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Surfactant protein-D and exposure to bioaerosols in wastewater and garbage workers

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ABSTRACT

Purpose Bioaerosols and their constituents, such as endotoxins, are capable of causing an inflammatory reaction at the level of the lung-blood barrier, which becomes more permeable. Thus, it was hypothesized that occupational exposure to bioaerosols can increase leakage of surfactant protein-D (SP-D), a lung specific-protein, into the bloodstream.

Methods SP-D was determined by ELISA in 316 wastewater workers, 67 garbage collectors and 395 control subjects. Exposure was assessed with four interview-based indicators and by preliminary endotoxin measurements using the Limulus amoebocyte lysate assay. Influence of exposure on serum SP-D was assessed by multiple linear regression considering smoking, glomerular function, lung diseases, obesity, and other confounders.

Results Overall, mean exposure levels to endotoxins were below 100 EU/m^3. However, special tasks of wastewater workers caused higher endotoxin exposure. SP-D concentration was slightly increased in this occupational group and associated with the occurrence of splashes and contact to raw sewage. No effect was found in garbage collectors. Smoking increased serum SP-D. No clinically relevant correlation between spirometry results and SP-D concentrations appeared.

Conclusions These results support the hypothesis that inhalation of bioaerosols, even at low concentrations, has a subclinical effect

on the lung-blood barrier, the permeability of which increases without associated spirometric changes.

Keywords: endotoxin; garbage; sewage; smoking; surfactant protein-D; wastewater

Introduction

Lung-specific proteins (Hermans and Bernard 1999) are synthesized predominantly in the airways and/or lung and comprise, among others, surfactant protein (SP)-A, SP-B, SP-C, SP-D and CC16. SP-D and SP-A are hydrophilic collectins with a structure and function distinct from those of SP-B and SP-C. They probably play a role in the regulation of innate immunity and are assumed to play a role keeping the lung in an uninflamed state despite daily exposure to microbes and their derivatives (Haczku 2008, Yamazoe et al. 2008, Kuroki et al. 2007).

After inhalation bioaerosols can bring about organic dust toxic syndrome (ODTS), a toxic alveolitis (Radon 2006, Rylander 2002). Cell wall components of bacteria (peptidoglycans, lipopolysaccharide and lipoteichoic acid) are important causes of this disease (Hoogerwerf et al. 2008, Poole et al. 2008, Ikegami et al. 2007) characterized by a flu-like syndrome and a pulmonary interstitial inflammation affecting the lung-blood barrier with reduced diffusing capacity and increased permeability to plasma proteins (Maris et al. 2005, George et al. 2003, O'Grady et al. 2001, Jagielo et al. 1996; Herbert et al. 1992). It has been suggested that the inflammation-induced increased permeability would be bidirectional. Besides a leak of plasma proteins into the epithelial lining fluid a leak of lung-specific proteins into the blood would also occur (Hermans and Bernard 1999). As a consequence, the plasma concentration of lung-specific proteins should increase as found for the Clara cell protein (CC16) after experimental inhalation of lipopolysaccharide (LPS) in volunteers

(Michel et al. 2005). Moreover, studies have shown that low lipopolysaccharide or lipoteichoic acid concentrations are capable of causing a subclinical inflammatory reaction (Hoogerwerf et al. 2008, Michel et al. 1997). Thus, lung-specific proteins leakage may be a sensitive and early sign of exposure to bioaerosols containing microbes and their toxins.

Bioaerosols have not only inflammatory but also toxic and irritative effects (respiratory symptoms and reduced pulmonary function) (Radon 2006, Rylander 2002). Thus, chronic exposure to bioaerosols could damage lung protein epithelial secretory cells, reducing their number and/or integrity. The decreased protein synthesis would then be reflected in less lung-specific protein leaking into the blood and decreased serum concentrations. Although this hypothesis has been examined for CC16 and silica in humans (Bernard et al. 1994), it has been examined for bioaerosols only in rats (Arsalane et al. 2000).

