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POTENTIAL MECHANISMS FOR ACUTE HEALTH EFFECTS AND LUNG RETENTION OF INHALED PARTICLES OF DIFFERENT ORIGIN

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ABSTRACT

Background: Environmental particle exposure is known to have negative health effects. There is limited knowledge about how size and origin of particles influence these effects. There is also little known regarding the fate of ultrafine particles (particles in nanosize;< 100 nanometers in diameter) after being inhaled.

Aim: The main objective of this thesis was to study acute health effects in humans and their potential underlying mechanisms, resulting from exposure to particles of different origins. Another aim was to develop a method for measuring human lung retention (clearance) of ultrafine carbon particles.

Methods: In this human exposure study, twenty healthy volunteers and sixteen individuals with sensitive airways diagnosed with mild asthma were exposed to a subway environment and a control environment for two hours each. Acute health effects in the airways and blood were measured using different markers indicating inflammation, effects on coagulation and lung function.

A new exposure method was developed for the study of lung retention of inhaled ultrafine particles Carbon particles were labeled with radioactive indium-111. The labeling allowed one week of follow up of particulate retention in ten healthy volunteers. One volunteer was followed for totally 29 days.

Results: After exposure to a subway environment, healthy individuals had significant increase in fibrinogen (coagulation factor) and regulatory T-cells expressing CD4/CD25/FOXP3 in peripheral blood. In asthmatics we found a statistically significant increased frequency of CD4 cells expressing T-cell activation marker CD25 in bronchoalveolar (lung) lavage fluid but no significant increase of regulatory T-cells in blood.

We developed a method for labeling and generating ultrafine carbon particles with a radioactive isotope indium-111 for use in human studies. A follow-up of healthy volunteers who inhaled the particle aerosol found a limited deposition of particles in the central airways. Seven days after exposure, measured lung retention was 91% at the group level. After correction for free radioactivity leaching from urine and blood samples, respectively mucociliary clearance from the central airways, the cumulative lung-particle retention was approximated to 96%. There was little translocation of particles from the lungs to the blood circulation (0.3%). The volunteer who was followed up for a total of 29 days demonstrated 10% further clearance of particles from the lungs.

Conclusion: Healthy individuals and asthma patients display different inflammatory responses following exposure to a subway environment. The health effects were not as pronounced in comparison to our previous studies performed in a road-tunnel environment with similar mass levels of particles with diameters <2.5 μm and <10 μm ($PM_{2.5}$ and PM_{10}), but with a higher number concentration of ultrafine particles, nitrogen monoxide and dioxide than in the subway. The different results show that health-risk assessment cannot be based solely on mass concentration information such as $PM_{2.5}$ and PM_{10}. More complex measurements

of particles are needed, and should include the number concentration levels of ultrafine particles as well as knowledge about the source of the particles.

Results from deposition and retention studies indicate limited translocation to circulating blood in healthy lungs during the first week, with faster clearance from central lung regions compared to the peripheral regions. This is probably due to mucociliary clearance from the larger airways.

SAMMANFATTNING

Akuta hälsoeffekter och möjliga mekanismer av inhalerade partiklar med olika ursprung, samt retention i lungan

Bakgrund: Det är känt att exponering för partiklar via omgivningsluften ger upphov till negativa hälsoeffekter. Det är emellertid mer oklart i vilken grad partiklarnas storlek och ursprung påverkar effekterna på hälsan. Ett annat tämligen okänt område är i vilken omfattning ultrafina partiklar (nanopartiklar, <100 nm i diameter) tas upp i kroppen efter inandning.

Syfte: Syftet med studien var att kartlägga och förklara akuta hälsoeffekter hos människa till följd av exponering för olika sorters partiklar. Dessutom avsågs att utveckla en metod för bestämning av retention (clearance) av ultrafina kolpartiklar (grafit) i lungan.

Metod: Tjugo friska försökspersoner samt sexton individer med känsliga luftvägar, diagnostiserade som personer med lindrig astma, deltog i studien. Personerna exponerades för omgivningsluften i tunnelbanan respektive en kontrollmiljö i två timmar vardera. Akuta hälsoeffekter studerades genom att mäta lungfunktion och genom analys av olika inflammatoriska markörer i blod och bronksköljvätska. Dessutom studerades effekt på koagulation i blod.

En ny metod för exponering utvecklades för att användas i studien av retention av inhalerade ultrafina partiklar. Kolpartiklar inmärkta med radioaktivt indium-111 möjliggjorde uppföljning av huruvida aerosolpartiklar stannade kvar i lungan hos tio friska individer i sju dagar. Utvecklingen hos en av försökspersonerna följdes i totalt 29 dagar.

Resultat: Efter exponering för miljön i tunnelbanan uppvisade friska individer en signifikant ökning av fibrinogen (koagulationsfaktor) och regulatoriska T-celler (CD4/CD25/FOXP3) i blod. Hos astmatikerna påvisades en signifikant förhöjd frekvens av CD4 celler som uttrycker
T-cellsmarkören CD25 in bronksköljvätskan, men däremot ingen signifikant ökning av regulatoriska T-celler i blodet.

En metod utvecklades även för att skapa ultrafina kolpartiklar, inmärkta med den radioaktiva isotopen indium-111, som lämpar sig för att användas i studier med försökspersoner. En uppföljande studie med friska frivilliga som andades in aerosolen endast kunde påvisa en begränsad deponering av partiklar i de centrala luftvägarna. Sju dagar efter exponering mättes motsvarande 91% av radioaktiviteten i lungorna. Efter korrigering för icke-partikelbunden radioaktivitet i blod- och urinprover, samt för mukociliär transport från de större luftvägarna, uppgick den kumulativa partikelretentionen till 94%. En obetydlig överföring (translokering) av partiklar till blodomloppet förekom (0,3%). Den försöksperson som studerades under totalt 29 dagar uppvisade ytterligare 10% minskning av mängden partiklar i lungorna mellan dag 7 och 29.

Slutsatser: Friska individer och personer med diagnostiserad astma uppvisar olika inflammatorisk respons efter att ha utsatts för tunnelbanemiljön. Resultaten skiljde sig även åt från tidigare studier genomförda i en vägtunnel med jämförbar massa av partiklar med diameter <2.5 µm och <10 µm ($PM_{2.5}$ och PM_{10}), men med en större andel ultrafina partiklar samt högre koncentration kvävemonoxid och kvävedioxid. De olika resultaten visar att hälsoriskbedömningar inte enbart kan grunda sig på information om total partikelmassa såsom angivelser för $PM_{2.5}$ och PM_{10}. Det är nödvändigt att utföra mer förfinade mätningar av partikelförekomst, vilket skall inkludera antalet ultrafina partiklar per volymsenhet liksom även kännedom om partiklarnas ursprung.

Resultaten från studierna av deposition och retention pekar mot att translokeringen av partiklar från lunga till blod är begränsad under första veckan efter exponering. Borttransporten var snabbare från de centrala regionerna av lungan jämfört med perifera delar, troligen beroende på mukociliär transport från de större luftvägarna.

LIST OF PUBLICATIONS

The thesis is based on the four following papers, which are referred to in the text by their Roman numerals (I–IV).

I. **Klepczyńska Nyström A**, Svartengren M, Grunewald J, Pousette C, Rödin I, Lundin A, Sköld CM, Eklund A, Larsson BM. Health effects of a subway environment in healthy volunteers. Eur Respir J. 2010 Aug; 36(2):240-8.

II. **Klepczyńska-Nyström A**, Larsson BM, Grunewald J, Pousette C, Lundin A, Eklund A, Svartengren M. Health effects of a subway environment in mild asthmatic volunteers. Respir Med. 2012; 106:25-33.

III. Sanchez-Crespo A, **Klepczyńska-Nyström A**, Lundin A, Larsson BM, Svartengren M. ^{111}Indium-labeled ultrafine carbon particles; a novel aerosol for pulmonary deposition and retention studies. Inhal Toxicol. 2011 Feb; 23(3):121-8.

IV. **Klepczyńska-Nyström A**, Sanchez-Crespo A, Andersson M, Falk R, Lundin A, Larsson B-M, Svartengren M. The pulmonary deposition and retention of indium-111 labelled ultrafine carbon particles in healthy individuals. *Manuscript*.

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CONTENTS

LIST OF ABBREVIATIONS

BAL-fluid	Bronchoalveolar lavage fluid; retrieved from peripheral lungs
Bq	Becquerel
Sv	Sievert
BW-fluid	Bronchial wash fluid is retrieved from central airways
DEP	Diesel exhausts particles
FEV_1	Forced Expiratory (exhaled) Volume in the first second
FOXP3	Forkhead box protein 3
FVC	Forced vital capacity
IL	Interleukin
^{111}In	Radioactive isotope indium-111
kD	kilodalton
NAL	Nasal lavage
NK cell	Natural killer cell
NO_x	Nitric oxides incl. nitric monoxide (NO), nitric dioxide (NO_2)
PAI-1	Plasminogen activator inhibitor-1
PM_x	Particulate matter, with aerodynamic diameter of <X µm
PEF	Peak Expiratory Flow
^{99m}Tc	Radioactive isotope technetium-99m
Th cell	T helper cell
TNF	Tumor necrosis factor
UF particles	Ultrafine (manufactured) particles, 1-100 nm in diameter

1 GENERAL INTRODUCTION

1.1 OUTDOOR AIR POLLUTION - PARTICLES

High particle levels in the subway environment

Air pollution has negative health effects. Combustion exhaust from motor traffic is a major contributor to ambient air pollution. One way of limiting overall air pollution exposure related to motor exhaust is to expand the subway underground system. However, in Stockholm, the levels of aerosol mass concentration levels of particulate matter Pin the subway are 5–10 times higher[1] than at street level in the inner city [1] and are comparable to particulate mass concentrations measured in a road tunnel in the same city [2]. Particulate mass concentration levels in the subway change little from day to day. The concentrations of airborne of particles in the subway system in Stockholm are within levels reported elsewhere worldwide like in Amsterdam [3], Helsinki [4], London [5], New York [6], Rome [7] and Seoul [8].

Specific size and composition of subway particles

In the subway particles are rather large, more than 1 micrometer in diameter, and mainly originate from tracks and wheels, with a high content of iron. Compared to the concentration in the street level environment, the number concentration of ultrafine particles, with diameter less than 100 nanometers, however, has a much lower magnitude in the Stockholm subway environment.

Ambient particles vary not only in size, but also by source and chemical composition. They may consist of metals, as in the subway environment, or include other chemicals compounds such polycyclic aromatic hydrocarbons (PAH) [9, 10], elemental carbon [11, 12], naturally occurring dust, pollen, sea salt, endotoxins [13] or other motor combustion related compounds.

Assessment of ambient particle exposure

Particulate air pollution is usually monitored by gravimetric measurements of PM_1, $PM_{2.5}$ and PM_{10}, where PM stands for particulate matter and the subscripts for diameters of <1 μm, <2.5 μm and <10 μm, respectively. Motor exhaust is a major source of airborne nano-sized particles, also called ultrafine particles. These are rarely monitored. Because of their low mass, measurement of number concentration is more relevant in this case.

Air quality – existing legislation

Health effects are seen at very low levels after exposure, which make it hard to set threshold levels. The WHO guidelines for PM_{10} establish 50μg/m^3 averaged over 24 hours and 20μg/m^3 as an annual average for air quality [11]. Within the EU, Directive 2008/50/EC sets ambient air quality norms for Member States. The exposure limit to fine particulates ($PM_{2.5}$) is set at 20μm/m^3 (based on a 3-year average) and becomes legally binding in 2015. For PM_{10} the exposure limits are set at 50μg/m^3 as a 24-h average (not to be exceeded more than 35 times per year) and 40μg/m^3 as an annual average [14]. Stockholm currently does not fulfill the EU norm for PM_{10} in urban air.

