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Health effects of a subway environment in healthy volunteers. Experimental data.

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Abstract

Environmental particle exposure, often estimated as PM_{10}, $PM_{2.5}$ or PM_1, is known to have negative impact on the health of the population. Little is known about how size and origin of particles influence the effects. We have previously shown that exposure to a road tunnel environment causes cellular inflammatory response in airways of healthy individuals.

In the present study our aim was to investigate potential airway health effects from exposure to a subway environment.

Twenty healthy volunteers were for two hours exposed to a subway and a control environment, followed by measurements of lung function, inflammatory response in lower airways (bronchoscopy) and in peripheral blood.

No cellular response in the airways after exposure to the subway environment was found. In blood we found statistically significant increase of fibrinogen and regulatory T-cells expressing CD4/CD25/FOXP3.

The subway and road tunnel environments have similar levels of mass PM_{10} and $PM_{2.5}$, while concentration of ultra fine particles and NO_x are lower in the subway. Although no cellular response was detected, the findings indicate a biological response due to the subway environment. Our studies illustrate that using gravimetric estimates of ambient particulate air pollution alone may have clear limitations in health risk assessment.

Keyword

Air pollution, bronchoscopy, inflammation, inhalation exposure, particle size

Introduction

Particulate air pollution is considered as a potential health problem. Combustion exhaust from motor traffic is a major contributor to ambient air pollution and is probably also one of the main causes of cardiovascular morbidity and mortality observed in a number of epidemiological studies [1, 2]. The mechanisms behind these effects are still not fully understood. One suggested hypothesis is that inflammatory events induced by particle exposure can influence the coagulation system. Particles could also have a direct impact on the heart by causing changes in heart rate variability. A case-crossover study indicated that even moderate increase in air pollution (PM_{10}) has effects on ventricular arrhythmias in patients with implantable cardioverter defibrillator [3].

Particulate air pollution is usually monitored by gravimetric measurements of PM_1, $PM_{2.5}$ and PM_{10}, where PM stands for particulate mass of particles with a diameter <1 µm, <2.5 µm respectively <10 µm. Measurements of ultra fine particles (UFP), defined as particles with a diameter less than 100 nanometers, are rarely monitored. Because of their low mass, measurement of number concentration is more relevant for UFP. Motor exhaust is a major source of UFP in the urban environment and has generally increased in the city environments. Several studies show that UF particles are more potent in inducing inflammatory response [4-6]. In an *in vivo* study on rats, urban PM_{10} particles and ultra fine carbon black particles were installed in the rat lungs and followed by a bronchoalveolar lavage (BAL) after 6 hours. Both the PM_{10} and UF particles induced inflammatory response, but the latter gave a greater influx of neutrophils. [7]

One way of limiting air pollution related to motor exhaust is by encouraging the use public transportation. Particle exposure measured as PM_{10} has however been found to be high in the subway environment. In Stockholm the PM levels in the subway are 5-10 times higher than at street level in the inner city area [8] and comparable to those found in the road tunnel [9]. This is also the case in several other cities, such as London [10, 11], Rome [12], New York [13] and Seoul [14]. In the subway, airborne particles are usually > 1 µm and originate mainly from wearing of tracks and wheels, which leads to a high content of iron. Compared to the concentration in the street level environment, the number of UF particles is however of a much lower magnitude in the subway environment.

Assessment of personal PM_{10} exposure has been performed on personnel working in the Stockholm subway. Exposure levels for subway drivers were compared with platform workers. The latter group was exposed to four times higher levels of particles than the train drivers [15]. This suggests that the subway environment exposure also could have an impact on the citizens using the subway. Another study showed that inflammatory response, measured as blood plasma concentrations of plasminogen activator inhibitor 1, interleukin-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. [16]

In an earlier *in vivo* study performed in a road tunnel, we have shown that particles derived from motor traffic induces inflammatory reactions in the lower airways of

healthy individuals, such as increased concentration of inflammatory cells in BAL fluid [9].

The aim of this study was to assess whether or not exposure in the subway environment would induce acute inflammatory effects in the lower airways in healthy individuals, as was observed in the road tunnel study. The two environments have similar $PM_{2.5}$ and PM_{10} mass concentration levels but differ in number concentration and composition.

The comparison of acute biological response derived from inhaled particles in relation to size and composition, could give guidance to what kind of particles that impose the most potent risk factor to human health.

The study design was chosen in order to facilitate direct comparison with results obtained in the previously mentioned road tunnel exposure study. Cellular response and inflammatory mediators were explored by performing BAL and the effects on lung function were measured by spirometry. Blood samples were taken to investigate impacts on the coagulation system (fibrinogen and plasminogen activator inhibitor-1).