Therefore, the aim of this study was to examine the effect of exposure to organic dust on serum SP-D concentration in a population of garbage and sewage workers occupationally exposed to bioaerosols.

Subjects and methods

The study took place in the frame of a mandatory risk assessment of the workplace requested by Swiss law. The study protocol was approved by the Swiss National Accident Insurance Fund. All workers were informed about the purposes of the risk assessment and gave written consent. A detailed description of population and methods has been given previously with the results of the SP-A, SP-B and CC16 measurements (Steiner et al. 2005, Widmeier et al. 2007), so only the main aspects will be briefly summarized here.

Subjects

Eligible were municipal manual workers from the Canton of Zurich. All workers exposed to garbage dust from the two largest cities and all workers exposed to wastewater in the Canton of Zurich had the opportunity to participate, whereas the groups of control subjects were approached one by one and asked for participation, until enough control subjects had entered the study..

Workers from the garbage collecting units (n = 86; 28 % participation rate) and workers from wastewater plants (n = 355; 90 % participation rate) were compared to control subjects from other administrative “units”: garden work (n = 197; 76% participation rate), waterway maintenance (n = 52; 79% participation rate), public transport maintenance (n = 25; 15% participation rate), and forestry work (n = 63; 93% participation rate). Overall, 778 subjects entered the study (61% participation rate). The participation of garbage workers remained low in one

plant although they were given the opportunity three times to participate in the study. Because of data protection it was not possible to gain an insight into the reasons for not participating. As exposure indicator, individual lifelong occupational history was substituted for current plant or administrative “unit” to avoid exposure misclassifications.

The tasks of garbage collectors, wastewater workers, and control subjects have been described previously (Steiner et al. 2005). In wastewater workers, specific tasks suspected to represent peak exposures were spray removal from basins, tank walls, grids, or rakes.

Methods

Each physician examined both exposed and unexposed subjects. The coding of the answers was always reviewed by the study coordinator and divergences resolved by consensus. Respiratory symptoms (appendix) were assessed using questions, instructions and definitions from the SAPALDIA study (Zemp et al. 1999, Leuenberger et al. 1998, Ackermann-Liebrich et al. 1991). Smoking was assessed by using questions from the questionnaire of the European Community of Steel and Coal (revision 1967). Socioeconomic level was defined by the highest education level attained at age 20 (three levels: no apprenticeship / apprenticeship / university). Exposure to sewage or garbage during the whole working life was assessed for each job separately using a structured interview. In wastewater workers only, suitable masks were defined as personal protective equipment worn for at least 50 % of the working time, adequate for work with wastewater and

maintained correctly. Unexpectedly, in the course of the study occupational histories showed that former or current (beside main job) farming jobs were not unusual. This was considered using the surrogate “ever exposed in farming” using definitions from the Swiss census (codes 111.01 and 111.02) (Meier 1996).

Spirometry was carried out with Microlab spirometers (MicroLab ML 3300 and 3500; Micro Medical Ltd., Kent, England) according to ATS criteria (American Thoracic Society 1994). Predicted values and 5th percentiles of forced vital capacity (FVC) and forced expiratory volume in the 1st second (FEV1) were calculated according to Quanjer et al (Quanjer et al. 1993).

A detailed examination of exposure to bioaerosols and endotoxins was carried out in wastewater workers because some clinical histories suggested a higher exposure to endotoxin in this group (Oppliger et al. 2005). Further measurements were then collected in garbage collectors (2005), in gardeners and forestry workers (2006 and 2007) to confirm the lower exposure suggested by clinical history. No measurements were carried out in workers maintaining waterways, whose jobs were similar to those of gardeners and forestry workers, or in the small group of public transport workers. The filtration method was used to assess the airborne concentration of endotoxins. Thus, sample of air were collected with the aid of pocket pumps (MSA Escort Elf, Mine Safety Appliance Company, Pittsburgh, USA or SKC pocket pump 210-1002, SKC Inc., USA) at a flow rate of 2 L/min, and loaded onto polycarbonate filter in a ready to use polystyrene cassette (endofree cassette, Aerotech Laboratories Inc., Phoenix, USA).

