cabin air filter with strong potential to limit unfavourable diesel exhaust effects.

This was done by screening the oxidative and proinflammatory activity of DEP, which had been filtered by several different types of filtering devices. These data together with information on the filtering efficacy of the devices, were used to select two filter devices for an experimental study in humans, based on the preceding study by Rudell et al (103), which trough measurements of a range of surrogate markers for health effects would identify the best filter. This filter was estimated by the study sponsor Renault to go into manufacture for their vehicle fleet. The study was performed under an unrestricted grant with the company involved in the study design, but not in data analysis, interpretation of the data or draft of the manuscript.

The two in-vitro models used were motivated by oxidative stress and proinflammatory effects, especially related to neutrophilic inflammation, being strongly linked to DE and traffic-related health effects (86, 95, 96, 163, 164). DE induced airway inflammation has been demonstrated to be regulated by redox-sensitive transcription factors like nuclear translocation of nuclear factor-Kappa B (NF-κB), activated protein-1 (AP-1) with downstream activation of mitogen-activated protein kinases (MAPk) (89, 156) which leads to IL-8 production as well as upregulation of adhesion molecule expression such as intracellular adhesion molecule-1 (ICAM-1) which is important for cell recruitment and migration. Blocking of NF-κB has been shown to dissipate proinflammatory cytokine production by DEP (165-167), suggesting an interrelation between the proinflammatory cascades and the oxidative stress reaction following exposure to DE.

IL-8 is one of the key neutrophil chemoattratants with bronchial epithelial cells as a major source, with inflammatory cells as complementary sources. IL-8 stimulates neutrophil chemotaxis by activation of the contractile cytoskeleton, and enhances adherence of neutrophils to endothelial cells, matrix proteins and upregulates the cell surface expression of CD11b/CD18,
combined with transendothelial migration and degranulation of MPO, MMP-9 and other components. IL-8 is a robust and well validated marker for in-vitro assessments using cell cultures for the proinflammatory potentials of DEP and was therefore chosen before other neutrophil chemoattractants such as GRO-α and ENA-78 are upregulated by DE in-vivo. IL-6 is an example of a more generic proinflammatory component that may also reflect DEP effects. They were both suitable for evaluation of DEP effects in-vitro with a focus on responses in A-549 cells reflecting the alveolar responses. Primary epithelial cells or bronchial explants would have been interesting alternatives but would have added considerably to the complexity of performing the study.

The in-vitro study was done with equal mass basis, which means that similar diesel particle concentrations were used to assess their oxidative potential irrespective to particle filtering capacity of each filter. The majority of the in-vitro studies have used equal mass basis as exposure metric (168, 169), however it is insensitive to differences in the filtering capacity of the filters, which had to be taken into account at a secondary step when an overall capacity of the filters to reduce proinflammatory and oxidative potential was considered.

The oxidative potential of the unfiltered and filtered DEP was investigated by incubating them with antioxidants in order to mimic the reactions in respiratory lining fluid (RTLF). The direct oxidative potential of the DEP from a current diesel engine and fuel was moderate and the examined filters had little effect on the oxidative activity of the DEP. It is well known that PAHs from DEP may undergo reactions with phase-1 and phase-2 enzymes to make them more water soluble, but on the same time making them more oxidative by generation of semi-quinones and quinones. This secondary generation of oxidative capacity was not measured by the present assay. In summary, the oxidative potential of the DEP was largely depending on the filtering capacity of the different devices. There were no indications that the filters enhanced or reduced the oxidative potential of the DEP on a mass equivalence basis, in contrast to what was seen for proinflammatory potential by measuring IL-8 in cell cultures.
Results discussion

Based on the *in-vitro* cell study results, it was clear that Filter B which contained active charcoal had a filtering capacity that was superior to the other filters in reducing the NO$_2$, hydrocarbons, DEP mass and number concentration, and the filtered DEP were less prone to induce IL-8 release and cytotoxicity. These data were in-line with the Rudell et al 1999 study (103), which showed the addition of active charcoal to be critical for reduction of DE induced symptoms why Filter B was taken forward to the human exposure study. Particle Filter A, which was composed of the same medium as Filter B, but without active charcoal compound, was chosen as a reference. Unfiltered exhaust was included as positive control and the human exposure study was designed to detect significant effects by filtering with Filters A and B. Filtered air was included as a negative control to enable an appreciation of the magnitude of effects.