Methods

Volunteers

Twenty healthy, non-smoking volunteers (7 women) with a mean age of 27 years (range 18-46) participated in the study. All volunteers underwent a routine physical examination (table 1). Chest X-ray and lung function tests were normal. None of the volunteers had any symptoms in the airways. An *in vitro* screening for the presence

of specific IgE antibodies against common inhaled allergens (Phadiatop®, Pharmacia-Upjohn, Uppsala, Sweden) showed that 90% of the volunteers did not have any IgE antibodies.

Airway infections were not allowed within six weeks before exposures occasions. All participants had given their informed consent to participate in the study. The study was approved by the local ethical committee at the Karolinska Institutet.

Study design

The study was carried out between mid October until mid March in Stockholm. The study had a randomised cross-over experimental design that involved exposure to both a subway environment and an office environment (control). The volunteers served as their own controls. As shown in figure 1 the volunteers were randomized into starting with exposure in either subway or control environment. The second exposure followed with at least three weeks interval. Fourteen hours after each exposure bronchoscopies and blood sampling were performed.

During participation in the study the volunteers were told to perform ordinary daily activities. However, no transportation using the subway system was allowed during the whole study period. The volunteers were also encouraged to avoid heavy physical activities during the days of measurements and to avoid staying in areas with heavy air pollution.

The study design was chosen to enable comparison with a previous exposure study performed in a road tunnel were inflammatory effects were seen [13]. Our study includes the same type of study population regarding age and sex, the same

exposure time and sampling time, as well as the same methods regarding clinical and exposure measurements.

Location of exposures

The subway exposures were performed during the afternoon rush hours (4-6 PM) at the platform of a subway station (Odenplan) in the central part of Stockholm. The exposure took place in a room located few meters below the subway platform level with the corresponding air quality to the platform level (data not shown). The room had two doors on each opposite sides, which were open to the adjacent platform during the exposure session. Control exposures were performed during corresponding hours in an office environment.

During the exposure sessions the volunteers alternated 15 minutes intervals of moderate exercise on a bicycle ergometer with 15 minutes of rest. The ergometer resistance was adjusted in order to achieve an individual ventilation rate of 20 liter air per minute and m^2 of the body surface. Self stated symptoms were recorded during exposure sessions (see below for details).

Environmental exposure measurements

Exposure levels were monitored during each exposure session. Sampling of $PM_{2.5}$ and PM_{10} particles (with an upper 50 % cut-off aerodynamic diameter of 2.5 respectively 10 μm) in the subway environment, was performed using Harvard impactors (Air Diagnostics and Engineering Inc., Maine, USA) equipped with Teflon filters (Air Diagnostics and Engineering Inc., Maine, USA) with a pore size of 2 μm and at a flow rate of approximately 10 L/min. The filters were weighed after 24 hours of conditioning using a Mettler Toledo MT5 scale (Mettler, Greisensee, Switzerland),

but also analyzed for a range of metals using an inductively coupled plasma mass spectrometry (ICP-MS).

The number concentration of airborne UF particles with an aerodynamic diameter of 14 - 100 nm, was determined using a Scanning Mobility Particle Sizer (SMPS) system consisting of Electrostatic Classifier model 3081 (TSI, USA) in combination with a Condensation Particle Counter (CPC) model 3010 (TSI, USA). Nitric oxide (NO_x) levels were monitored using a chemiluminescent instrument (AC 31M, Environnement, Poissy, France).

During the control exposures portable logging instruments were used. To enable comparison regarding particle exposure levels, these instruments were also used during exposure sessions in subway environment. The two portable logging instruments were DataRAM (MIE pDR1000), that measures the mass of particles between 0.1 and 10 ($PM_{0.1-10}$) µm in diameter, and P-Trak, a particle counter (TSI) estimating the number of particles between 20-1000 nm in diameter.

Self reported symptoms

During the exposure sessions self reported symptoms of irritation from eyes, upper and lower airways as well as experience of disturbing smell were recorded before and every 30 minutes throughout the exposure. The intensity was graded from 0 to 10, where 0 corresponded to no symptoms and 10 to severe symptoms, according to a modified Visual Analogue Scale (VAS).

Lung function tests

Lung volume was measured with a spirometer (Jaeger Masterscope, Würzburg, Germany) which included vital capacity (VC), forced vital capacity (FVC) and forced expiratory volume during the first second (FEV_1). We also measured exhaled NO levels (Aerocrine, Niox Mino). Measurements were performed immediately before bronchoscopies.

Measurements of peak expiratory flow (PEF) were performed with a portable lung health monitor (PIKO-1, Ferraris, Louisville, US) just before the exposure session, after one hour as well as straight afterward the two hours long session. Each volunteer was instructed to repeat measurements at around 8 PM, 10 PM, 6-7 AM as well at the clinic at 7:30-8 AM in the following morning.