Airflow was calibrated before and after field sampling. The air was sampled continuously for the duration of shift or of special tasks, as appropriate. All filter samples were transported in their cassettes in a cold box to the laboratory within three hours where they were stored at -20°C for 1-3 months to await endotoxin measurement. Endotoxins were extracted by shaking the filters at room temperature for one hour in 10 ml of pyrogen-free water in a 50 ml conical polypropylene tube. The filter extracted solutions were vortexed vigorously prior to drawing a sample which was analysed for endotoxin content using a quantitative kinetic chromogenic Limulus Amoebocyte Lysate (LAL) assay (Kinetic-QCL endotoxin kit, BioWhittaker, Cambrex Bio Sciences Verviers, Belgium) at 37°C with an automated microtiter plate reader. *Escherichia coli* O55:B5 endotoxin was used as a calibration standard to calculate endotoxin concentration in the experimental samples. Results were expressed in units of endotoxin (EU) per cubic meter of air. Concentrations were always determined in the same laboratory using the same kinetic LAL assay.

As the study was planned, technical reasons precluded determinations of SP-D, and measurements of lung-specific proteins were restricted to SP-A, SP-B and CC16. Later SP-D determinations became available and this determination was added to get a comprehensive picture of the effect of bioaerosols on lung-specific proteins. SP-D determinations were carried out between June to September 2008 and January 2009 to March 2009 on serum samples frozen at - 20°C and already used once for the determination of another lung-specific protein. Determinations were performed by ELISA (BioVendor, Modrice,

Czech Republic) according to the manufacturer's instructions and quality controls. SP-D measurements were carried out in batches comprising samples from exposed and control workers. The laboratory knew neither exposure status nor clinical history.

Determinations of serum creatinine (S-creatinine), CC16, SP-B and SPA have been described elsewhere (Steiner et al. 2005, Widmeier et al. 2007).

Data analyses

The normality of the distribution was tested and, if necessary, logarithmic transformations done or non parametric tests used However, to be consistent with previous results (Oppliger et al. 2005) endotoxin concentrations are given as mean and range. The linear multiple regression models are those already used for SP-A, SP-B and CC16 (Steiner et al. 2005). Independent variables were age (years), gender (0: male; 1: female), height (metres), pack-years, time elapsed since smoking cessation (years), currently asthma and/or symptoms of bronchitis (0: absent, 1: present), BMI (kg/m^2), S-creatinine ($\mu mol/ l$), and exposure. Exposure category was defined as non exposed / exposed (coded 0/1) to garbage or sewage aerosols. Exposure duration (years) was calculated for all consecutive exposed jobs as a sewage or garbage worker over the whole life. Occurrence of splashes was categorized as never exposed to sewage, never more than 20 splashes of raw sewage in any job, or at least one job with more than 20 splashes (codes 0, 1 and 2, respectively). As for exposure to raw sewage categories were never exposed, exposure 1-5 times monthly or more than 5 times monthly in at least one job (codes 0, 1, and 2,

respectively). For analyses restricted to wastewater workers, none of the subjects had a code of 0 for contact with raw sewage or splashes. Therefore, the codes 1 and 2 were recoded as 0 and 1, respectively. A surrogate for use of personal protective equipment was wearing a mask (0: yes / 1: no). This variable was available in wastewater workers only. All classifications were laid down without knowing the results. To test for the development of tolerance (Rylander 2002), the number of days at work since the last day on leave was included in additional analyses (codes 0 for > 5 days to 5 for 0-1 day). Collinearity and residuals were examined and logarithmic transformation done if appropriate. All calculations were done with SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC, USA).