Exposure to unfiltered diesel exhaust induced symptoms, which were associated with mucus membrane such as eye irritation, nasal irritation, unpleasant smell, throat irritation, bad taste as well as headache. Almost all these symptoms improved after exposure to the DE filtered with active charcoal containing Filter B, whereas particle Filter A, in spite of reducing the particle mass and number almost by half, failed to improve these symptoms. This was also true previous studies evaluating a ceramic particle trap, placed at the end of the tail pipe to filter the DE and reduced the particle number by about 50% without significant improvements of symptoms, bronchoconstriction and inflammatory markers in the bronchoalveolar lavage (BAL) (85, 104).

The use of the modified Borg scale, has been shown to be useful in quantifying symptoms related to DE exposure and a recent study has confirmed the current model of blinding the exposure for the research subjects (170). The current study used a relatively large cohort of subjects in order to have a sufficient power to detect significant filtering effects on the primary symptom parameters, and also enable a reasonable power for secondary parameters, for which intervention data were few or lacking.

Exposure to DE has been shown to enhance the production of inflammatory cytokines along with upregualtion of endothelial adhesion molecules both in human experimental studies (86, 97) and *in-vitro* studies (166). Indications of a spill over of the inflammatory response to DE to the systemic circulations were found in a previous exposure study where IL-6 and TNF-α were increased at the time point of blood collection 24 hours after exposure.
Since the proinflammatory response in the lungs have appeared to peak around 6 hours after exposure, it was estimated that an increase in blood markers would also be present around this time point and that the present population could be large enough to detect a difference in the levels. This time point was also for logistical reasons more appealing since collection of blood samples on the following day would have added substantial additional load to an already complex and staff demanding study. In hindsight, it is possible that a later time point could have been needed to detect any evident inflammatory response as estimated in the peripheral blood. A modest but significant change was found for s-ICAM1, an immunoglobulin superfamily adhesion molecule shown to play an important role in the neutrophil-endothelial and epithelial cell adhesion. Since no significant increase occurred after unfiltered DE vs. filtered air, the significant difference between Filter B and unfiltered DE could be a chance finding.

Standard spirometry parameters were shown to be unaffected by DE in this study. Exposure to DE has previously been demonstrated to significantly increase airway resistance when using the more sophisticated and sensitive whole body plethysmography technique (104). The logistical difficulties prevented this technique from being applied and standard spirometry was performed. It is worth to mention that the changes in FEV1 and FVC using standard spirometry to evaluate acute effects of traffic-related air pollution only been shown in the study by McCreanor et al 2007 (29). The study investigated a large cohort of healthy and asthmatic subjects exposed during walk along the busy Oxford street in London, where diesel exhaust was expected to be the dominating source of air pollution, with exposure in a nearby park as reference. It is possible that differences in populations and previous exposure may have accounted for the differences, but it is also possible that other components in the polluted Oxford street air, such as break wear dust or other coarse particles, could have played a role.

Limitations

Ideally the study should have incorporated sensitive cardiovascular investigations such as bilateral forearm plethysmography with infusions of vasoactive agents, which has been shown to powerful to detect adverse effects by diesel engine exhaust. Such techniques could potentially have given a much broader view of the ability of filters to protect against noxious effects by DE when exposed in traffic. Unfortunately it was beyond the scope and resources available for the project to undertake such investigations in a four-arm exposure study. Blood pressure and pulse rate were recorded but were not significantly affected. Other investigators have shown effects of DE when subjects were resting during the exposures. In this study intermittent
exercise was used to enhance the breathing and minute ventilation, and this has likely given carry-over effects into the resting periods when pulse and blood pressure were measured.

Conclusions

Taken together Study III indicates that vehicle cabin air inlet filters should not only contain an efficient ultrafine filter component, as the addition of active charcoal component was necessary to reduce the proinflammatory potential of the DEP, as well as to improve symptoms. The DE exposure level used has previously been demonstrated to cause worsening of bronchoconstriction in asthmatics, impaired fibrinolysis, increased arterial stiffness, thrombocyte adhesion, ex-vivo thrombus formation in healthy individuals as well as ST-T segment changes in subjects with coronary heart disease. It is possible that the best filter in the study Filter B, has the capacity to affect also these parameters, but this will demand complementary research to determine.

There are now two cabin air inlet filter studies with well-established results. A question is whether there is potential for further improvement of filters. In the Rudell et al 1999 study (103), there were differences shown even between to filters with different types of active charcoal components, suggesting that the design and manufacturing of active charcoal filters may give varying efficacy to reduce health related efficacy.