Peripheral blood

Peripheral blood was sampled in connection to bronchoscopy. Cell differential counts were performed using an autoanalyser (Advia 120 Hematology System, Bayer). Fibrinogen in plasma is involved in the coagulation system. It was analyzed by a kinetic fibrinogen assay. Plasminogen activator inhibitor-1 (PAI-1) was analyzed using Enzyme-Linked ImmunoSorbent Assay (ELISA) (Chromolize PAI-1, Biopool, Trinity Biotech, Bray, Ireland). Blood was also used for immunostaining and flow cytometric analysis.

Bronchoscopy, bronchoalveolar lavage and bronchial wash

Bronchoalveolar lavage (BAL) was performed by inserting a flexible fiberoptic bronchoscope, (Olympus, Olympus Optical Co. Ltd, Tokyo, Japan) under local anaesthesia, as described in detail by Eklund & Blaschke [17]. 250 ml sterile

phosphate buffered saline was instilled into the middle lobe in 5 aliquots of 50 ml each. After each installation the fluid was gently aspirated and collected and kept on ice.

The BAL fluid (BALF) was filtered through a single layer of Dacron net (Millipore, Cork, Ireland), centrifuged at 400g for 10 minutes at 4^{o}C. The pellet was resolved in RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma-Aldrich, UK). Cells were counted and total cell viability (mean 94.4 %) was determined by trypan blue exclusion, using a Bürker chamber. BALF cell pellet was also used for immunostaining and flow cytometric analysis (see below).

The supernatant was analyzed for inflammatory cytokines interleukin IL-1B, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor-α (TNF-α). Cytokine analyses were done by flow cytometry (FACSCalibur, Becton Dickinson) using CBA (Cytometric Bead Array) technique (Becton Dickinson).

Bronchial wash was done in a segmental bronchus in the upper lobe by installing 2 x 10 ml sterile phosphate buffer saline. Each installation was followed by dental aspiration of fluid.

Immunostaining and flow cytometric analysis

Lymphocyte subsets were determined in BALF and erythrocytel-ysed human peripheral blood using the TBNK 6 color Multitest. TBNK consists of a combination of fluorochrome conjugated monoclonal antibodies for T-cells, B-cells and NK-cells. All of them have synonyms expressed as CD (cluster of differentiation). We also used a

set of monochlonal antibodies specific for markers of T cell activity and of T cell regulatory functions.

For each analysis, $1x10^6$ BALF cells were incubated with antibodies for 30 minutes in the dark and kept on ice at 4^oC, while blood was kept at room temperature. Blood samples were lysed with lysing solution and washed twice with cellwash (BD Biosciences, San Jose, CA, USA). All samples were analyzed by a flow cytometer using an 8 color FACSCanto II (BD Franklin Lakes, NJ USA). Approximately $2.5x10^5$ cells were collected from each sample. Lymphocytes were gated by forward and side scatters. For the TBNK Multitest a gate was set for CD45 (white blood cells). For the T-cell activation and T-regulatory panels a gate was set for CD3 cells (T-cells). Isotype matched negative control antibodies always stained less than 1%. Antibody combinations used in BALF and blood are shown in appendix 1.

All monoclonal antibodies were from BD Biosciences, San Jose, CA, USA, with the exception of FOXP3 which was obtained from eBiosciences, San Jose, CA, USA (appendix 1).

Statistical analysis

Statistical analysis was carried out with SPSS version 15.0 on a Windows based PC platform (SPSS Inc, Chicago, Illinois, USA). Individual changes in different parameters for subway and control exposure were analyzed using Wilcoxon´s non-parametric 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.

Results

Environmental exposure measurements

During totally ten subway exposure occasions the mean levels of $PM_{2.5}$ (mean level ± SD) and PM_{10} were 77±10 μg/m^3 and 242±40 μg/m^3, respectively. The mean number concentration of ambient airborne particles with an aerodynamic diameter below 100 nm was 8283±1716 particles/ml. The mean level for NO was 58±12 μg/m^3 while for NO2 it was 24±3 μg/m^3. For comparison with exposure measurements in the road tunnel see table 2.

All but three metals (iron, barium and manganese) were below the detection limits (ICP-MS). The content of metals in the PM_{10} fraction (mean level ± SD) was 58.6±21.0 % of iron, 1.0±0.4% of barium and 0.5±0.2% of manganese. The content of metals in the $PM_{2.5}$ fraction was to a large extent below the detection limit. No analysis of endotoxins were performed, but an *in vitro* study showed that subway particles contained endotoxin below the detection limit (0.01 pg/ml) [18].

For the control exposure the particle the concentration mean level of $PM_{0.1}$-PM_{10} (using DataRAM) was 16±4 μg/m^3 (mean level ± SD), while the number of particles between 20 and 1000 (using P-Trak) was 1007±660 particles/ml. The corresponding levels in the subway environment were 162±25 μg/m^3 and 10549 ±1453 particles/ml, respectively.