Results

Three subgroups were constituted according to current exposure to bioaerosols (table 1). Whenever exposure defined by the plant differed from individual occupational history, misclassifications were corrected. The 26 workers with only former exposure were not exposed any longer and, therefore, were included in the control group. In these workers, time elapsed since last exposure to wastewater or garbage was fairly long (25^{th} percentile, median and upper limit of range: 3, 11, and 31 years, respectively). All 19 drivers of compactor trucks (four with previous exposure to garbage dust), who did not load garbage were considered as presently not exposed. Sixteen subjects currently exposed to both garbage and wastewater had nearly the same duration of exposure to wastewater (median 11; percentile 5^{th} – 95^{th}: 0.5 – 21.0 years) and garbage (median 11; percentile 5^{th} – 95^{th}: 0.5 – 22.5 years). They were assigned to the group of garbage collectors.

Preliminary measurements of endotoxin showed the highest concentrations in wastewater workers during special tasks (table 2). The lowest concentrations were found in drivers of compactor trucks (without loading garbage) and for control tasks/office work. In forestry workers, the upper range of endotoxin concentrations reached about 350 EU/m^3. However, this was found in summer only and mainly due to four outliers (endotoxin concentrations > 200 EU/m^3) carrying out unexpected tasks (three workers cutting grass at the roadside and one worker cleaning out a pipe containing sludge). After excluding these four cases mean

concentration decreased from 111.4 to 57.8 EU/m^3 (range 20-141). Importantly, a history of clinical symptoms supporting an excessive endotoxin concentration had been found in wastewater workers only (Steiner et al. 2005, Jeggli et al. 2004).

In the whole population, median SP-D concentration was 74.4 ng/ml (5^{th} and 95^{th} percentile: 29.8-178.6). In the highly selected subgroup of never smokers without cough, expectoration, dyspnoea, wheezing, any asthma, history of lung disease, and with measured FVC, FEV1, and FEV1/FVC ≥ 5^{th} percentile and normal S-creatinine (< 141 μmol/l), SP-D concentrations changed very little (median: 71.4 ng/ml; 5^{th} and 95^{th} percentile: 29.2-149.5; n = 168). No statistically significant correlation with age ($p > 0.9$) and no statistically significant association with current (n = 19) or ever asthma (n = 59), symptoms of bronchitis (n = 100), or clinical history of pneumonia (n = 27) on SP-D was found ($p > 0.10$). The correlations with FVC and FEV1 (percent predicted and litre or litre/sec) were rather low ($0.03 < \text{Spearman } \rho < 0.12$). In contrast, the pattern of SP-D concentrations (table 3) suggested an effect of current smoking and possibly of exposure to aerosols. However, these associations might have been confounded by correlations between SP-D and BMI (Spearman $\rho = -0.09$; $p = 0.01$; n=732) or serum creatinine (Spearman $\rho = 0.06$; $p = 0.09$; n=728). The prevalence of

Table 1 Characteristics of the study population

Characteristic	Control workers (n = 395)	Wastewater workers (n=316)	Garbage workers (n=67)
Age (years)	42 (22-59)	46 (30-60)	43 (27-57)
Gender (male)	367 (93)	315 (99)	66 (99)
Education level			
Low	74 (19)	39 (12)	30 (45)
Middle	302 (77)	274 (87)	37 (55)
High	15 (4)	3 (1)	0 (0)
Nationality			
Swiss	319 (81)	286 (91)	39 (58)
Other countries	76 (19)	30 (9)	28 (42)
Smoking			
Never smoker	169 (43)	97 (31)	11 (17)
Ex-smoker	89 (23)	95 (30)	21 (32)
Current smoker	136 (35)	124 (39)	34 (51)
Pack-years (in smokers only)	16 (1-66)	20 (1-64)	20 (1-60)
Time since giving up smoking (years)	10 (0-35)	15 (1-32)	6 (1-30)
Height (m)	1,75 (1,63-1,86)	1,75 (1,65-1,86)	1,72 (1,63-1,86)
Weight (kg)	78 (60-103)	81 (65-110)	80 (63-102)
BMI (kg/m²)	25.3 (20.7-32.1)	26.5 (21.6-34.3)	26.3 (20.9-32.9)
Respiratory symptoms or diseases			
Symptoms of bronchitis	64 (16)	30 (10)	12 (18)
Ever asthma	40 (10)	22 (7)	5 (7)
Current asthma	14 (4)	7 (2)	1 (1)
Duration of exposure (years)			
Sewage	0	11 (1-28)	0 (0-16)
Garbage	0	0	10 (0.5-21)
Wearing a mask (yes)	NA	85 (27)	NA
Farming exposure	67 (17)	29 (9)	13 (19)
Job change prior to current job because of any health problem	34 (9)	37 (12)	6 (9)