[1] $PM_{2.5}$ and PM_{10}

Other air pollutants
Outdoor ambient air pollution contains not only particulate matter but also gaseous compounds such as nitrogen oxides (NO and NO^2), sulfur dioxide (SO^2), ozone (O^3). All are associated with negative health effects.

1.2 HISTORIC PERSPECTIVE, EPIDEMIOLOGY AND HEALTH EFFECTS OF PARTICLES

The awareness of air pollution and its health effects was pointed out already in 1661, when John Evelyn published a report on air pollution and its negative effects on health. Almost 300 years later, on 6 December 1952, extremely heavy smog in London led to increased mortality and hospitalization.). Earlier, in 1930´s, a similar event occurred in Meuse valley, in Belgium [15]. Despite this, British epidemiologists concluded in 1979 that there was no negative health effect from particulate air pollution [16]. However, in 2006 the U.S. Environmental Protection Agency (EPA) reported that "Inhalation of fine particles is causally associated with premature death at concentrations near those experienced by most Americans on daily basis" [17].

Epidemiological studies
Epidemiological studies agree nowadays that particulate matter air pollution is associated with health effects. The associations have been found in both short-term studies, based on daily variation in air pollution and health parameters, and in long-term studies where individuals have been followed over a longer period of time. An advantage in short-term studies is that the population may serve as it´s own control. One limitation is to make assumptions that the exposure is representative for larger population [18].

Two frequently cited studies are the APEAH (Air pollution and Health: a European Approach), which included 10 European cities [19], and NMMAPS (the National Morbidity and Mortality Air Pollution Study), covering cities in the US [20, 21]. Through coordination between different cities, meta-analyses were performed strengthening earlier studies. In the U.S. there was a strong association between $PM_{2.5}$ and general risk of death and caused by cardiovascular and respiratory illnesses. In western European cities the risks of daily deaths from cardiovascular and respiratory diseases increased with elevated concentration of black smoke and in sulfur dioxide levels. No relations with daily mortality and nitrogen dioxide were shown.

Acute health effects - inflammation, respiratory and cardiovascular illnesses
Acute health effects may be measured with different markers indicating signs of increased inflammation, increased coagulation and decreased lung function. Specifically, particulate matter (PM) air pollution has been identified in several epidemiological studies as associated with adverse health effects, both short and long term, including mortality [20, 22, 23], lung cancer [24], cardiovascular disease [24-27] respiratory illnesses [25, 27]. On the other hand, subway employees and other professional drivers do not show any increased relative risk for myocardial infarction [28].

Particular matter has been associated with mortality and hospital admission due to respiratory and cardiovascular diseases, but the mechanisms behind these effects are still not fully understood. One theory is that airborne particulate matter increase inflammatory factors that increase coagulation. Particulates could also have a direct impact on the heart causing changes in heart-rate variability. [29] High airborne mass concentration may lead to "lung overload" with failed clearance leading to inflammation [30].

Earlier *in vivo* studies from our group have shown that particulate air pollution from city traffic induce inflammatory reactions in the lower airways. Larsson *et al* showed increased amount on inflammatory cells in the bronchial alveolar lavage (BAL) fluid in a study on 16 healthy individuals. [2]

Susceptible population – asthmatics
Individuals with asthma have a chronic inflammation in their airways and are more vulnerable to air pollutants than a healthy population, and inhaled particulate air pollution may exacerbate pre-existing lung inflammation [31]. Asthma symptoms and increased need for medication are most strongly associated with ultrafine particles and with $PM_{2.5}$ rather than PM_{10} [32-35].

Approximately 10% of Swedish adults are diagnosed with asthma. Asthma is defined as a chronic inflammation in the airways with variable lung function, also called "airway obstruction." The symptoms of asthma: episodes of wheezing, cough, chest tightness and breathlessness, are caused by swelling and increased mucus production in the airways. Many inflammatory cells are involved in asthma pathogenesis, particularly mast cells and eosinophils. Also T cells are also likely to play an important role involving various cytokines. For details, see "Immune defence" below.

Asthma is more common in children than in adults. Atopic (allergic) diseases such as atopic eczema and allergic rhinitis are considered to be risk factors for development of asthma. Approximately 80% of asthmatic children and 40–50% of asthmatic adults have atopic asthma [36]. Other risk factors are genetic predisposition, smoking parents as well as indoor and outdoor environmental factors (air pollution).

Asthma is less common in Eastern Europe, rural Africa, India and China. In the Westernized countries asthma has increased since the early 1960s. Genetic predisposition alone cannot explain the increase. The increase may be due to

increased asthma awareness. However, focus has been put on changes in environmental factors such as air pollution.

Previous exposure studies with asthmatics
A corresponding *in vivo* exposure study performed in a road tunnel in Stockholm, including 14 asthmatics exposed for two hours, showed increased levels of inflammatory mediators, such as the pro-inflammatory IL-12 and TNF-α as wells the anti-inflammatory IL-10, in a nasal lavage for a subgroup of seven asthmatics without ongoing medical corticosteroid treatment [37]. The same road tunnel was used in another exposure study with 20 asthmatic volunteers that were exposed for only 30 minutes. In comparison to a control (clean) environment, the road tunnel enhanced asthmatic reaction (reduction of lung function) to inhaled allergens.[38]

Short-term exposure (1 hour) to diesel exhaust (with high PM_{10} levels) has also been shown to increase bronchial hyper responsiveness in asthmatic subjects, which is an important feature of asthma. The increase was detected one day post exposure. There was also an induction of the pro-inflammatory IL-6 in mucus that is coughed up from the lower airways (sputum). [39] In London, 60 adults with asthma were exposed (2 hours) for a busy street environment, as well as for a park environment for equally long time. The exposure for city air pollution reduced the lung function (FEV_1 and FVC). The reduction was most associated with ultrafine particles and elemental carbon.[40]

Children and elderly
Other susceptible groups are children and the elderly. The first pediatric studies (late 1980s) were based on airborne emissions from a steel mill in Utah Valley. Associations were seen between low levels of PM_{10} and fewer hospital admissions in children. [41] A four-year follow-up cohort of 1 678 children from Southern California revealed negative effects of air pollution (NO_2, $PM_{2.5}$ and elemental carbon) on lung function development [42]. Also, a four-year prospective birth cohort of 4 089 Swedish children has found a relation between traffic indicators (NO_x and PM_{10}) and development of airway disease and sensitization for allergens in children [43].

The elderly are also considered to be a susceptible population to air pollution. A study by Cakmak S et al estimated a mortality rate associated with ambient air pollution and observed an association between PM_{10} and the mortality of population over 85 years (three times higher) in comparison to those under 65 years. Mortality related to PM_{10} was three times higher for the elderly group. [44]

1.3 IMMUNE DEFENCE – RESPONSE TO PARTICULATE AIR POLLUTION

Airborne particulates from air pollution may induce inflammatory responses. Induction of inflammation or anti-inflammatory agents may vary from minutes to days.

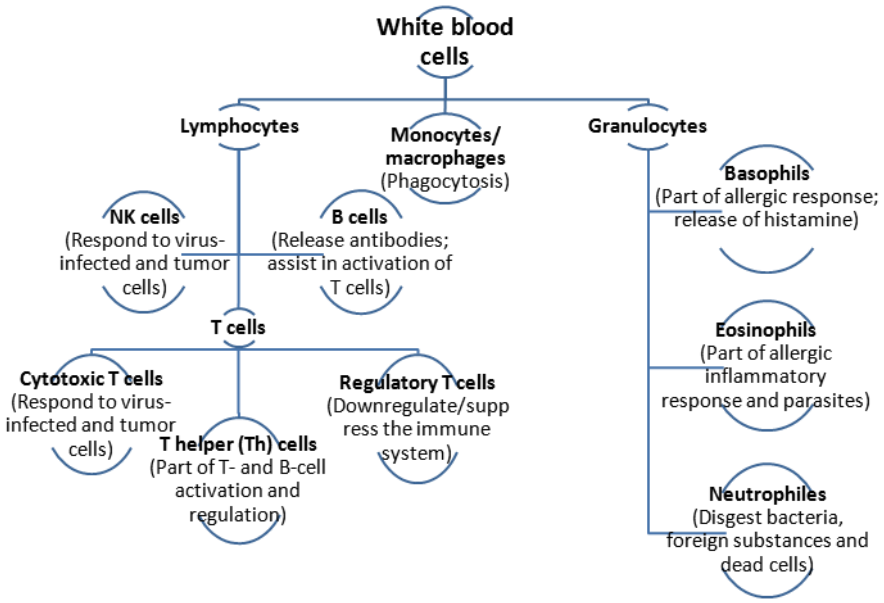
White blood cells
Blood consists of 55% plasma and 45% blood cells. Cells circulating in blood are: blood platelets (thrombocytes), red blood cells (erythrocytes) and white blood cells (leukocytes), where the latter are involved in the immune defence. Only a small percent of the leukocytes are found in blood. They are mainly located in other tissues, such as lung tissues. An increased number of leukocytes may indicate an ongoing inflammatory process or a down-regulated immune defence.

Leukocytes are usually divided into granulocytes (neutrophils, eosinophils, basophils), lymphocytes (B-, T-, NK-cells) as well as monocytes (macrophages and dendritic cells). Figure 1 summarizes the white blood cells and their role in the defence systems.

Innate and adaptive immune system
The immune system is a network of organs, tissues, cells and inflammatory mediators that protects the body from pathogens. It is usually divided into the nonspecific (innate) and specific (adaptive) immune system, with secreted mediators that overbridge the two systems. The first line of defence is performed by the nonspecific immune system. The leukocytes involved there specialize in digesting (phagocytosis) foreign material. The cells in the innate defence system that have phagocytic ability are neutrophils, monocytes, macrophages and dendritic cells.

The adaptive immune response has two main cell types: B and T cells. They are able to distinguish individual pathogens (infectious agents) or other agents by using cell-surface receptors. An useful protocol for identifying whether molecules (receptors or cell adhesion) on the surface on white blood cells are present or not, is the cluster of differentiation (CD) nomenclature. There are several hundred CD markers, for example CD3 denotes T cells. T cells are further divided into regulatory T cells, cytotoxic T cells and T helper (Th) cells. The CD markers that are most frequently discussed in this thesis are found in the list below. Sometimes symbols "+"(pos) or "-"(neg) are used in connection to CD markers. This is used to indicate whether a certain cell expresses or lacks the specific CD molecule.

Figure 1. A summary the white blood cells in the immune system, with their main functions.



A useful protocol for identifying whether molecules (receptors or cell adhesion) on the surface on white blood cells are present, or not, is the cluster of differentiation (CD) nomenclature. There are several hundred CD markers, for example CD3 denotes T cells. T cells are further divided into regulatory T cells, cytotoxic T cells and T helper (Th) cells. The CD markers that are most frequently discussed in this thesis are found in the list below. Sometimes symbols "+"(pos) or "-"(neg) are used in connection to CD markers. This is used to indicate whether a certain cell expresses or lacks the specific CD molecule.

General markers	
CD45	Defines white blood cells (leukocytes)
CD69	Used to detect early activation (1-2 hours) of lymfocytes
T-cell markers	
CD3	Defines T cells
CD4	Defines T helper (Th) cells
CD8	Is expressed on cytotoxic T cells
CD25	Used to detect early activation of activated T cells. If the response is high it may indicate presence of regulatory T cells, on which CD25 is also expressed
FOXP3 (fork head box protein 3)	Transcription factor[2] for regulatory T cell, preferentially CD4posCD25bright.
B-cell marker	
CD19	Defines B-cells
NK-cell marker	
CD56posCD16pos	Defines NK cells or NK T cells

Inflammatory mediators

There are numerous signaling protein molecules (cytokines and chemokines) that are secreted by cells to facilitate intercellular communication during inflammatory responses. In this thesis, both pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, TNF-α) and an anti-inflammatory cytokine
(IL-10), are discussed.