Symptoms

In comparison with the control exposures, self reported symptoms of irritation from the lower airways were significantly higher during the exposure to the subway environment. Experience of disturbing smell was also higher. Irritative symptoms from the nose had a tendency to be elevated during exposure to the subway environment (p=0,054), while the irritative symptoms from the eyes were not affected.

Lung function tests

No significant changes between subway and control exposures were observed in regard to VC, FVC, FEV_1, exhaled NO or PEF. For additional data, see appendix 2.

Peripheral blood

The total number of cells, cell concentrations or distribution of different cell populations in blood did not differ when comparing control and subway exposures. Fibrinogen levels in the peripheral blood increased from 2.2 (interquartile range 2.00-2.38) g/l after control exposure to 2.3 (2.21-2.58) g/l after the subway exposure (p =0.026). Levels of PAI-1 were often below the detection limit of <5 kilounits/l (14 volunteers after the subway exposure respectively 13 after the control). In order to perform statistical analysis, these cases were assigned a value of 3. No significant changes were found in the levels for the PAI-1 (appendix 2).

Brochoalveolar lavage fluid and bronchial wash

No significant changes were seen in BALF or bronchial wash fluid between subway and control with regard to recovery, cell viability, the total number of cells, cell concentration or distribution of different cell populations. Neither were any significant

changes seen in cytokines expression in the BALF. For additional data, see appendix 2.

Immunostaining and flow cytometric analysis

The T-cell (CD4)/T cytotoxic-suppressor (CD8) ratio in BALF and blood was similar when compared after control respectively subway exposure. In BALF the median was 2.2 (control) versus 2.0 (subway), and in blood 2.0 (control) versus 2.2 (subway). NK-T-cells ($CD3^{pos}CD56^{pos}CD16^{pos}$) remain unchanged. Subway exposure had a tendency to increase NK-cells ($CD3^{neg}CD56^{pos}CD16^{pos}$) in blood ($p<0.09$), but not in BALF, of the volunteers.

In blood the % frequency of CD4 cells in blood expressing T-cell activation marker CD69, synonym to $CD4^{pos}/CD69^{pos}$, also increased significantly after exposure in the subway ($p<0.003$). The median changed from 2.9 (2.3-3.3) after control to 3.5 (3.0-4.0) after subway exposure. The same was for $CD4^{pos}$/HLA-DR^{pos} that increased ($p<0.03$) from 6.5 (5.8-8.0) to 7.0 (6.3-8.3). A tendency of increased ratio of $CD4^{pos}/CD25^{pos}$ ($p<0.07$) was observed. The $CD8^{pos}/CD69^{pos}$ increased significantly after exposure in the subway ($p<0.01$), from 2.9 (2.2-3.7) after control to 3.8 (2.5-5.5) after subway exposure. In BALF no such expressions were seen.

The frequency of CD4 cells expressing the T regulatory marker FOXP3 or $CD25^{bright}$/FOXP3, increased significantly in blood after subway exposure ($p<0.03$ for both), which is illustrated in figure 2a and 2b. No such difference was seen in BALF after subway exposure.

Discussion

We have found that a two hours long exposure in the subway environment leads to significantly increased expression of markers for regulatory T (Treg) cells as well as elevated levels of fibrinogen in peripheral blood in healthy volunteers. The subway environment also seemed to induce irritative symptoms in the lower airways.

In our earlier *in vivo* study we have shown that exposure to particles derived from city traffic increased amount of inflammatory cells in the BALF of healthy individuals [9]. Since the exposure levels regarding gravimetric parameters like $PM_{2.5}$ and PM_{10} are in the same levels when comparing the two studies, one could expect that the inflammatory effects observed in the road tunnel study, would also be seen after exposure in the subway environment. However, no signs of cellular inflammatory response in the lower airways assessed by bronchoscopy were found after exposure to the subway environment, regardless if cells were retrieved from peripheral (BALF) or central (bronchial wash) airways. NO_2 levels need to be taken into account when comparing the subway and road tunnel environment, as NO_2 is suggested to be a marker for air pollution. NO_2 is not present to any higher extent in the subway environment. WHO states that healthy humans rarely are influenced (lung function and inflammation) by short term NO_2 exposure levels such as in the subway environment (< 1ppm = 1.880 $\mu g/m^3$) [19].

Analysis of metal content in PM_{10} from the subway station show high metal content, mainly iron, which derives from wear of wheels, rails and brakes. Another important difference between the two environments is that the number concentration of UFP was ten times higher in the road tunnel. There were also high levels of NO_x in the road tunnel, mainly from exhaust fumes, which are not present to a major extent in the subway. These differences in exposure might perhaps explain the discrepancies in inflammatory events between the two studies.