Values are median (5^{th} to 95^{th} percentile) or number (percent). BMI: body mass index; NA: not available.
Pack-years and time since giving up smoking are calculated in the group of ever cigarette smokers or ex-smokers only, respectively. Definitions of respiratory symptoms and asthma: see under methods. Exposure duration (years) was calculated in currently exposed workers for all successive exposed jobs as sewage or garbage worker over the whole life. Sixteen garbage collectors were also exposed to sewage (details in text).

Table 2 Preliminary assessment of exposure to endotoxins (EU/m^3)

	Wastewater	Garbage	Gardeners	Forestry
Winter				
Indoors	29.7 (1.7-81) (11) [a]	NA	NA	32.2 (18.8 – 48.3) (5)
Driving		3.4 (0.7-13.8) (16)		
Outdoors	8.8 (1.4-29.0) (11) [a]	8.1 (2.5-15.7) (17)	11.3 (3.3-21.6) (16)	32.7 (23.5-48.7) (12)
Summer				
Indoors/ Driving	52.6 (7.1-158) (11) [a]	NA 3.6 (1.0-12.6) (17) [c]	NA	NA
Outdoors	29.8 (2.3-103) (11) [a]	11.0 (7.5-26.3) (18)	13.8 (2.1-51.2) (17)	57.8 (20-141) (12) [e]
Special tasks	98.6 (1.4-497) (15) [b]	8.0 (2.0-16.9) (3) [d]	NA	See text
Control tasks	7.3 (0.4-21.4) (9) [b]	See under driving	NA	NA
Office work	0.9 (0.1-1.7) (2)		5.1 (2.7-8.4) (4) (winter) 4.8 (4.0-5.6) (2) (summer)	NA

Values are mean, range, and number of subjects; NA: not applicable or not available (in some groups no mere control task was found); all measurements were made through personal sampling (295 -530 min according to shift duration), unless explicitly stated otherwise.
Indoors/Driving: indoor tasks for wastewater workers or only driving of compactor trucks for garbage workers (sitting in the driver's cab; no garbage loading), respectively. In forestry workers, indoors is work in repair shop.
[a] stationary sampling, 4 h
[b] personal sampling, 22 – 170 min. (special tasks) or 4 h (control tasks)
[c] one outlier excluded (89.4 EU/m^3)
[d] Slitting open the garbage bags for control of contents ("garbage police")
[e] After excluding four outliers (see text). With outliers: 111.4 (18.0-346) (16).

Table 3 SP-D concentrations (ng/ml)

	Control workers	Wastewater workers	Garbage workers	p
Never smokers	68.0 (27.5-155.9) n=161	77.0 (39.0-157.0) n=94	90.6 (18.6-167.6) n=11	0.2
Ex-smokers	67.8 (29.9-167.1) n=81	67.8 (25.7-140.5) n=87	68.0 (32.3-152.7) n=20	1.0
Current smokers	81.3 (26.8-159.8) n=127	89.6 (40.0-236.3) n=119	86.9 (36.5-186.1) n=33	0.1

Figures are median, 5^{th} and 95^{th} percentiles, and subgroup size (as some SP-D determinations are missing total is less than 778); p: level of significance of the differences between occupational subgroups in the same smoking category (Kruskal-Wallis test).

FVC, FEV1 and FEV1/FVC under the 5th percentile was low and similar in all three exposure groups ($p > 0.1$). The difference in serum SP-D between groups with values above and below the 5th percentile was at most of borderline significance ($0.03 < p < 1.0$).