1.4 PREVIOUS EXPOSURE STUDIES IN SUBWAY ENVIRONMENT

***In vitro* results**

An *in vitro* study showed that particulate air pollution in subway air was more genotoxic than in street-level air, as subway particles induced oxidative stress in cultured human lung cells [45]. In two other *in vitro* studies a cytokine release (IL-6, IL-8 and TNF-α) could be stimulated from human macrophages by particles derived from a subway station air as well by particles from air along a heavily trafficked urban street. Street-level airborne particulate air pollution was the most potent stimulators. [46, 47] Similar results regarding cytokine release were shown in an *in vitro* animal study using a murine macrophage-like cell line). Induction of lipid peroxidation, arachidonic acid release and formation of ROS (reactive oxygen

[2] A transcription factor is a molecule/protein that binds to a specific DNA sequences.

species) were however, stronger for subway air particles than for urban street air particles. [48]

***In vivo* results**

To our knowledge only one study has been performed investigating acute health effects in humans after exposure to a subway environment and none regarding asthmatics. Personal PM_{10} exposure has been assessed in personnel working in the Stockholm subway environment. Exposure levels for subway drivers were compared with platform workers. The latter group was exposed to four times higher levels of particulate matter than the train drivers. [49] This suggests that the subway environment exposure potentially impacts citizens using the subway. Another study showed that inflammatory response, as measured as blood plasma concentrations of plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6) and fibrinogen, had a tendency to be higher for subway platform workers than for train drivers and subway ticket sellers. Measurements were performed on non-smoking, healthy workers after two non-working days, and a second sample after two working days. [50]

1.5 DEPOSITION AND RETENTION OF INHALED PARTICULATE AIR POLLUTION

Particle surface area deserves serious consideration. Ultrafine particles have a greater surface area per mass unit compared to larger sized particles, and are thereby likely to be more reactive and to promptly initiate an inflammatory process [51-53]

In vivo human lung retention data obtained under controlled exposure conditions play a fundamental role in understanding the biological pathways of particulate pollutants in the human body. How do ultrafine particles in air pollution behave in the body after being inhaled? Do they remain in the lungs or are they eliminated (cleared) from the body by mucociliary clearance or other mechanisms? Is there a translocation to the blood circulation and further distribution to other organs?

Relevance of size and solubility of particles

Particles deposited in the lungs may accumulate or be eliminated by different biological mechanisms such as mucociliary transport, phagocyte action (ingestion) and pinocytosis (cells absorbtion and engulfment). However, clearance efficiency depends on particulate distribution within the lung structure and the physicochemical characteristics of the inhaled particles. Particularly, mucociliary clearance from human airways has been suggested to be particle-size dependent [54] rather than composition dependent. Ultrafine particles are also believed to have more toxic properties than larger particulate matter due to their ability to reach into the alveoli, where gas exchange occurs, and be further translocated to secondary organs [55, 56] Ultrafine particles have relative large surface, which increase effect per mass there are also results indicating that macrophages have a reliative inability to phagocytize nanosized particles quickly [57].

Another on-going discussion regarding the ultrafine particles is whether they impaire phagocytic ability of macrophages once they reach the alveoli region. *In vitro* studies with human and rat alveolar macrophages exposed to ultrafine carbon particles showed that a reduced phagocytic capacity when exposed to silica particles [58]. In an extended study, apart from the ultrafine carbon particles, also particles derived from diesel exhaust had the same inhibitory effect on the phagocytic ability human alveolar macrophages when exposed to silica particles and microorganisms. The authors concluded that this effect may result in more exacerbations of subgroups of chronic inflammation in their airways, as it may increase susceptibility to infections. [59]

Labeling of particles and follow up by gamma camera

Scintigraphic registration techniques (gamma cameras) may be used for lung deposition, retention and clearance studies. It is based on particles labeled with a radioisotope, inhaled and then followed externally by gamma counting. Various isotopes may be used for the labeling. Technetium-99m (^{99m}Tc) has long been used for human studies [60, 61]. Nonetheless, there are still few human studies on translocation using ultrafine carbon particulates aerosol that correspond to the size of particles in motor exhaust.

The risks of using ionizing radiation

Radiation of a substance is due to the instability of the atom nucleus (balance between protons and neutrons). If the nucleus is unstable, then the substance will be "radioactive" and accompanied by emissions of radiation energy. Once the atom reaches a stable configuration, no more radiation is emitted, and the material will become non-radioactive. The half-life is the amount of time that it takes for a substance undergoing decay to decrease by half.

Measurement of radioactivity depends on the objective, as shown below. Retention studies use activity measurements.

Unit	Abbreviation	Representing	Used to measure
Becquerel	Bq	Disintegrations per second	Radioactivity
Sievert	Sv	Absorbed radiation dose	Biological effect

Some examples of radiation levels are as follows: A medical x-ray lung examination delivers a mean dose of 0.01 mSv, a round-trip flight over the Atlantic results in a dose of 0.1 mSv, and a medical x-ray lung examination delivers a mean dose of 0.2 mSv (0.1-5 mSv), and a more extensive stomach x-ray delivers up to a dose of 20 mSv. This can be compared to the mean yearly dose of 4–5 mSv that each person in Sweden is exposed to due to natural background radiation (including radon). [62]

Radiation energy can liberate an electron from an atom and become an ion. There are various forms of ionizing radiation, such as beta (β) radiation consists of electrons, which can be stopped by an aluminum plate. For medical investigations α and β radiation should be avoided. ^{111}In generates gamma (γ) radiation that consists of energetic photons. This radiation is detected by gamma camera. Also

technetium-99m (^{99m}Tc) generates γ-radiation, which can be stopped by 5-mm thick lead shield.

1.6 EXPOSURE STUDIES WITH ULTRAFINE PARTICLES

Labeling with Technetium-99m isotope

Data from controlled exposure to ultrafine particles labeled with a radionuclide have provided basic information about the biological pathways of the ultrafine particles in the human body after inhalation. Insoluble carbonaceous ultrafine particles labeled with ^{99m}Tc have been frequently used in short-term human lung clearance measurements. Our previous human studies with ultrafine carbon have shown a slow clearance rate. Most particulates were retained in the lung region after two days. At 24 hours, no significant translocation of 100 nm carbon particles from the lungs to the blood was observed in either in healthy subjects or in a group of asthmatics or smokers [63]. Furthermore, Mills et al has shown that ^{99m}Tc particles remain in the lungs for at least 6 hours after inhalation [64]. The result contradicts interpretations from another study made by Nemmar *et al*[65]. In their study five healthy volunteers inhaled ^{99m}Tc particulate aerosol. The particles rapidly passed into the systemic blood circulation with a peak within 10-20 minutes. In the Nemmar *et al* study there may have been a large amount of non-bound radioisotope, as thyroid glands and bladder was visible in the whole body scan (normally non-bound ^{99m}T is translocation to that part of the body).

The above mentioned studies share a common problem with measurement error due to the poor sensitivity of gamma camera, as well as the physical properties of the radioisotopes that is used. The short physical half-life of ^{99m}Tc (six hours) only permits short-term clearance studies of couple of days post administration. There is a possibility that particles enter the lung tissue without blood translocation [66], which may explain the different results. A longer follow-up (900 days) period of Teflon labeled a gold-195 radioisotopes (^{195}Au) in ten healthy men, showed that there was a translocation within the thoracic region with accumulation of particles in lymph nodes [67].

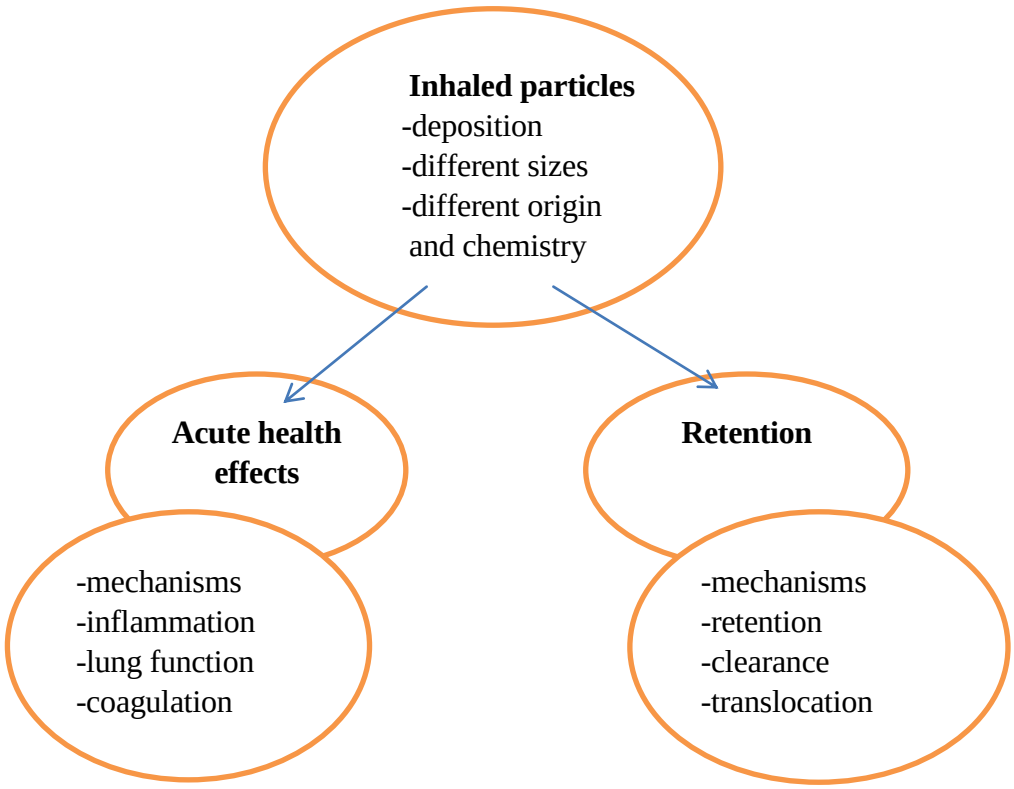
Indium-111 – a suggested isotope for retention studies

Both the time-dependent stability of the chemical bound between particle and radioisotope and the limited sensitivity of the radiation detectors hamper accuracy in long-term lung clearance studies in humans when using scintigraphic techniques. Indium-111 (^{111}In) has been suggested as a candidate to replace ^{99m}Tc for labelling of carbonaceous particles in human exposures. Traditionally, ^{111}In complexes (physical half-life 2.8 days) have been routinely used for in vivo diagnostic nuclear medicine procedures such as for localization of primary and metastatic neuroendocrine tumours bearing somatostatin receptors (indium-pentetreotide), for evaluating patients with fever of undetermined origin (indium-granulocyte), etc.

2 GENERAL SCOPE – HUMAN EXPOSURE STUDIES

The title of this thesis is: "Potential mechanisms for acute health effects and lung retention of inhaled particles of different origin".

The thesis consists of human exposure studies with particles of different origin, sizes and chemical composition. The topic can be described schematically as below.



Papers I and II focus on wear particles occurring in a subway environment. Exposure studies were performed in a subway environment ("Odenplan Station" in Stockholm, Sweden) with healthy volunteers respectively asthmatics. The set-up was similar to previously performed exposure studies in a road tunnel ("Söderledstunnel" in Stockholm).

Paper III describes a method to label nanosized (ultrafine) particles with a radioactive isotope, indium-111, for use in human lung deposition and translocation studies. The method was used for the exposure study on healthy volunteers described in **Paper IV**.

3 AIMS AND SPECIFIC RESEARCH QUESTIONS

The primary objective of this thesis was to study acute health effects and their potential underlying mechanisms resulting from exposure to inhaled particles with different size and composition. The knowledge gained may also provide valuable information regarding the process of setting limit values for airborne particles or particulate matter and personal protection.

The specific research questions and aims were:

1) Does exposure to a subway environment cause airway inflammatory response?

2) Are there differences in acute health effects due to exposure to particles that originate from different sources or environments, such as from car traffic and a subway environment?