Epidemiological studies show that the risk of cancer, respiratory and cardiovascular disease can be related to exposure to combustion products [1, 2]. The associations are difficult to explain and the mechanisms are not fully understood, but particles generated for example by combustion are suspected to influence the heart by inflammation that leads to increased coagulation [20]. On the other hand, subway employees and other professional drivers do not show any increased relative risk for myocardial infarction [21]. The exposure levels have not been very high and the cohorts under study have been limited in numbers.

In two in vitro studies the cytokine release (IL-6, IL-8 and TNF-α) from human macrophages was stimulated by particles derived from a subway station and a traffic intensive urban street. Street particles were the most potent stimulators. [22, 23] Similar results regarding cytokine release were shown in an *in vitro* animal study using a murine macrophage like cell line [18]. Induction of lipid peroxidation, arachidonic acid release and formation of ROS (reactive oxygen species) were however, stronger for subway particles than urban street particles. In an *in vivo* study

C57Bl/6 mice were exposed to different type of particles (100μg), like subway PM_{10} particles and diesel exhaust particles (DEP) by intratracheal administration. Signs of inflammatory response were observed in bronchoalveolar lavage fluid at 8 hours after exposure, with increased number of neutrophils (subway and DEP) as well as inflammatory mediators TNF-α and MIP-2 (macrophage inflammatory protein) (subway). [24] The discrepancies with our results could be explained by the exposure methods used, intratracheal administration versus normal inhalation of larger particles, which targets different lung compartments.

There are to our knowledge only one study performed that has investigated acute health effects in humans caused by exposure in the 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 non-smoking, healthy train drivers and subway ticket sellers [16]. In our study the plasma levels of fibrinogen also increased after the subway exposure. No such effect was seen in the road tunnel study. The increases is however very modest and within normal range. The findings are supported by a study in London with more than six thousand office workers involved. Increased levels of urban PM_{10} correlated with increased levels of plasma fibrinogen [25]. A prospective longitudinal study of over one thousand survivors from myocardial infarction showed similar results. Here also a correlation between particle number concentration and IL-6 levels in blood of the survivors was found. [26] In our study we did not detect any significant release of inflammatory cytokines after the subway exposure.

Blood CD4 cells showed a small but statistically significant increased expression of activation markers CD69 and HLA-DR after the subway exposure, and similar results applied to CD8 cells regarding the activation marker CD69. Such small increased levels of CD69 and HLA-DR in blood cells might not have been detected in the BALF compartment because they already express high levels of activation markers, or because the time point of analysis might be inadequate.

Regulatory T cells are important regulators of immune tolerance. In order to delineate T cells with regulatory functions, we analysed the CD4 cell expression of FOXP3 alone or in combination with $CD25^{bright}$, which were the markers available for the time being. We found an up-regulation in peripheral blood but not in BALF after subway exposure. It would have been interesting to analyse the expression of CD127 as an additional Treg marker. However, we were unable to add this marker at the time of our study [27]. Subway particles may generate oxidative stress [22], but we can not exclude that psychological stress itself may affect our results. Psychological stress may increase the proportion of T regulatory cells, which was shown in a previous study where a group of students were followed during an exam respectively a control situation [28]. No differences were observed in the NK and NK-T-cell populations in blood or BALF after subway exposure, while in our previous road tunnel study both decreased in BALF after exposure.

The relative young study population (only four volunteers were older than 30) could perhaps have influenced the results. However, we have previously been able to demonstrate a cellular response after road tunnel exposure in a study population with similar age and sex distribution as in the present one. Every subject also served as

their own control, which also strengthens the findings of this study. Choice of sampling time could also have affected the outcome, but on the other hand, we found positive results in the road tunnel study with the same sampling timepoint (14 hours post-exposure).

The results from our study show that a two hours long exposure of healthy individuals to subway environment leads to an up-regulation of T-cells with a phenotype compatible with T regulatory functions, and a limited increase of fibrinogen levels in blood. 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. The function role of the T regulatory cells is not quite clear. Whether this imposes a real health risk to the population, is still too early to conclude. Further studies are needed to evaluate these effects.

It is clear that even if exposure environments are similar regarding mass concentration like $PM_{2.5}$ and PM_{10}, a risk assessment cannot be based solely on this exposure information since the particle characteristics can differ substantially regarding size and composition. More complex measurements of particles, which include number concentration levels of UF particles as well as knowledge of the source of particles, are needed.

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Table 1. Basal characteristics for the twenty healthy volunteers.