In multiple linear regression, smoking and exposure to wastewater bioaerosols increased serum SP-D, whereas time since quitting smoking decreased it (table 4). Using the same models, similar results were found for exposure to raw sewage (partial regression coefficient: 0.02, $p = 0.01$). In table 5, two different regression models illustrate the effects of several determinants of SP-D in wastewater workers only. The effect of the number of days at work since last day on leave did not point to a tolerance effect. Not wearing a mask increased serum SP-D. In all models the distribution of the residuals was good.

SP-D correlated weakly or not at all with serum CC16, SP-A, and SP-B concentrations ($0.005 <$ Spearman $\rho < 0.08$; $0.05 < p < 0.9$; $n = 712, 690, 725$, respectively).

BMI was mostly more tightly associated with serum SP-D than body height or creatinine and correlated inversely with FVC and FEV1. However, multiple regression models including these spirometric tests hardly differed from those presented in table 4. In particular, FEV1 and FVC never reached the significance level of 0.05.

Selection before entering the study and participation may have biased the results. However, job change prior to current job because of any health problem ($n = 77$) was not associated with

exposure subgroup (χ^2; p = 0.4). After excluding both plants with low participation (table 4) or lumping together formerly with currently exposed workers results did not change (details not shown).

Table 4 Determinants of SP-D (log transformed)

	Model 1 (n = 717)	Model 2 (n = 717)	Model 3 (n = 703)	Model 4 (n = 695)
Intercept	1.67 < 0.0001	1.66 < 0.0001	1.66 < 0.0001	1.45 < 0.0001
Gender (male=0; female=1)	- 0.03 0.5	- 0.05 0.4	- 0.04 0.4	- 0.01 0.8
Age (years)	0.0003 0.8	0.0002 0.8	- 0.0001 1.0	0.0003 0.8
Height (meters)	0.18 0.2	0.18 0.2	0.20 0.1	0.28 0.05
Pack-years (number)	0.002 < 0.0001	0.002 < 0.0001	0.002 < 0.0001	0.002 < 0.0001
Time since smoking cessation (years)	- 0.002 0.05	- 0.002 0.05	- 0.002 0.1	- 0.002 0.05
BMI (kg/m^2)	- 0.01 < 0.0001	- 0.01 < 0.0001	- 0.01 < 0.0001	- 0.01 0.0002
Currently asthma and/or symptoms of bronchitis (no=0; yes=1)	- 0.02 0.5	- 0.02 0.3	- 0.02 0.5	- 0.01 0.7
Creatinine (μmol/l)	0.001 0.09	0.001 0.06	0.0009 0.2	0.001 0.05
Exposure to bioaerosols from:				
- wastewater	0.05 0.003	0.002 0.1	0.03 0.003	0.06 0.003
- garbage	0.03 0.4	0.002 0.4	NA	0.007 0.8
- farming	- 0.003 [a] 0.9	- 0.001 [b] 0.4	NA	- 0.008 [a] 0.8
Adjusted R^2	0.06	0.05	0.06	0.06

Figures indicate the partial regression coefficient with the corresponding significance level.
NA: not applicable
The models differ according to population and indicator of exposure to wastewater and garbage. Model 1: whole population, current exposure to garbage or sewage (yes/no); model 2: whole population, duration of exposure (years); model 3: whole population, exposure to wastewater splashes; model 4: both plants with low participation excluded, exposure to garbage or sewage (yes/no) with workers formerly and currently exposed to wastewater or garbage lumped together.
[a] ever worked as a farmer (see methods); [b]duration of work in farming jobs (years)