3) Do asthmatics respond differently in acute health effects from healthy population after exposure to a subway environment, and therefore constitute (imply) a special risk group?

4) Is PM_{10} mass concentration a sufficient indicator of general health risks from particles in different environments?

5) To develop a method for labeling ultrafine carbon particles with indium-111 that could be used in human pulmonary deposition and retention studies.

6) To study lung deposition and retention of inhaled ultrafine particles using above mentioned method and to
    a. estimate translocation of the particles
    b. analyze regional differences in the larger airways and in the alveolar region
    c. analyze interindividual differences related to sex, age and BMI.

4 METHODS

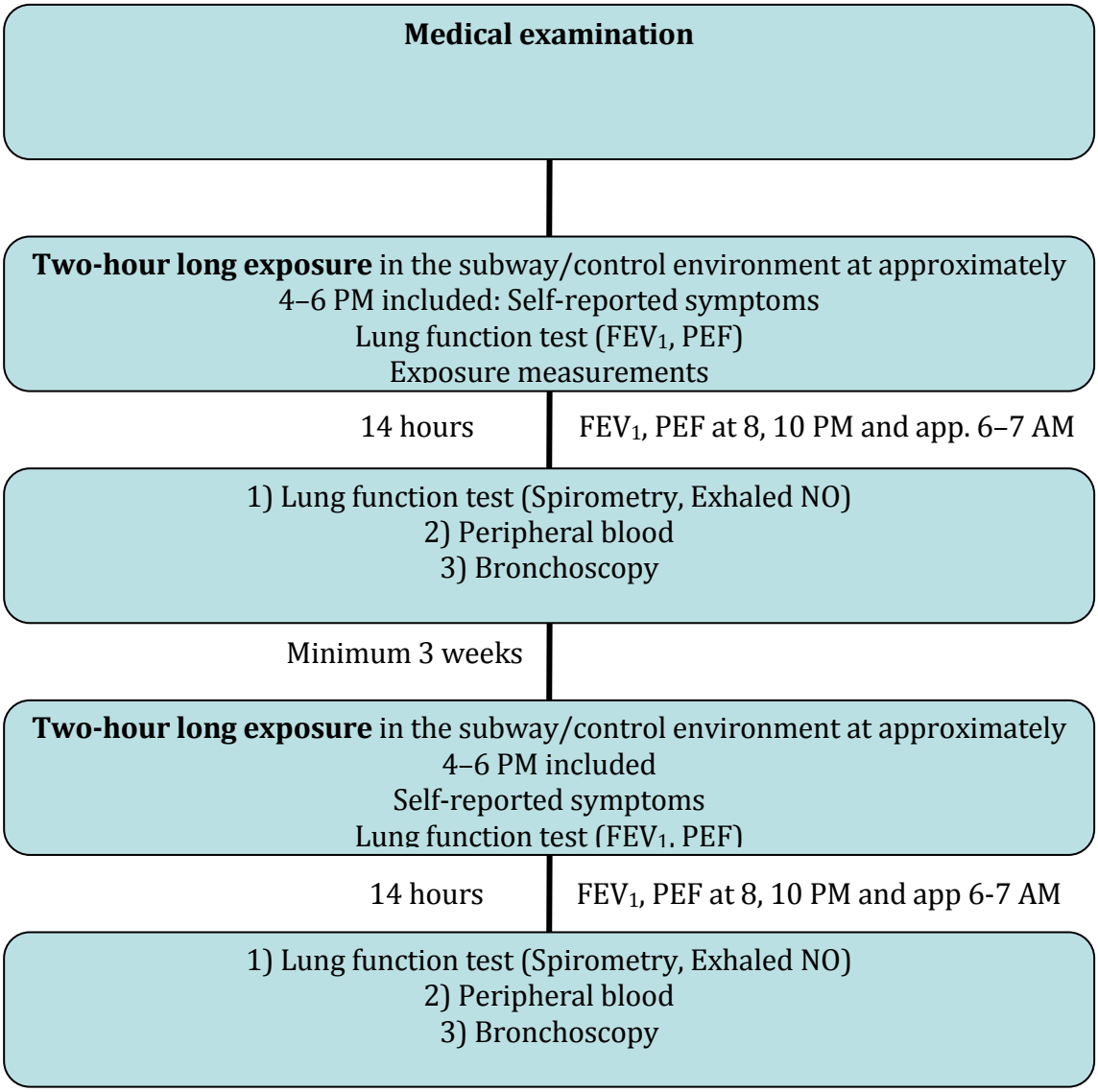
This thesis is based on a series of human exposure studies. All participants gave their informed written consent to participate in each study. The studies were approved by the Regional Ethical Review Board at the Karolinska Institutet. Studies with ultrafine particles (papers III and IV) were also approved by local Radiation Protection at Karolinska University Hospital in Stockholm (Solna).
The first two studies (**papers I and II**) with healthy volunteers and asthmatics, respectively, focused on exposure to "wear" particles (from wheel and brake wear etc.) occurring in the subway environment in Stockholm. These particles are generally larger than 1 micrometer in diameter and have a high content of metals, mainly iron. In the third study (**paper III**) we developed a method to produce an aerosol of ultrafine carbon particles labeled with radioactive indium (^{111}In). This permitted the study of long-term deposition and translocation of inhaled nanosized carbon particles in healthy volunteers (**paper IV**). Additional information and more detailed description of each study are given in papers I–IV

4.1 HUMAN EXPOSURE STUDIES IN STOCKHOLM SUBWAY ENVIRONMENT

Study design
A randomized, cross-over experimental design was used to study exposure to both a subway environment and an office environment (control). The volunteers served as their own controls. The design of the subway studies was similar to the previously mentioned road-tunnel exposure study [2] in order to facilitate a direct comparison with the road-tunnel study results.

The Odenplan subway station in central Stockholm was selected for exposing subjects to a subway environment, as it is a representative subway station regarding particle exposure in Stockholm, with limited addition of motor combustion exposure. Subjects were exposed over a two-hour long period during the afternoon rush hour (4–6 P.M.), with the volunteers alternating 15 minutes of moderate exercise on a bicycle ergometer with 15 minutes of rest. The ergometer resistance was adjusted in order to achieve a standardized individual ventilation rate of 20 liters of air per minute and m^2 of body surface area. Control exposures took place during corresponding hours in an office environment. The starting order of exposure (office vs subway environment) was randomized. The second exposure followed with an interval of at least three weeks. Bronchoscopies and blood sampling were performed 14 hours after each exposure. See Figure 2 below.

Figure 2. Study design: randomized crossover experimental design with exposure to a subway environment and an office environment (control).



Volunteers

The participants in the two studies were all non- smokers: 20 healthy and 16 individuals diagnosed with asthma, respectively. All volunteers underwent routine physical examinations including a chest X-ray, an allergy screening for common inhaled allergens (Phadiatop test) and lung function tests. For basal characteristics of the study population, see Table 1. None of the healthy volunteers had any airway symptoms. For asthmatics an including criteria was to test positive in a bronchial hyperreactivity lung test (metacholine provocation). They were not allowed to use inhaled corticosteroids (anti-inflammatory medication) or any other anti-inflammatory drugs for the last 3 months preceding their participation in the study. Short-acting non-corticosteroid treatment was permitted, however, when needed.

The volunteers were not allowed use the subway on regular bases for at least two months before inclusion, or throughout the study period. They were told to perform ordinary daily activities, to avoid heavy physical activities during the days of measurements and to avoid staying in areas with heavy air pollution. During their participation in the study, the volunteers were without any symptoms of cold or other inflammatory symptoms for at least four weeks before each exposure.

Table 1. Basal characteristics of the participants in the subway study.

Characteristics	**Healthy volunteers**	**Asthmatics**
Number of volunteers	20	16
Women	7	11
Mean age (range) years	27 (18–46)	26 (18–52)
Positive for tested allergies	2	14
Chest ray	All normal	All normal
FEV_1% pred ± SD	109 ±12	104 ±14

Abbreviations: % pred = % predicted; FEV_1 = forced expiratory volume in 1 sec.

Environmental exposure measurements

Extensive exposure measurements were performed during the subway exposure sessions: $PM_{2.5}$ and PM_{10}, particle number, nitrogen dioxide and carbon monoxide concentrations, humidity and temperature. The equipment used for particle measurements is presented in Table 2. The volunteers wore a passive sampler for measuring background nitrogen dioxide background exposure over 24 hours (including exposure).

Particle mass levels ($PM_{2.5}$ and PM_{10}) were collected on filters using Harvard impactors. Sampled filters were also used for a further analysis of a range of metals. The number concentration of airborne UF particles was determined using a Scanning Mobility Particle Sizer (SMPS) system (for details see Papers I and II). During the control exposures, portable logging instruments were used to assess exposure. To enable comparison regarding particle exposure levels, these instruments were also used during exposure sessions in the subway environment. The two portable logging instruments used were a DataRAM, for measurements of mass of particles, and a P-Trak, a particle counter. For details see Table 2.

Table 2. Equipment used for particle measurements.

Equipment	Measurement of	Additional information
Mass concentration (ug/m^3)		
Harvard impactors	$PM_{2.5}$ and PM_{10} particles	Equipped with Teflon filters with a pore size of 2 μm
DataRAM	Particles between 0.1 and 10 micrometer (μm) in diameter	Calibrated by the manufacturer against a standard dust (Arizona road dust)
Particle number concentration (particles/ml)		
Scanning Mobility Particle Sizer (SMPS) system	Ultrafine particles between 10–100 nanometers (nm) in diameter	Consisting of Electrostatic Classifier model 3080 and Condensation Particle Counter (CPC) model 3010
P-Trak	Particles between 20–1000 nm in diameter	

Measurements of acute health effect

Self-reported symptoms

During the exposure sessions, self-reported symptoms of irritation from eyes, nose and lower airways, as well as experience of disturbing noise and smell, were recorded prior to and every 30 minutes throughout exposure. The intensity was graded from 0 to 10, where 0 corresponded to no symptoms and 10 to severe symptoms.

Lung function tests

Lung volumes (FEV_1, FVC) were measured with a spirometer (Jaeger Masterscope). Measurements were performed immediately before bronchoscopies. In addition, a portable monitor (PIKO-1) was also used to measure forced expiratory volume (FEV_1) and peak expiratory flow (PEF). Measurements with the PIKO-1 were collected immediately preceding the exposure session, after one hour of exposure, and immediately following the two-hour session. Each volunteer was also instructed to repeat the measurements at around 8 PM, 10 PM, 6–7 AM, as well as at the clinic at 7:30-8 AM in the following morning, corresponding to 2, 4, 12–13 and 14 hours after exposure.

Peripheral blood

Peripheral blood was sampled in connection to bronchoscopy. Cell differential counts were performed, as well as an analysis of fibrinogen in plasma and plasminogen activator inhibitor-1 (PAI-1), both of which involve the coagulation system. Blood was also used for immunostaining and flow cytometric analysis, described further below.

Bronchoscopy, bronchoalveolar lavage and bronchial wash
Bronchoalveolar lavage (BAL) was performed in the middle lobe with 5x50 ml sterile phosphate buffer saline by inserting a flexible fiberoptic bronchoscope under local anaesthesia. A BAL fluid (BALF) cell pellet was used for immunostaining and flow cytometric analysis. For details, see Papers I and II. The supernatant was analysed for inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70) and tumor necrosis factor-α (TNF-α). Bronchial washing was performed in a segmental bronchus in the upper lobe by instilling 2 x 10 ml sterile phosphate buffer saline.

Immunostaining and flow cytometric analysis
Lymphocytes from the BALF and peripheral blood samples were analyzed with a TBNK 6-color Multitest. TBNK reagent consists of a combination of antibodies for T cells, B cells and NK cells. We also used a set of monochlonal antibodies specific for markers of T-cell activity and of T-cell regulatory functions. For a list of the used markers, see General introduction (section Immune defence – response to air pollution).