Volunteer no	Age	Phadiatop positive	Sex	VC (pred*%)	FVC (pred*%)	FEV_1 (pred*%)
1	26	-	M	87	104	105
2	28	-	F	125	120	104
3	27	-	F	135	121	120
4	18	+	M	108	107	121
5	22	-	M	106	112	88
6	25	-	M	105	108	106
7	42	-	M	110	115	111
8	31	-	F	100	91	88
9	25	-	M	88	87	86
10	25	-	F	110	104	105
11	18	-	M	120	134	115
12	18	-	M	120	124	125
13	25	-	F	107	106	102
14	36	-	F	142	140	124
15	25	-	M	101	112	115
16	26	-	M	117	123	116
17	29	-	F	105	101	91
18	26	+	M	115	129	115
19	27	-	M	120	124	114
20	46	-	M	118	116	120
Summary	**Mean age 27 years**	**2 Phadiatop positive**	**7 female**	**Median 112 ± 14**	**Median 114 ± 13**	**Median 109 ± 12**

* pred = predicted

Figure 1. Study design; randomised cross-over experimental design with exposure to a subway environment and an office environment (control).

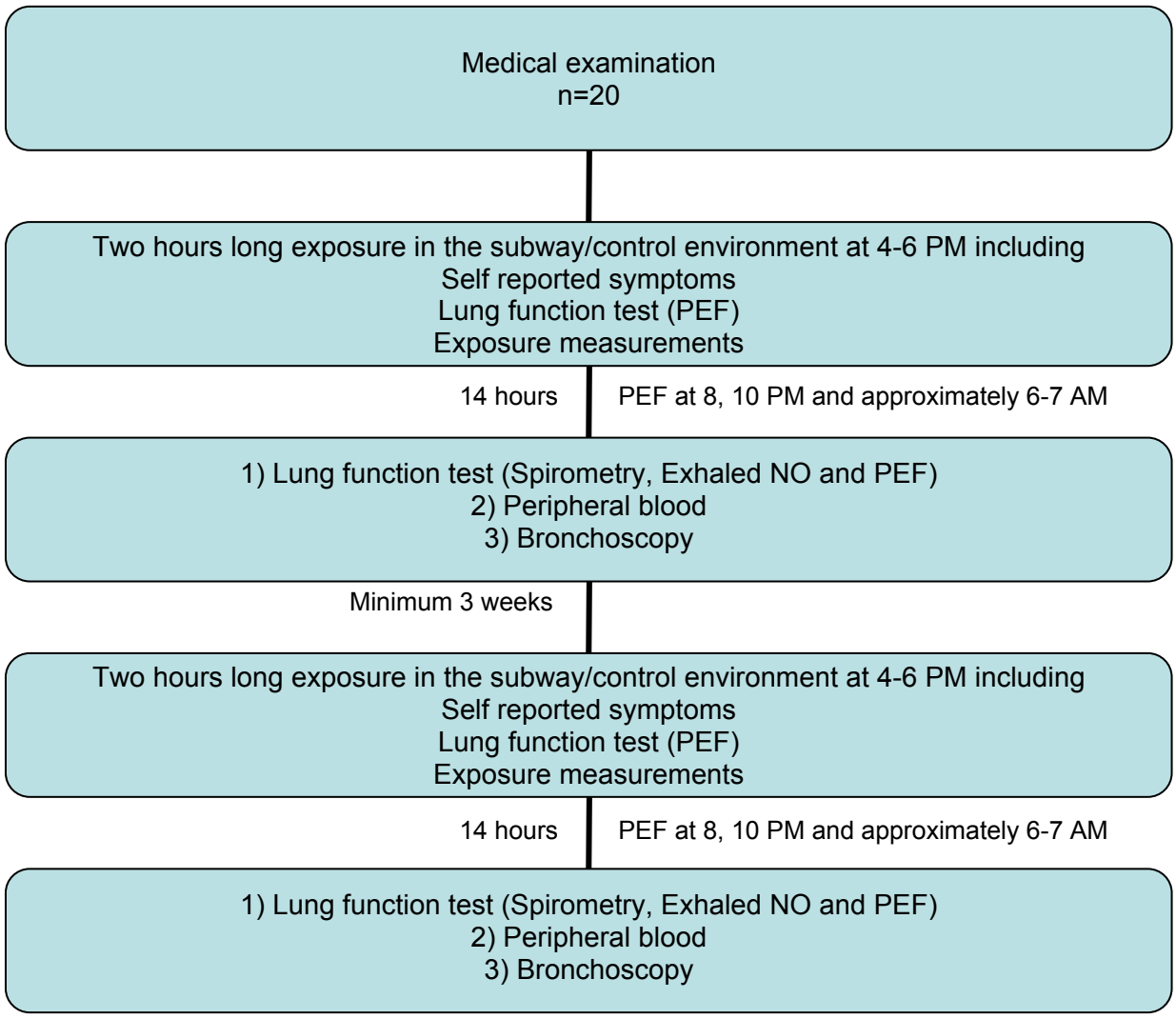

Table 2. Median values for environmental exposure measurements in the subway in comparison to levels in a previously investigated road tunnel in the same city and during the same seasons. (Larsson, 2007)