Table 5 Determinants of SP-D (log transformed) in wastewater workers only

	N = 281	N = 279
Intercept	0.85 0.04	0.79 0.06
Age (years)	0.0003 0.9	0.0005 0.8
Height (meters)	0.53 0.01	0.58 0.009
Pack-years (number)	0.003 0.001	0.003 0.0004
Time since smoking cessation (years)	- 0 .005 0.007	- 0.004 0.009
BMI (kg/m^2)	- 0.006 0.07	- 0.007 0.07
Currently asthma and/or symptoms of bronchitis (no=0; yes=1)	0.02 0.7	0.02 0.6
Creatinine (μmol/l)	0.002 0.06	0.002 0.1
Number of days at work since last day on leave	0.001 0.9	NI
Wearing a mask (yes = 0; no = 1)	0.09 0.002	0.11 0.0003
Duration of exposure (years)	NI	- 0.001 0.6
Occurrence of splashes	NI	0.05 0.1
Contact with raw sewage	NI	- 0.03 0.5
Adjusted R^2	0.10	0.12

Figures indicate the partial regression coefficient with the corresponding significance level. NI: not included in this run. Number of days at work since last day on leave, occurrence of splashes and contact with raw sewage: see methods for definitions and coding. Total is less than 316 because of missing values.

Discussion

This is the first study examining the effect of occupational exposure to garbage or wastewater bioaerosols on serum SP-D concentrations. This lung-specific protein was increased dose-dependently by smoking and occupational exposure whereas it decreased with BMI and time after quitting smoking. However, the explained variance remained small.

These findings suggest weak subclinical effects of endotoxins on the lung-blood barrier despite a low endotoxin exposure. Indeed, exposure was mostly below 50 EU/m^3, the lowest occupational limit proposed to date (Douwes et al. 2003), and few workers had clinical symptoms of endotoxin exposure (Steiner et al. 2005, Jeggli et al. 2004). Exposure surrogates could suggest that splashes and wearing a mask are more important determinants of serum SP-D than duration of exposure.

A small SP-D increase may also have been brought about by methodological factors, bias and confounding. A systematic bias due to the long sample conservation is unlikely as a conservation bias must have induced changes associated with smoking, time since quitting smoking and exposure despite analyses being carried out blind in batches comprising samples from exposed and control workers. Moreover, the outcome corresponded to the study hypothesis, exposure indicators gave consistent results, and further statistical analyses did not disclose unexpected biases or confounders. The same calculations will be done with the data from the follow-up of this cohort to see whether they can be confirmed.

The study has limitations. Although a systematic approach with an already developed questionnaire was used, the reconstruction of exposure was only possible with fairly crude anamnestic indicators. Moreover, the relationship between these indicators and the short term tasks with higher endotoxin exposure as they were actually measured is unknown. Therefore, bias due to differential misclassification may have distorted the results of multiple regression analyses. Secondly, the study was cross sectional and, therefore, the population subjected to complex and uncontrolled selection mechanisms. Thus, interpretation must be cautious until confirmation of these findings by the results of the follow-up.

A weak increase of the CC16 concentration has been described in the same wastewater workers (Steiner et al. 2005), but no clinically relevant correlation between SP-D and other lung-specific proteins was found (highest correlation coefficient 0.07; $p = 0.06$ for SP-A). This may be an argument against the hypothesis of damage to lung-blood barrier, which should increase permeability for all lung specific proteins. However, differences in physico-chemical properties, metabolism, and solubility between lung specific proteins may explain our findings (Hermans and Bernard 1999). Furthermore, recent literature suggests a physiological difference. Indeed, the hydrophilic SP-D and SP-A, in contrast to SP-B and SP-C, could play a role in maintaining the lung in a relatively uninflamed state despite daily exposure to a significant amount of microbes and their constituents (Kuroki et al. 2007). Thus, an increased secretion of these hydrophilic collectins may be a regulatory mechanism that does not affect other lung specific proteins.

Appendix: definition of asthma and bronchitis

Symptoms of bronchitis: positive response to one of the questions "Do you usually cough during the day, or at night?" and/or "Do you usually bring up any phlegm from your chest during the day, or at night?" *Ever asthma:* positive response to both questions "Have you ever had asthma" and "Was this confirmed by a doctor?" *Current asthma:* positive answer to at least one of the two following questions as well: "Are you currently taking any medicine for asthma" or "Have you had an attack of asthma in the last 12 months".

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