The scope of using highly efficient filters may in the future potentially be recommended not only for personal cars, heavy vehicles and collective traffic. Moreover, the usage may be extended to houses, especially for sensitive groups (171). It should be stated that the potential for filtering of air pollutants should not eliminate the need to regulate, manage and reduce the traffic-related air pollution emissions, which should be a priority for society.
Study IV

Methods discussion

The previously demonstrated ability of DE to cause vascular dysfunction makes studying endothelial Nitric Oxide (eNO) kinetics to be an attractive field of research, as it has a central role in vascular tone maintenance (172). The protocol for study II has was designed to continue the investigation of the proposed effect of NO bioavailability disturbance and vasomotor dysfunction after DE exposure, as shown in human exposure studies previously (90, 91). In order to extend the time course investigation of the impaired vasomotor function following DE exposure, an identical study was done with a vascular study at 24 hours post exposure (97). The study demonstrated the endothelium-dependent vasodilatation by acetylcholine to be reduced, with a tendency for bradykinin, but without effect from endothelium-independent vasodilatation. The later has been shown to be affected when the vascular investigation was performed 2 hour post exposure, hence the current study conducted at this time course.

The use of controlled exposure to a predefined air pollutant, in this case DE PM, reduces the risk of concomitant effect of other component and thereby reduces the potential confounders which may induce oxidative stress and endothelial Nitric Oxide synthase uncoupling. Moreover, this study was conducted by crossover design. Therefore, not only the power to detect minimal changes increased but also the effect of potential inter-subject variations was minimal.

Results discussion

One of the main findings in these studies were the equal vasoconstriction after exposure to DE and filtered air in the presence of local NO synthase inhibition. In addition, there were similar vasomotor responses to endothelium dependent and independent vasodilators, which were attenuated significantly after DE in previous studies (90, 173). The absence of a significance effect of DE on the vasomotor function, in the presence of NO-clamp, suggests that basal peripheral resistance vessel tone is maintained by a balance in the NO generation and consumption. Furthermore, the ability of NOS inhibition to reverse the vasoconstrictor effect of DE suggests a decrease in the NO bioavailability either by upregualtion of the expression of inducible NOS (iNOS) or via uncoupling of endothelial NOS (eNOS) leading to increased in oxygen free radical species formation (174). Free radical formation has been suggested to cause
further vascular impairment and by using superoxide dismutase (SOD), the inhibitory effects of DE PM to vasodilators was partially reversed (98).

In the presence of systemic NOs inhibition, the central vascular resistance and blood pressure were increased followed DE exposure, while the systemic vascular resistance was increased both after exposure to DE and filtered air. It could potentially have been that a decreased NO bioavailability in the conduit vessels was so pronounced, that it could not be compensated by an increased basal NO release, as in these vessels, the vascular tone is depending predominantly on endothelial NO generation (175).

The plasma nitrite concentration was increased combined with decrease in the NO precursor L-arginine that may indicate increase in the NO production. However, it has been estimated that as little as 1.2% of plasma arginine is used for whole body production of NO (176). In addition, human endothelial cells can recycle L-citrulline to L-arginine (177). Therefore, it is unlikely that increase NO production would cause the decrease in plasma arginine.

It is worth to mention that there are another mechanisms for vasodilatation, especially in the peripheral vessels such as via endothelium-derived hyperpolarizing factor (EDHF) and prostaglandins (178), and that might explain why the response to acetylcholine infusion (endothelium dependent vasodilator) was the same in both exposures in spite of presence of NOS inhibitors.