Type of exposure	**Subway environment**	**Road tunnel environment**
Ultra fine particles (number concentration)	8 266/ml	110 000 /ml
$PM_{2.5}$ (mass concentration)	76 µg/m^3	64 µg/m^3
PM_{10} (mass concentration)	237 µg/m^3	176 µg/m^3
NO	59 µg/m^3	874 µg/m^3
NO_2	24 µg/m^3	230 µg/m^3

Appendix 1. Three antibody combination panels were used for determination of lymphocyte subsets in bronchoalveolar lavage fluid and blood.[1]

Antibody combination panel 1: **TBNK multitest**	**Antibody combination panel 2:** **Activation markers**	**Antibody combination panel 3:** **T regulatory cell markers**
CD3	CD3	CD3
CD4	CD4	CD4
CD8	CD8	CD8
CD45	CD69	CD25
$CD56^{pos}CD16^{pos}$	HLA-DR	CD69
CD19	-	FOXP3

TBNK: T-, B- and NK-cells; **CD3**: T-cell marker; **CD4**: T-helper/inducer; **CD8**: T-cytotoxic/suppressor; **CD25**: Anti-IL-R-activation; **CD45**: white blood cells (leucocytes); **CD69:** early activation; **$CD56^{pos}CD16^{pos}$**: NK-cells or NK-T cells; **CD19**: B-cells; **HLA-DR**: MHC II activation; **Forkhead box P3 (FOXP3)**: T regulatory cell, preferentially $CD4^{pos}CD25^{bright}$

[1] All monoclonal antibodies used for phenotypic characterization of T-cells and T-cell activation are from BD Bioscience, apart from FOXP3 that was purchased from eBioscience.

Appendix 2. Comparisons between outcome parameters from control and subway exposure in lung function is presented as mean ± SD. Data for cellular response in blood, and for bronchoalveolar lavage and bronchial wash findings, as well as for PAI-1 in blood, are presented as medians (interquartile ranges).

Outcome parameter	**Control exposure**	**Subway exposure**
Lung function		
VC (predicted %)	109 ± 14	111 ± 13
FVC (predicted %)	112 ± 13	112 ± 14
FEV_1 (predicted %)	105 ± 12	105 ± 14
Exhaled NO (ppb)	18 ± 8	17 ± 9
PEF total after exposure (l/min)	537 ± 138	522 ± 113
PEF quotient (after exposure/before exposure)	1.0	0.96
Blood cell concentration (10^9/l)		
Leukocytes	5.7 (5.1-6.7)	5.6 (5.2-6.5)
Neutrophils	2.8 (2.6-4.0)	2.9 (2.5-3.6)
Eosinophils	0.1 (0.1-0.2)	0.1 (0.1-0.2)
Basophils	0.1 (0.1-0.1)	0.1 (0.1-0.1)

Lymphocytes	2.4 (2.0-2.6)	2.4 (1.9-2.7)
Monocytes	0.4 (0.3-0.4)	0.4 (0.3-0.4)
Blood differential (%)		
Neutrophils	50.8 (46.5-54.8)	51.7 (45.8-54.9)
Eosinophils	2.0 (1.8-3.0)	2.0 (1.8-3.4)
Basophils	1.8 (1.5-2.0)	1.8 (1.6-1.9)
Lymphocytes	40.2 (35.1-43.9)	40.5 (35.9-45.2)
Monocytes	5.9 (5.3-7.0)	6.1 (5.0-7.1)
Bronchoalveolar lavage (BAL) findings		
Recovery (%)	74.0 (71.2-78.2)	70.7 (64.5-75.4)
Viability (%)	95.3 (91.3-98.1)	95.5 (94.3-97.7)
Total number of cells (*10^6)	14.3 (12.3-17.8)	12.7 (10.1-17.0)
Total cell concentration (*10^6/l)	79.5 (67.8-98.9)	75.1 (59.2-96.2)
Number of mast cells[2]	1.5 (0.0-5.0)	1.5 (1.0-4.0)