Statistical analysis
Statistical analysis was carried out with SPSS versions 15.0 (paper I) and 17.0 (papers I and II). Individual changes in different parameters for subway and control exposure were analyzed using Wilcoxon's nonparametric rank sum tests. A paired t-test was performed for lung-function data and exposure measurements. Values of $p<0.05$ were regarded as significant. Descriptive statistical analysis was used in paper II-III, as well as a lineal least square fitting or linear regression model of the data.

4.2 HUMAN EXPOSURE STUDIES WITH ULTRAFINE PARTICLES

Generation of indium-111 labeled carbon particle aerosol
To study lung deposition and retention of ultrafine particles, we needed to develop a method to label particles with a radioactive isotope, indium-111 (^{111}In). The half-life of ^{111}In is 2.83 days. The main emitted radiation is gamma (γ) radiation, but there is also some beta (β) radiation. ^{111}In enables study of deposition and retention in humans for up to 30 days.

A commercially available solution of ^{111}In in hydrochloric acid was slowly heated using a silicon oil bath in a nitrogen atmosphere. This was done to remove the hydrochloric acid from the solution. After evaporation to dryness, distilled water was added, and the evaporation to dryness process was repeated (for a total of three times). Thereafter, the remaining ^{111}In (free from hydrochloric acid) was dissolved in 99% ethanol. The solution was then placed in the graphite crucible of a commercially available, but slightly modified, Technegas generator and simmered for 15 minutes in pressurized air to cause indium oxidation (chemical reaction that involves transfer/loss of electrons).The crucible was then refilled with the indium solution and the simmering in air repeated. Thereafter, the indium-labeled ultrafine carbon particles were generated using a one-second crucible burning time at about 2500°C in order to keep the aerosol particle size as small as possible. To minimise particle agglomeration after generation, the aerosol

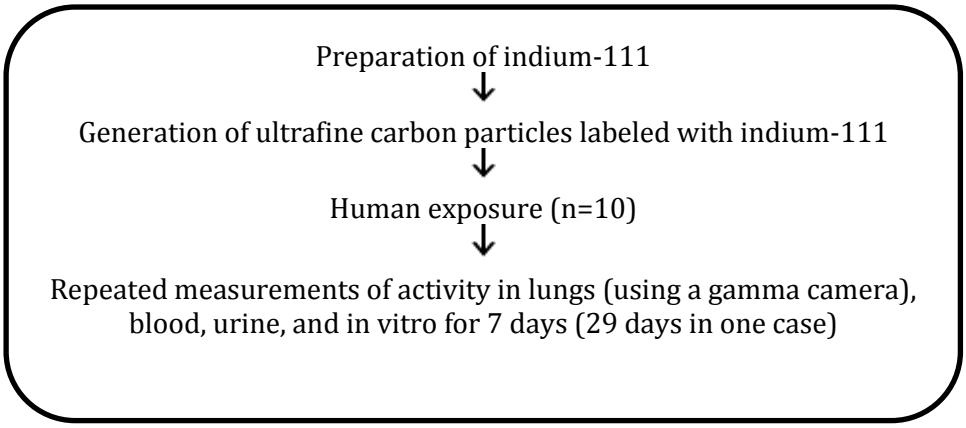
was directly diluted in a 70-liter flexible and conductive bag half-filled with clean air. Aerosol particle sizes and concentrations were measured with a Scanning Mobility Particle Sizer Spectrometer (SMPS) consisting of a Classifier 3080 and a Condensation Particle Counter (CPC) 3022A.

Study design for human exposure studies

After generation and dilution of particles in the flexible bag, the aerosol was administered to ten volunteers under spontaneous and normal tidal breathing. Each volunteer wore a nose clip and breathed through a mouthpiece. A calibrated pneumotachometer coupled to a pressure transducer was used to measure the inhaled and exhaled aerosol volume. A glass microfibre filter attached to the pneumotachometer was used to collect the exhaled aerosol. The total activity in this filter was measured using an ionization chamber.

At each exposure event, the volunteers inhaled an air aerosol containing ultrafine particles labeled with the radioactive isotope indium-111. The radioactivity dose used was 4.8 mSv per person. A calibrated radiation protection monitor was used during aerosol inhalation to register the amount of radioactivity deposited in the lungs. Inhalation was terminated when the measured radiation reached a preset calibration value corresponding to approximately 1 MBq (the maximum permitted amount of radioactivity). After aerosol inhalation the subject rinsed the mouth with water to avoid ingestion of activity deposited in the oral cavity. Activity in lungs, blood, urine and in vitro was followed for 7 days (29 days in one case). The count median diameter (CMD) of the particle size distribution during the full exposure was estimated from the distributions of the aerosol samples taken immediately after dilution in the conductive bag and after administration to the subject. After administration, the remaining aerosol in the bag was filtered through a teflon filter using a vacuum pump. A sample of this filter was used for in-vitro follow up of the activity leaching using the membrane diffusion technique. The study design for human exposure and follow-up shown in figure 3.

Figure 3. Flow chart for human exposure studies with ultrafine carbon particles labeled with radioactive isotope indium-111.



Human exposure
Ten non-smoking healthy volunteers (5 women) with a mean age of 29 years (range 20–54) participated in the study. All volunteers underwent a routine physical examination, including a lung function test performed with a spirometer. They were also screened for the presence of specific IgE antibodies against common inhaled allergens. Two of the ten volunteers had IgE antibodies to some radioallergosorbents (cladosporium, birch, cat, horse, etc).

Stability: the bonding between indium-111 and ultrafine particles
When tracing particles using a radiolabel, the degree of stability of the bond between particle and label must be assessed. An unstable bond will "leach" activity as free ^{111}In, which will lead to difficulties in the interpretation of particle clearance. Dialysis was used to detect the levels of free (unbound) ^{111}In.
After each exposure, the remaining aerosol was filtered through a teflon membrane filter. The radioactivity of a piece of filter was measured in a sodium-iodine well detector, and added into a 45-mm dialysis tube with pore size of 12–14 kDa[3]. The tube was then sealed with belonging clips and covered by 100 ml of 0.9% NaCl equilibration buffer, and the radioactivity was monitored for one week. "Leaching" is defined as the percent of radioactivity in the buffer compared to initial radioactivity in the teflon filter.

Aerosol deposition, retention and clearance
Pulmonary retention in lungs was monitored every 24 hours for a week using an image of the chest region (thorax, thyroid and upper abdomen) using a two-headed gamma camera. Image acquisition was gradually incremented from 10 minutes directly after the exposure to 25 minutes on the final day. For comparison a chest phantom was used filled with 1 MBq of ^{111}In. Gamma camera image could then be used to correct the radioactivity in the lung versus the radioactivity on corresponding Teflon filter.

Measurements in the chest region detected by gamma camera were not sufficient to evaluate whether the radioactivity was particle-bound or free in the body. In combination with the gamma pictures, blood and urine were also sampled every day for one week post exposure. These samples were dialyzed in a similar way as the filters. Dialysis of blood samples showed whether there was a translocation of labeled particles or free radioactivity from the lungs to the blood or not, and in urine samples whether there was a clearance of labeled particles from the body via urine.

Extended lung retention follow-up for one volunteer
For one female volunteer, an extended follow-up was performed using a whole-body scanner with sodium iodide detectors at The Swedish Radiation Safety Authority. This method is more sensitive than using a gamma camera. The retention was normalized between these two modalities at day seven post exposure. One to four repeated measurements were performed at each occasion

[3] kDa = kilodalton. A unit for molecular weight or mass, where 1D is approximately 1.661×10^{-27} kg.

at days 7, 14, 22 and 29, both for the volunteer and reference material containing ^{111}In. The volunteer was 47-years old with a normal height (171 cm) and weight (72 kg) and with no history of pulmonary diseases or allergies.

5 RESULTS

The main results from the four studies comprising this thesis are summarized below. Additional information and more detailed description of the study results are given in the paper I-IV.

5.1 HUMAN EXPOSURE STUDIES IN SUBWAY ENVIRONMENT

Healthy volunteers (**paper I**) were exposed for two hours to both a subway and a control environment in Stockholm. Acute health effects were monitored, such as lung function, inflammatory response in the lower airways (using bronchoscopy) and in peripheral blood, as well as fibrinogen as a marker of coagulation. No cellular (inflammatory) response was observed in the lower airways after exposure to the subway environment, although in peripheral blood we found a statistically significant increase of fibrinogen (coagulation marker) and increased levels of regulatory T-cells expressing CD4/CD25/FOXP3.

We have previously shown that exposure to a road tunnel environment causes cellular inflammatory response in airways of healthy individuals. The subway and road tunnel environments have similar levels of mass PM_{10} and $PM_{2.5}$, while the number concentrations of ultrafine particles, nitrogen monoxide and nitrogen dioxide are lower in the subway environment. Another difference between the two environments was that half of the PM_{10} fraction in the subway mainly consisted of iron, but also less than 1% of barium, manganese and copper. For details see Table 3.

Table 3. Median value for environmental exposure measurements during exposure of healthy volunteers in the subway in comparison with levels in a previously investigated road-tunnel environment in the same city and during the same season.

Type of exposure	**Subway environment**	**Road tunnel environment**
Ultrafine particles (number of particles/ml)	8 266	85 000[4]
Approximate ultrafine particle surface area concentration ($\mu m^2/ml$)	84[5]	732
$PM_{2.5}$ ($\mu g/m^3$)	76	64
PM_{10} ($\mu g/m^3$)	237	176
NO ($\mu g/m^3$)	59	874
NO_2 ($\mu g/m^3$)	24	230

[4] 110 000 particles/ml (20-1000 nm)

[5] The approximation may be compared to another more recent measurement of the particle surface area in Odenplan subway station, with a diffusion charging particle sensor (LQ1-DC (Matter Engineering AG). The mean particle surface area concentration for particles up to 10 µm in the subway was 70 (50-100 $\mu m^2/cm^3$). (Midander *et al*, 2012; accepted for publication) [77]

A corresponding study with asthmatics (**paper II**) showed a statistically significant increased frequency of CD4 cells expressing T-cell activation marker CD25 in bronchoalveolar lavage fluid, furthermore, unlike in the study with healthy volunteers there was no significant increase of regulatory T-cells in blood. This means that inflammatory responses after exposure in subway environment differ between asthmatic and healthy humans.

For other comparisons of acute health effects that were monitored, such as lung function, inflammatory response in the lower airways (using bronchoscopy) and in peripheral blood, as well as fibrinogen as a marker of coagulation, see table 4. When analysing lung function, as measured by a portable monitor (PIKO-1), only peak expiratory flow (PEF) was used, because of the potential measures, PEF was considered have more qualitative repeat- measurement accuracy in the absence of assisted supervision.

Table 4. Dissimilar health effects in healthy and mild asthmatics after exposure to a subway environment (**paper I-II**), where ↗ indicates an increased significant difference between subway and control exposure ($p<0.05$) and **ns** indicates a no significant difference between subway and control exposure.

Parameter	**Healthy volunteers**	**Asthmatics**
Lung function: VC, FVC, FEV_1, PEF	ns	ns
Cells in brochoalveolar lavage (BAL) and in in bronchial wash (BW): • recovery • viability • number of cells • cell concentration • % cell differentiation	ns	ns
Blood cells: • cell concentration, • % cell differentiation	ns	ns
Cytokines in BAL fluid: IL-1B, IL-6, IL-8, IL-10, IL-12p70, TNF-α	ns	ns
Lymphocyte subsets in BAL fluid	ns	**sign ↗** CD4+CD25
Lymphocyte subsets in blood	**sign ↗:** CD4+CD69 CD4+HLA-DR CD8+CD69 CD4+FoxP3 CD4+CD25+FOXP3	ns
Symptom (irritation)	**sign ↗:** lower airways disturbing smell	**sign ↗:** eye, nose, disturbing smell
PAI-1 in blood	ns	ns
Fibrinogen in blood	**sign ↗**	ns

5.2 HUMAN EXPOSURE STUDY WITH ULTRAFINE PARTICLE AEROSOL

Method development – labeling of ultrafine particles with indium-111
We developed a method to produce stable carbon particles in nanosized-labeled indium-111 (^{111}In) (**paper III**). To minimize the naturally occurring agglomeration of ultrafine particles, the particle aerosol was diluted and stored in a 70-liter conductive bag filled with clean air. The agglomeration rate of a typically sized carbon particle labeled with ^{111}In is shown in figure 4 below. The agglomeration followed a linear regression.