**Mechanisms of combustion related cardiovascular disease**

In addition to vascular dysfunction, there are other mechanisms by which exposure to ambient air, particularly traffic-related PM may exert its acute and chronic effects on the cardiovascular system (159). It has been shown that direct instillation of PM10 in Watanabe hyperlipidemic rabbits was resulted in development of vulnerable plaque in coronary vessels and aorta (179). Similar findings were seen after chronic exposure to concentrated ambient PM2.5, which caused progression in aorta plaques in apolipo-protein–E-knockout mice (180, 181). A cross sectional study by Künzli and co-workers revealed an increase of carotid intima-media thickness (CIMT) by 6% for every 10 µg/m³ increase in PM2.5 (182). The CIMT has been used frequently for cross sectional and longitudinal studies for investigation the role of air pollution in atherogenesis (38, 183).
Progress of plaque formation requires a further disturbance in coagulability status. Significant changes in circulating markers of platelet and endothelial cell activation, such as von Willebrand factor (vWF), p-selectin and CD 40L, were found in relation to increase exposure to PM$_{2.5}$ in healthy subjects during Beijing Summer Olympics game (184). Moreover, in-vivo impaired endogenous thrombolysis and enhanced ex-vivo thrombus formation have been demonstrated after exposure to DE in healthy subjects (90, 92). Rupture of the vulnerable atherosclerotic plaque, enhanced thrombosis generation and impaired vascular function are important pathophysiological predisposing factors for the development of acute myocardial infarction. Case-crossover and time series studies have linked exposure to traffic-related to trigger acute cardiovascular events (39). Recent study suggest the risk for ST-Elevation Myocardial Infarction (STEMI) to increase by 18% with each 7.1 μg/m$^3$ increase in the PM$_{2.5}$, one hour before onset of STEMI, whereas there was no such risk with Non- ST-Elevation Myocardial Infarction (NSTEMI) (185). Plaque rupture is mandatory in both STEMI and NSTEMI, however, the former needs further activation of coagulation's pathways to cause total artery occlusion. The impaired endogenous fibrinolysis and/or enhanced thrombus formation at vessel's injury sites can explain the proposed relationship between PM and onset of STEMI in the presence of plaque rupture. Moreover, impaired endothelial and non-endothelial dependent vasodilation, combined with vascular constriction and change in the arterial stiffness may contribute to the development of acute coronary syndrome.

**Limitations**

The study was designed to investigate the effect of NO bioavailability after exposure to DE. However, there are other different mechanisms that may be involved in the endothelial vasomotor dysfunction after DE exposure such as via autonomic neural signalling, prostaglandins and endothelium-derived hyperpolarization factor. As a possible confounder, uptake of nitric oxide from the diesel exhaust itself could contribute to the elevated nitrite levels observed in this study (186). As always in experimental studies with healthy subjects, there is a concern that an investigation of more susceptible individuals with co-morbidities could have yielded stronger effects (187). On the other hand, healthy subjects do not have vascular dysfunction, which could limit the ability to study some mechanisms. Complementary studies with other groups of individuals may therefore be of interest.
Conclusions

In the presence of a local NO clamp diesel exhaust exposure did not result in vascular dysfunction, whereas the exhaust exaggerated the vasoconstrictive effect of systemic NO synthase inhibition along with increased in basal nitrite concentration. Reduced NO bioavailability appeared as the predominant mechanism behind the vascular dysfunction, at the examined time point.
Final Comments

The newly published data from WHO are fearful with indications of 7 million annual deaths as a consequence of air pollution exposure, in addition to the high burden of worsening of respiratory and cardiovascular diseases. Therefore, it is urgent to undertake measures to reduce air pollution exposure and to understand the mechanisms behind the adverse health effects.

The present thesis intended to explore the mechanisms behind the adverse health effects of wood smoke and diesel exhaust; two major contributors to air pollution. The first two papers shed new light on the deposition of wood smoke particles in the human lung and the subsequent respiratory and systemic effects. The investigated wood smoke particles deposited less than diesel exhaust particles, but nevertheless caused inflammatory effects in the bronchial mucosa, albeit in an unexpected fashion. There were also findings of airway and systemic immunomodulation that may relate to the adverse health effects of biomass combustion reported in the epidemiological studies. Combustion of wood and other biomass can generate smoke with considerable variations in particle and chemical composition. Hence, extensive additional research is needed to improve the understanding of the underlying respiratory and cardiovascular mechanisms and their relation to adverse health effects.

The two subsequent papers were based on diesel exhaust exposures, with the first exploring the potential of air inlet filters to reduce the exposure to diesel exhaust while travelling in vehicles. The addition of an active charcoal component to the filter potentiated the filtration efficacy and reduced airway and mucosal symptoms. Whilst reduction of primary combustion emissions should always be the main approach, the study points to the potential possibility to reduce adverse health effects by using efficient filters in different settings, especially to protect vulnerable individuals.

An improved understanding of how diesel exhaust mediates its respiratory and cardiovascular effects is important due to the extensive negative health consequences related to this pollutant, especially as up-to-date medical treatments have failed to fully protect vulnerable groups. It was therefore of interest to further explore the role of nitric oxide-related mechanisms for the maintenance of vascular function when exposed to diesel exhaust. Two complementary studies were performed and generated new valuable information on the bioavailability of nitric oxide. Future research may
elucidate new possibilities to medically prevent the well-documented adverse effects of motor engine exhaust.
Conclusions

From this thesis, it is concluded that:

- Sooty PAH-rich wood smoke particles has a higher deposition probability in human lungs compared to particles generated from a pellet burner during complete and incomplete combustion, but lower than for diesel exhaust. For wood smoke particles in the size range of 50-300 nm the deposition probability is tended to be higher in subjects with COPD compared to healthy subjects.