[2] Enlarged 16 times/10 visual field

Cell concentrations in BAL fluid (10^6/l)		
Macrophages	66.8 (56.7-84.9)	60.4 (50.1-86.1)
Lymphocytes	8.8 (5.6-17.2)	8.2 (6.0-13.5)
Neutrophils	1.4 (0.9-1.9)	1.7 (1.1-2.4)
Eosinophils	0.1 (0.0-0.4)	0.1 (0.0-0.5)
Basophils	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Cell differential in BAL fluid (%)		
Macrophages	85.9 (80.4-89.4)	85.3 (81.9-88.3)
Lymphocytes	11.3 (8.3-17.9)	11.4 (8.2-15.9)
Neutrophils	1.9 (1.2-2.2)	2.2 (1.4-3.0)
Eosinophils	0.2 (0.0-0.4)	0.2 (0.0-0.6)
Basophils	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Bronchial wash (BW) findings		
Recovery (%)	40.0 (37.0-50.0)	40.0 (35.0-50.0)
Viability (%)	82.0 (67.0-94.0)	75.0 (58.0-86.0)
Total number of cells (*10^6)	0.4 (0.2-0.8)	0.7 (0.3-1.0)
Total cell concentration (*10^6/l)	52.5 (30.0-99.1)	85.0 (36.0-133.0)

Number of mast cells[3]	2.0 (0.0-4.5)	2.0 (0.0-3.0)
Cell concentrations in BW fluid (10^6/l)		
Macrophages	51.9 (51.7-52.2)	57.8 (57.8-57.8)
Lymphocytes	2.2 (2.1-2.3)	6.9 (6.9-6.9)
Neutrophils	14.7 (3.3-26.0)	5.2 (5.2-5.2)
Eosinophils	0.1 (0.1-1.2)	0.1 (0.1-0.1)
Basophils	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Cell differential in BW fluid (%)		
Macrophages	64.6 (56.8-73.0)	55.8 (41.3-76.8)
Lymphocytes	5.6 (3.5-8.2)	5.7 (3.6-10.0)
Neutrophils	28.0 (18.2-34.5)	38.3 (11.4-54.0)
Eosinophils	0.2 (0.0-0.5)	0.3 (0.0-0.7)
Basophils	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Cytokine concentrations in BAL fluid (pg/l)		
IL-1B	79.8 (57.8-165.9)	64.9 (53.1-139.8)
IL-6	394.9 (263.2-963.0)	391.9 (272.0-686.4)

[3] Enlarged 16 times/10 visual field

IL-8	8569.5 (4646.8-19802.3)	4901.0 (3696.0-14260.0)
IL-10	15.2 (5.5-21.2)	9.6 (6.3-17.3)
1L-12p70	14.8 (10.6-24.0)	13.7 (8.0-22.1)
TNF-α	8.9 (1.0-58.6)	8.2 (0.0-49.0)
PAI-1 in blood (kilounits/l)		
PAI-1	3 (3-11)	3 (3-11)

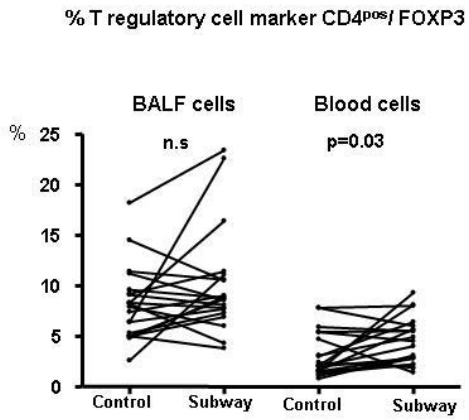

Figure 2a. The figure shows the frequency of CD4cells expressing the T regulatory marker FOXP3 in bronchoalveolar lavage fluid (BALF) and peripheral blood after control respectively subway exposure. In blood the $CD4^{pos}$/FOXP3 ratio increased from 2.1 (1.5-5.4) after control to 4.3 (2.5-5.9) after subway exposure. In BALF the increased ratio from 8.1 (5.2-9.4) to 8.8 (7.5-10.9) was non-significant (n.s.).

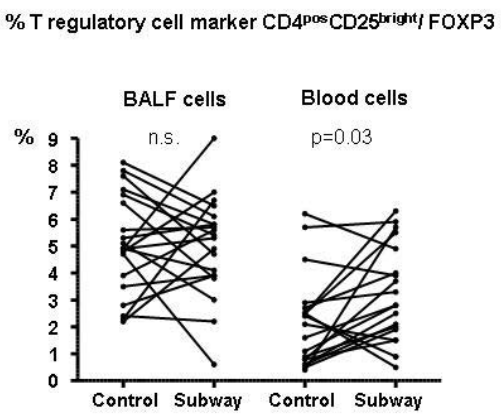

Figure 2b. The figure shows frequency of CD4 cells expressing the T regulatory marker $CD25^{bright}$/FOXP3 cells from bronchoalveolar lavage fluid (BALF) and peripheral blood after control respectively subway exposure. In blood the $CD4^{pos}CD25^{bright}$/FOXP3 ratio increased from 2.1 (0.8-2.7) after control to 2.8 (1.7-4.5) after subway exposure. In BALF the increased ratio from 4.9 (3.7-6.8) to 5.4 (3.9-6.0) was non-significant (n.s.).