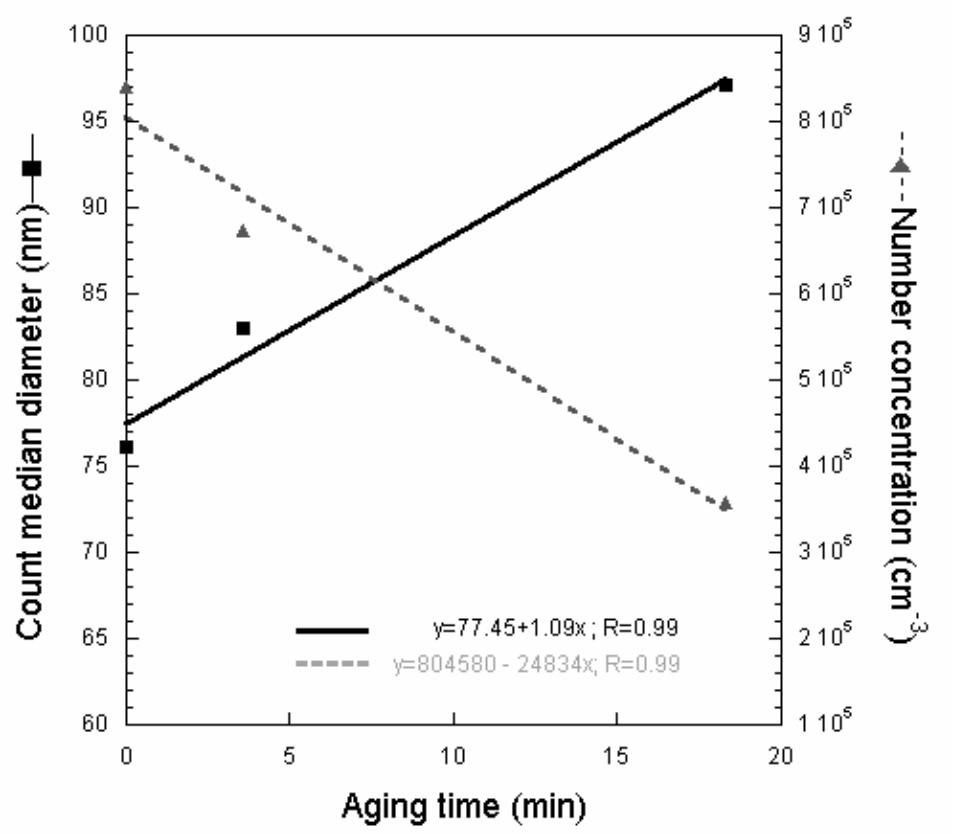


Figure 4. Aging effect on indium-111 labeled particles; size agglomeration after generation and during storage in a 70-liter bag filled with clean air.

Stability of particle label

In order to use the developed method for human studies, it was important to generate carbon particles with a stable bond to indium-111. For tracing the degree of stability of the bond between particles and the isotope, we have used an in vitro dialysis technique. The generated particle aerosol was filtered through a teflon membrane filter and added to a dialysis tube. They were placed together in a sodium chloride buffer. The radioactivity detected in the buffer indicated "leaching" free (unbound) ^{111}In. Figure 5 shows examples for the cumulative ^{111}In radioactivity leaching from the carbon particles as a function of time after generation for three different initial particle sizes. This figure shows that more than 98% of the generated ultrafine particles were initially bound. Moreover, figure 5 shows that the radioactivity-leaching rate is particle-size dependent. For 100 nm particles, the leaching was 0.01% per hour, for 70 nm it was 0.02% per hour and for 50 nm particles it was 0.18% per hour. Seven days after generation, the cumulative radioactivity leaching varied from 2 % to 5 % for 100 nm and 50 nm initial particle sizes, respectively, this was satisfactory for use in human studies (**paper III**). Our aim in human studies was to generate 100 nm carbon particles.

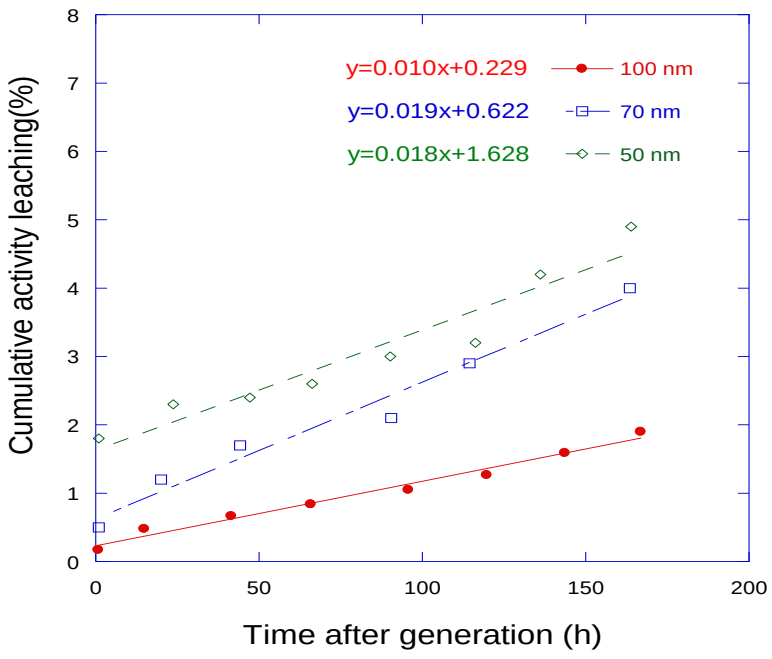


Figure 5. Cumulative indium-111 radioactivity leaching rate from carbon particles for three different particle sizes.

Human exposure – study of pulmonary deposition, retention and clearance

The inhaled ultrafine particles

It took in average 1 min 50 s for the ten healthy volunteers (**paper IV**) to reach the goal of inhaling 1 MBq ^{111}In labeled carbon aerosol. Particle size increased with time during exposure. Estimated mean value for the median of all exposures was 84 nm (range 58-124), with a geometric standard deviation of 2.0 (1.6 – 2.2). The mean number concentration was 460 * 10^3 cm^{-3} (350-870).

Stability of indium-111 labeled ultrafine particles used in human exposure studies

In vitro dialysis we found that the bond between ^{111}In and ultrafine carbon particles decreased linearly during the week. At the end of the week, the in vitro leaching of free unbound particles was 2.10%±1.60% at the group level. The cumulative in vivo leaching, calculated from measurements of free radioactivity in blood and urine, also gradually decreased. For urine it was 0.6%±0.4%, while in blood it was 1.5%±1.5%.

Pulmonary deposition, retention and clearance of the inhaled particles

Approximately 31.4% (±11%) of the inhaled original, particle-bound indium radioactivity was deposited in lungs of the volunteers. There was an even distribution over the lungs due to a low, central-airway impaction during administration. Retention of radioactivity in the pulmonary region was followed using gamma images of the chest region. The decreasing radioactivity in the pulmonary region corresponds to the amount of ^{111}In labeled carbon particles that was cleared from the lungs. The elimination rate was faster in central regions (see figure 6) in comparison to peripheral, which is most likely due to the fact that mucocilliar transport is the dominant process in the central region.

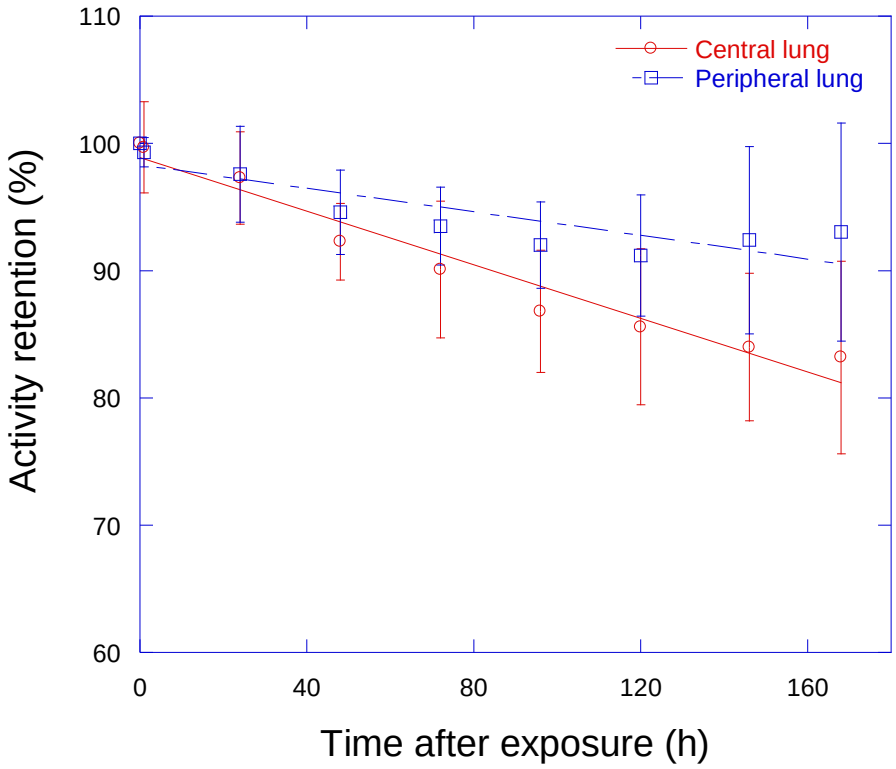


Figure 6. Radioactivity retention, as measured with a gamma camera, in the central and peripheral lung regions as a function of time after administration of the ^{111}In labeled ultrafine carbon particles.

Seven days after exposure, lung retention as measured by gamma camera was 91%±8.3% at the group level. After correction for free radioactivity leaching from urine and blood samples, respectively mucociliar clearance from the central airways, the cumulative lung-particle retention was approximated to 96.4%±7.1%. The values at each time point represent a group-level average. The plot in figure 7 also presents the error bars corresponding to the fully corrected estimates of lung retention. Neither, sex, age nor BMI seemed to influence retention or clearance.

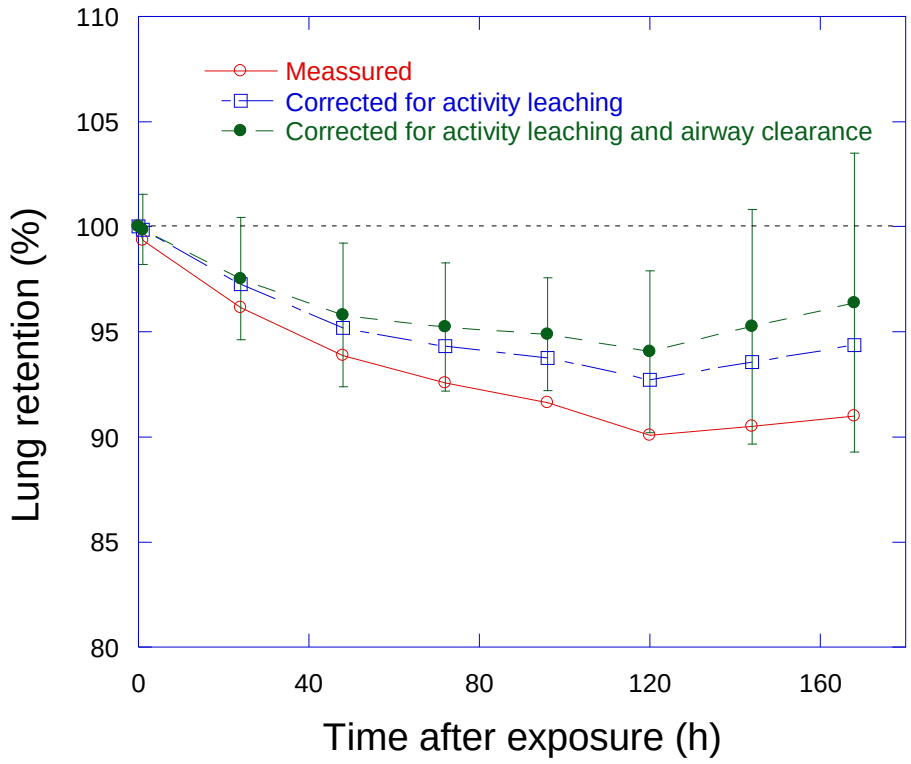


Figure 7. Pulmonary retention of inhaled ^{111}In labeled carbon particles with and without corrections for radioradioactivity leaching (unbound ^{111}In) and clearance from the central airways.