- Exposure to sooty and PAH-rich wood smoke generated during incomplete combustion is associated with airway inflammatory responses, characterized by infiltration of lymphocytes and mast cells, in the absence of concomitant neutrophil recruitment at the investigated time point, 24 hours after exposure.

- The rationale for the observed reductions in bronchoalveolar lavage cell numbers is unclear, but could potentially be associated with wood smoke-induced impairment of cellular function or cell death.

- By using cabin air inlet filters the amount of diesel exhaust entering the vehicle cabin can be reduced. The addition of an active charcoal component to the filter medium can substantially improve the filter’s efficacy and has the potential of reducing traffic-related adverse health effects.

- The diesel exhaust-induced impairment of endothelial vasomotor function is associated with reduced NO bioavailability.
Acknowledgements

The writing of this dissertation has been one of the most significant academic challenges I have ever had to face. Without the support, patience and guidance of the following people, this thesis would not have been completed. It is to them that I owe my deepest gratitude.

Prof. Thomas Sandström, who undertook to act as my main supervisor despite his many other academic and professional commitments. Your wisdom, immense knowledge and generosity to the highest standards inspired and motivated me. Throughout these years, I have also learned a lot about your extraordinary human qualities. Thank you for your concern about my family.

Prof. Anders Blomberg, my co-supervisor, thanks for setting me off on further roads, and for your good influence. I am happy for all your support. Thank you for unforgettable moments during meetings and conferences all over the world.

Prof. Ellinor Ådelroth, I would like to take this opportunity to express my heartfelt thanks to you for your kind support.

Dr. Ragnberth Helleday, the head of dept. of Clinical Medicine in the university hospital, my appreciation to you is beyond words. Thank you, for giving me the opportunity to have all time needed for completing this research.

Dr. Annelie Behndig, my sincere thanks for endless support with new ideas during these years and for fantastic time during the conferences and meetings.

Dr. Jamshid Pourazar Thank you for all your help. Always giving the right advice and making thinks look easier.

Assoc. Prof. Christoffer Boman, Assoc. Prof. Jakob Löndahl, Prof. Erik Swietlicki and Assoc. Prof. Jenny Rissler, thanks for introducing me into aerosol science. I am extremely grateful for your kind patience and to be there when I need to ask.

Dr. Jeremy Langrish and Dr. Ian Mudway, thank you so much for your generosity, excellent feedback and great knowledge.
Dr. Jenny Bosson Damewood, thanks for your support and wonderful planning for meetings and conferences.

Assoc. Prof. Anders Bucht, Dr. Camilla Österlund and Åsa Gustafsson, my sincere appreciation for great discussions and collaborations.

Dr. Stephane Couderc and Dr. Anna Bion, Thank you for cooperation and meetings.

Anniika Johansson and Frida Holmström, our excellent research nurses, thanks!

Dr. Nirina Larsson, Dr. Jon Unosson, Dr. Greg Rankin, Dr. Maria Sehlstedt, Dr. Robert Linder, and Ann-Britt Lundström, my amazing research colleagues, for all nice discussions, chats and laughs throughout these years, my sincerest gratitude to you!

Lena Åström, without you, I could not imagine how all logistics needed would have worked. Many thanks!

I am grateful to the volunteers who participated in these studies. Without them, the research would not have been possible, and their tolerance, good humour and insight added much.

Dr. Kenneth Nilsson, Dr. Eva Norrman, Dr. Margareta Söderberg, Dr. Annika Wallin, Dr. Stefan Barath, Dr. Elisabeth Öberg Karlsson, Dr. Ulrika Öhman, Dr. Olga Pettersson, Dr. Kristina Hillring and Dr. Sofia Kemi I want to give you my deepest appreciation for supporting me and covering my position clinically during research time.

My Parents, sisters and brothers, thousands of kilometres are separating us, however, this is just a geographical fact. I have always felt that you are here with us. Thank you for your faith in me.

Azhar, my beloved wife, my deepest thanks to you for your support, encouragements, quiet patience, and unconditional love.

Seif, I thank you our lovely son for your sweet smile and understanding.

Sala, our daughter, you are giving us unlimited happiness and pleasure.
This work was generously supported by grants from the Swedish Heart-Lung Foundation, the County Council of Västerbotten, Swedish Energy Agency, The EU ERA-NET Bioenergy program (project BIOHEALTH) and Renault Technocentre.

I apologize in advance for any unintentional forget to anyone who helped me even in a word.
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