Limited translocation to blood

According to our estimation, the particle translocation from lungs, found in peripheral blood and urine, was rather small and did not change much during the week. After seven days it was 0.3%±0.2%.

Extended follow up for one volunteer
One volunteer was monitored for 29 days (unlike the remainder were monitored for 7 days) after exposure. This female volunteer had a further clearance of approximately 10% from the lung region, see figure 8.

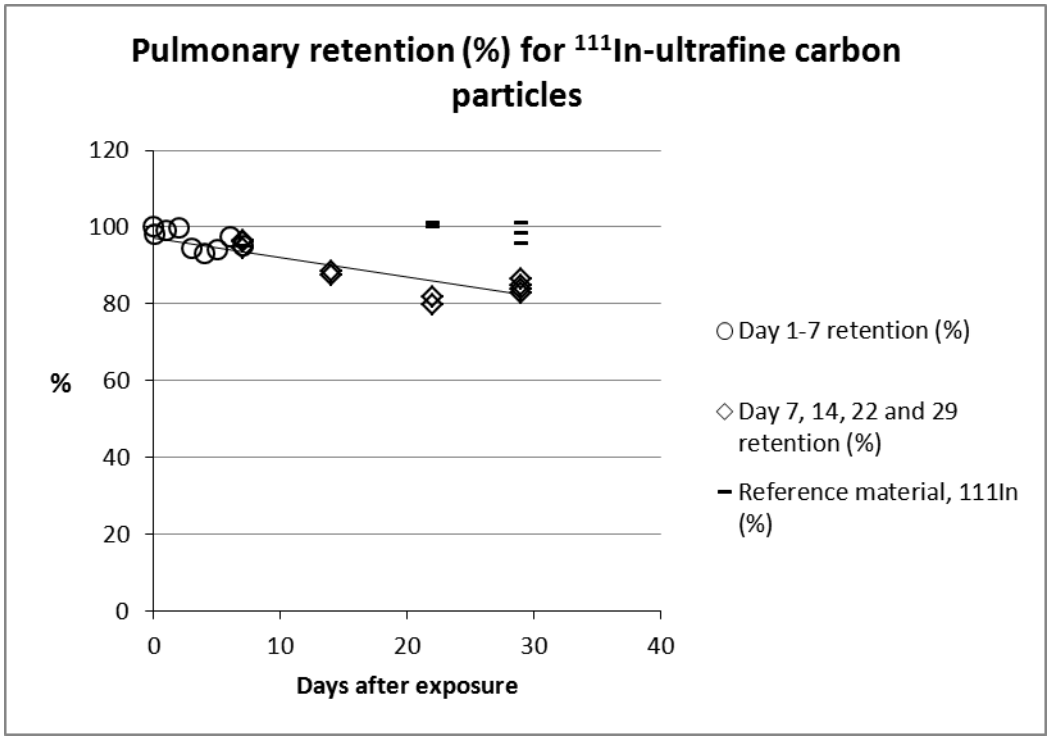


Figure 8. Lung-retention measurements (uncorrected for leaching) of ultrafine carbon particles labeled with ^{111}In with a sodium iodide scintillation detector for one person (◊), as well measurements of radioactivity for a reference material (-). The retention was normalized to previous measurements with a gamma camera (o) at day seven after exposure.

6 DISCUSSION

6.1 ACUTE HEALTH EFFECTS CAUSED BY DIFFERENT PARTICULATE AIR POLLUTION

Do acute health effects caused by particle exposure differ depending on size and composition? That was our primary objective when starting exposure measurements in an actual subway environment. Using an identical study protocol as in a previous study performed in a road tunnel environment enables us to compare the two environments regarding potential health effects. Analysis of the metal content in PM_{10} from the subway station showed a high metal content, mainly iron, which derives from the wear of wheels, rails and brakes (which is not the case in the road tunnel environment. An important difference between the two environments was that the number concentration of ultrafine particles was ten times higher in the road tunnel due to the motor exhausts, which mainly generate ultrafine particles. There were also high levels of NO_x in the road tunnel, mainly from exhaust fumes, which is not present to any major extent in the subway.

In vivo and *in vitro* studies show that ultrafine particles are more pro-inflammatory than $PM_{2.5}$ and PM_{10}. In one *in vivo* study, carbon black as well as titanium dioxide was installed in rat lungs in both the size of 14-29 nm (ultrafine) respectively 250-260 nm (fine particles). The ultrafine particles showed to be significantly more pro-inflammatory, increasing neutrophils in the bronchoalveolar lavage fluid. [52] In another *in vivo* study in rats, the neutrophil influx in bronchoalveolar lavage fluid was also higher with ultrafine carbon black particles, rather than with particulate city air pollution (PM_{10}) [51].

Healthy individuals

Our earlier *in vivo* study demonstrated that exposure to particles derived from road tunnel increased the amount of inflammatory cells (total cell number, lymphocytes and alveolar macrophages) in the BALF of healthy individuals. The participants also reported increased levels of irritative symptoms from eyes, upper (nose) and lower (lung) airways and an experience of disturbing odour. [2]

Since the exposure levels regarding gravimetric parameters like $PM_{2.5}$ and PM_{10} were at equivalent levels when compared to the subway environment, it is reasonable to expect that the inflammatory effects observed in the road tunnel study also would be observed after exposure in the subway environment. However, no signs of cellular inflammatory response were observed in the lower airways after exposure to the subway environment, assessed by bronchoscopy, regardless of whether the cells were retrieved from peripheral (BAL fluid) or central (bronchial wash) airways. The exposure resulted in a significantly increased expression of markers for regulatory T cells as well as elevated levels of fibrinogen in peripheral blood in healthy volunteers. The participants reported increased levels of irritative symptoms lower airways and an experience of disturbing odour.

Studies on regulatory T cells is quite novel. Generally, T-regulatory cells are important in balancing or suppressing immune responses. At the time of our road tunnel study, we were unable to analyse for the marker for regulatory T cells. In that sense the results cannot really be compared.

To our knowledge, there is only one previous study investigating acute health effects in humans caused by exposure in a subway environment. It showed that inflammatory response, measured as plasma concentrations of PAI-1, interleukin-6 and fibrinogen, had a tendency to be higher for subway platform workers than for nonsmoking, healthy train drivers and subway ticket sellers [50]. In our study the plasma levels of fibrinogen also increased after the subway exposure, but not PAI-1. The increase of fibrinogen levels was however very modest and within normal range. No such effect was seen in the road tunnel study. However another study performed in in London, involving more than six thousand office workers, showed increased levels of urban PM_{10} correlated with increased levels of plasma fibrinogen[68].

The inflammatory response due to different type of particles, including subway air particulate matter (PM_{10}) and diesel exhaust particles (DEP), has been studied *in vivo* in an animal study. C57Bl/6 male mice were exposed to particles (5-100μg/mouse) by intratracheal administration. Signs of inflammatory response were observed in bronchoalveolar lavage fluid 8 hours after 100μg/mouse exposure, with an increased number of neutrophils (due to subway particles and DEP) as well as pro-inflammatory cytokines TNF-α and MIP-2[6](due to subway air). [69] The discrepancies in inflammatory response with our subway results, with not as pronounced inflammatory response, may be explained by the difference in exposure methods. Intratracheal administration targets different lung compartments, versus normal inhalation of larger particulate matter.

Health effects in asthmatics compared to healthy participants

Inhaled particles in air pollution may exacerbate the already ongoing lung inflammation in asthmatics [31]. Our earlier *in vivo* study demonstrated that exposure to particles derived from road tunnel induced irritative symptoms from upper (nose) and lower (lung) airways, and decreased lung function (PEF) in asthmatic participants. In non-treated asthmatics there was an increase of pro-inflammatory cytokines IL-12 and TNF-α, as well as of the anti-inflammatory IL-10 in nasal lavage. [70]

Even though asthmatics are known to be more sensitive to air pollution than are healthy persons with increased symptoms and emergency visits due to air pollution [71], this could not be clearly demonstrated in the present subway study. No effect on lung function (PEF) was demonstrated, but mild asthmatics exposed to a subway environment showed statistically significant increase of recruited CD4 cells expressing the T cell activation marker CD25 (CD4pos/CD25pos) in lung in comparison to a control exposure. CD25pos is a general activation marker present on activated T cells in inflammatory reactions. This was not seen in healthy volunteers. In our study the CD4pos/CD25pos

[6] MIP-2= macrophage inflammatory protein

changes were seen in BAL, but not in blood, which suggest a local effect in the lungs. Our co-workers Lundström *et al* [72] have compared oxylipin levels in bronchoalveolar lavage (BAL) fluid from the same healthy and asthmatics, showing reduced anti-inflammatory response in asthmatics following exposure to the subway air.

In our study asthmatics reported significantly increased irritation in the eyes and the nose during the exposure to the subway environment, as well as an increased experience of disturbing odour. Similar to asthmatics, self-reported experience of a disturbing odour was registered in healthy volunteers, but without corresponding significant changes in irritation in the eyes or nose. Instead, healthy subjects reported increased signs of irritation in the lower airways, which we found interesting.

Regarding regulatory T cells that were induced in healthy but not asthmatic participants, there is at present limited knowledge regarding the role of regulatory T cells in asthma. Evidence suggests that asthma is characterized by a relative deficiency in regulatory T cells [73]. Such tendency was also seen in asthmatics in our study in comparison to the healthy participants.

Differences in response due to different environments

There is to our knowledge no other study that has investigated acute health effects in asthmatics caused by exposure in a subway environment. The acute health effects differ for asthmatics after exposure to road tunnel respectively subway environment. This may be due the presence of car exhaust in the road tunnel generating ten times higher levels of ultrafine particles and nitrogen oxides (NO and NO_2), than was monitored in the subway environment. Association between ultrafine particles and asthmatic symptoms, but not $PM_{2.5}$ and PM_{10}, as well as an increased need of medication has been demonstrated in previous studies [32-34].

Acute health effects response in asthmatics to environmental pollutants may differ depending on the composition and concentration of pollutants, as well as on asthma severity. For this study we chose a group of “mild asthmatics”, with no regular need of asthma medication, which could be one explanation to the weak responses observed in our study. In an *in vivo* study on asthmatics with a 2-hour exposure in a busy road in London found that road traffic exposure had a negative effect on the lung function of the asthmatics, as measured as FEV_1 and FVC in comparison to exposure in a park. The effects were greater in moderate asthmatics compared to mild asthmatics. They furthermore found neutrophilic inflammation and decreased pH in sputum. Associations were strongest with ultrafine particles and with elemental carbon. [40]

Acute health effects due to subway environment

The results from our study show that healthy individuals exposed to the subway environment for two hours resulted in an up-regulation of T cells with a phenotype compatible with T cell regulatory functions, important for the regulation of immune tolerance, and a limited increase of fibrinogen levels in blood. It is still too early to conclude what an up-regulation may imposes

regarding evaluation of real health risk for the population exposed to the subway environment.

One of the main result from our study shows that a two-hour exposure by asthmatic individuals leads to a different T cell response than for healthy volunteers in our previous study. There is a local activation of T cells in asthmatics in BAL fluid, while healthy volunteers demonstrated a systemic increase of regulatory T cells in blood that suppress the inflammatory response.
Overall, acute health effects after exposure to our subway environment were few. Although no cellular response or increased levels of inflammatory cytokines were detected in either blood or BALF, the findings indicate a minor biological response due to the subway environment. Further studies are needed to evaluate these effects.

Recently a new exposure study in the subway environment has been performed using repeated measurements to monitor possible earlier or later effects on the inflammatory responses. Since BAL is not a suitable method for repetitive measurements, repeated blood sampling was used. Blood sampling was also considered particularly suited for healthy volunteers, since the inflammatory effects were seen in their blood. The data are now being evaluated.

6.2 PARTICLE EXPOURE LEVELS AS A HEALTH RISK INDICATATOR

Monitored subway particle levels world-wide
Our measurements in Stockholm subway show that the levels of particles are within levels reported world-wide in scientific literature (according to the table 5 below). The subway system in Stockholm has only electrical driven subways and is almost free from motor exhaust coming from the ventilation system. This is not always the case in other subway systems. Even though particle levels may be similar world-wide and mainly contain iron, a risk assessment cannot be based solely on this exposure information since the chemical composition may still vary. Our study is however representative for the overall effects from rail wear particles. More complex measurements may be needed to cover other aspects of subway exposure.

Table 5. Particle levels in subway environments world-wide.

City	PM2.5 (µg/m³)	PM10 (µg/m³)	Ultrafine (particles/ml³)	References
Stockholm	63			[74]
Stockholm	260	470		[1]
Stockholm	*77*	*242*	*8 280*	*[75]* ***paper I***
Stockholm	*71*	*232*	*8 960*	*[76]* ***paper II***
Stockholm	60	160	12 000 (20-1000 nm)	[77]
Stockholm		100-500		[78]
Amsterdam	137	394	32 792	[3]
Helsinki	47 & 60		31 000	[4]
London	246			[5]
London	270-480	1000-1500	14 000- 29 0000	[79]
Mexico City	57			[80]
New York	39		17 416	[6]
Paris		68 resp 361		[69]
Prague		102		[81]
Rome		35-479		[82]
Seoul		126		[83]
Seoul	129	359		[8]

$PM_{2.5}$ and PM_{10} – inadequate for assessing health risks

Both the previous road tunnel environment and the current subway were found to have similar mass levels of particles at diameters <2.5 µm and <10 µm ($PM_{2.5}$ and PM_{10}), although the road tunnel had a higher number concentration of ultrafine particles as well as higher levels of nitrogen monoxide and dioxide. We found that different health impacts despite the similar $PM_{2.5}$ and PM_{10} mass concentrations, and which implies that health-risk assessment cannot solely be based on this exposure information, since the particle characteristics can differ substantially by size and composition. More complex measurements of particles, including number concentration levels of ultrafine particles as the source of particles, are needed.

6.3 PULMONARY DEPOSITION AND RETENTION IN HUMANS OF ULTRAFINE PARTICLES

Indium-111 labeled ultrafine particles: high retention and minor translocation

We successfully developed a method to generate indium-111 (^{111}In) labeled ultrafine carbon particles for use in human studies. Human inhalation time was 3.5 minutes to reach about 1MBq ^{111}In-particle exposure. During this period, the subject was exposed to four times higher particle number concentrations than those measured in road tunnels [2]. One week post-administration, the average pulmonary radioctivity retention at group level was 91%, with a marginal particle translocation (0.3%) rom the lungs to peripheral blood. A 29-day follow-up was

performed for one volunteer, which showed that retention was reduced by another 10%. When corrected for activity leaching and central airway clearance by mucocilliar transport, the particle retention after one week was 96.4±7.1% at group level. The clearance rate was within the expected range with previously performed human studies.

In our study the clearance was mainly from the central parts of the lungs. This was probably attributable to mucocilliar clearance from the larger airways. Normally, it is generally assumed that mucociliary transport is a process that terminates within a few days, which was also clearly seen in our studies. During the first 4 days after exposure, there is a fast clearance rate, clearly representing mucocilliar transport, which then tapered off. Our previous human studies with 6, 8 respectively 10 μm teflon particles also showed a faster clearance from large ciliated airways, while the more peripheral parts (ciliated bronchioles) had almost 100% retention after 24 hours. In both regions the retained fraction was independent of particle size. [84]

No inter-individual differences

Neither the retention nor clearance was affected by age, sex or BMI. Our research team has previously performed several human exposure studies on deposition, retention and clearance of particles, mainly on larger teflon (4-16 μm) particles labeled with ^{111}In. Ultrafine particles differ not only in chemical properties but also in surface properties, which makes it harder to compare the results. The surface area of a nano-sized atom or molecule has is much larger per given mass than for a micro-sized molecule. An increased area can also act as a carrier for more co-pollutants. Some general aspect can be concluded from those previous studies; for instance, retention within a group may vary more than that for each individual [60] and that long-term clearance (21 days) from the small airways decreases with age [85].

Marginal translocation

There was marginal translocation of carbon particles from lungs to blood, which supports our earlier results on with ^{99}Tcm-labeled ultrafine carbon particles with a size of 35 nm [86]. But as discussed by Wiebert *et al*, even small translocation from lungs into the circulation may have harmful health effects. In this context one needs to consider that low radioactivity levels are connected with possible uncertainties in the detection accuracy.

There are limited human studies on translocation using ultrafine carbon particles. Due to safety aspects, are all short-term studies and show either no or limited translocation or major translocations of up to 1%. A 1% translocation lies at the interpretation threshold at which some authors referring to it as limited translocation [63, 64, 86-88] while another author may refer to it as a major translocation [65].

Potential health problem due to the nano-size and solubility of particles
Altogether, the results from our human study with high pulmonary retention of ultrafine carbon particles and with marginal translocation to blood, are consistent with previously performed short-term, follow-up studies by Wiebert *et al* [63, 86]using 99mTc-labelled UF carbon particles.

A potential health problem with the high pulmonary retention of ultrafine carbon particles is that they may have an inhibitory effect the phagocytic ability of alveolar macrophages, similar to particles derived from diesel exhaust. [59] The mentioned study was an *in vitro* study with human alveolar macrophages exposed to particles, which showed to have impaired attachment and ingestion process to silica particles and microorganisms. The authors concluded that this effect may result in more exacerbations of subgroups of chronic inflammation in their airways, as it may increase susceptibility to infections.

In this context it is important to remark that in deposition studies the used carbon particles are non-soluble in the body liquids/tissues. However, their chemical property may differ from particles present in outdoor ambient air may comprise different chemical forms and hence be partly soluble in body fluids, and thereby result in different health effects. Furthermore, they can carry organic molecules of various types, possibly correlated to the onset of inflammatory processes in the body in relation to their accumulation in the body. The solubility of particles being able to affect measurements of clearance and deposition was mentioned back in 1973 [89]. Furthermore, Philipson *et al* [90] showed that if insoluble particles reach the alveoli they will probably mainly be cleared by macrophagial phagocytosis and be transported to the ciliated airways where they are cleared out, a process that may take several years.

Limitations – due to low radioactivity
There are limitations of the technique because of the low radioactivity level used to study particles. In the (gamma camera) scintigraphic technique, the noise errors concerning particle distribution in the lungs increased with time. The low amount of inhaled radioactivity also represents a challenge regarding radioactivity-leaching measurements in blood and urine. The buffer to sample volume ratio in the dialysis process is crucial. In this work, we use a 100:1 buffer to sample the volume ratio. Larger buffer volumes are still desired, since the larger the buffer volume, the greater the diffusion gradient. However buffer volume is limited by the minimum detectable radioactivity concentration of the radiation detector used. Hence, a general approach for a better estimation of radioactivity leaching is to measure the radioactivity of the sample before and 24 hours postdialysis rather than buffer samples. In this way, the buffer can be renewed to ensure a good diffusion capacity throughout the entire period.

Low leakage or protein binding?
Measurements of retention and clearance are highly dependent on the quality of the aerosol labeling, hence radioactivity-leakage studies are mandatory when using radiolabeling particles to study lung retention [91]. In our study, the bonding between particle and radionuclide was very stable. Seven days after exposure, the *in vitro* activity-leaching test showed a 3.2% free activity in the aerosol sample (at group level). This correlates well with the corresponding *in vivo* values of free activity measured in blood and urine (2.1% seven days after exposure).

Leaching measurements with the dialysis-diffusion technique in blood cannot differentiate aerosol-bound radioactivity from free radioactivity bound to blood cells or proteins. Hence the dialysis method is still inconclusive for determining the presence of extrapulmonary aerosol particles. There was also a good correlation between the *in vitro* leaching test and with corresponding *in vivo* values of the free radioactivity measured in blood and urine, meaning that there probably was little other biological bonding to consider. Furthermore, this method, incorporates gamma camera imaging of the thorax and abdomen) may additionally offer a good indication of the nature of the bound radioactivity.

Future research - effect on deposition and retention in injured lungs
Little is known about how underlying inflammation or lung diseases may affect the deposition and retention of ultrafine carbon particles. What happens in the case of exposure with ultrafine particles? Do people with injured lungs have an increased translocation of particles into the blood circulation? Inconclusively, our previous studies with healthy and asthmatics found no difference in translocation of ^{99m}Tc labeled carbon particles after 24 hours (Wiebert, Sanchez-Crespo et al. 2006). A study in COPD patients exposed to ^{99m}Tc-labeled carbon particles (larger than nanosize) showed a slightly increased deposition in comparison to a healthy subgroup. After 24 hours, there was a tendency toward increased retention in COPD patient (67%) compared to healthy subjects (64%) [87]. Furthermore, retention studies with larger particles (teflon labeled with indium-111) in patients with damaged lungs, e.g., patients with chronic bronchitis, showed higher pulmonary retention after 3 days [92], as well as after 21 days [93]. The method developed here, namely ^{111}In-labeled nanosized particles, enables longer follow-up studies of retention as well as translocation in individuals with underlying inflammation or lung diseases.

7 MAIN CONCLUSIONS

1) Subway environment did not cause a classic inflammatory response (cellular response).

2) The health effects after exposure to subway environment differed and were less pronounced from those observed after exposure to a road-tunnel environment. The different reactions indicate that other mechanisms might be relevant for health effects in humans.

3) Our study shows that responses after exposure in subway environment differ between asthmatic and healthy humans. There was no convincing indication that asthmatics have a stronger reaction compared to healthy individuals. Asthmatics had changes locally in lower airways, while in healthy volunteers the signs indicated some systemic changes in blood.

4) It is clear from our studies that even if different exposure environments have similar $PM_{2.5}$ and PM_{10}, mass concentrations, a health-risk assessment cannot solely be based on this exposure information, since the particle characteristics can differ substantially regarding size and composition. More complex measurements of particles, which include number concentration and composition of particles, are needed.

5) The current study demonstrated that it is possible to generate and administer an indium-111 (^{111}In)-labeled ultrafine graphite (carbon) aerosol to humans. Compared to previously presented methods based on Technegas aerosol, the ^{111}In labeled carbon particles also showed improved physicochemical properties. This allows extended follow-up assessments of particulate retention in healthy individuals, as well as in individuals with obstructive lung disease.

6) Results from lung-deposition and retention studies indicate:
    a. There is limited translocation to the bloodstream in individuals with healthy lungs in the first week
    b. Clearance from the central lungs is probably attributable to mucocilliar clearance from the larger airways
    c. There is no association between pulmonary retention (clearance) and sex, age or BMI.

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