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PLASMA C3D LEVELS OF YOUNG FARMERS CORRELATE WITH RESPIRABLE DUST EXPOSURE LEVELS DURING NORMAL WORK IN SWINE CONFINEMENT BUILDINGS

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Abstract: Work in swine confinement buildings leads to an inflammatory response and may be associated with increased levels of acute phase proteins. We compared the inflammatory response of a control group of young former farm workers with age-matched former farm workers who had previously developed the lower airway symptoms of wheeze, cough, tightness of the chest during work in swine confinement buildings, and because of these symptoms had stopped work. Both groups were subjected to an experimental exposure in a swine confinement building for 3 hours. Complement activation and acute phase proteins were measured in blood samples and broncho-alveolar lavage. Plasma C3d levels correlated with respirable dust, significantly so for individual cases and for the whole cohort. Plasma C3, fibrinogen and α_1-acid glycoprotein peaked 1 and 6 h after exposure start, mannan-binding lectin, C-reactive protein and α_1-antitrypsin peaked after 2 h. Surfactant protein D (SP-D) and α_2-macroglobulin were downregulated. In lavage, only SP-D, α_2-macroglobulin and fibronectin were detected. FEV_1, FVC, TLC and FEV_{25-75} did not vary during exposure. There was complement activation in response to respiratory dust, more so amongst cases than in the control group. Acute exposure, with work related levels of organic dust containing endotoxin, leads to a weak systemic inflammatory response.

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INTRODUCTION

Exposure to organic dust leads to local and systemic inflammatory responses mediated primarily by granulocytes and complement, and may be associated with increases of hepatic acute phase protein levels [24, 28, 43]. Upon long-term exposure to work conditions in swine confinement buildings (SCB), lung capacity is lost more rapidly than in control populations [11, 12]. The development of lower airway symptoms (cough, wheeze and tightness of chest)

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Figure 1. Exposure Plan. Participants attended 2 overnight and 2 ambulatory visits, all spaced by approximately 1 week. The first overnight visit served to collect baseline data and the second was for exposure. The ambulatory visits were for follow-up 1 and 2 weeks after exposure. On day of exposure, repeated measurements were taken as indicated. BAL: bronchoalveolar lavage, PD_{20}: metacholine provocation, blood: blood sample, bp: body pletysmography.

has been described as a chronic response to this environment [30, 45], the long-term effects of which are not yet known.

The acute cellular response to inhaled organic dust is thought to be provoked mainly by pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) and myristic acid, which signal an imminent infection [23]. These compounds activate the innate immune system through Toll-like receptors. The innate humoral response to infection is through complement, activated via 3 related pathways: the classical pathway, the alternative pathway and the recently described mannan-binding lectin (MBL) pathway [22, 42]. Insufficiency in serum levels of MBL due to mutations in the MBL gene have been associated with an increased incidence of acute infections in the respiratory tract during childhood [14, 40]. Surfactant protein D (SP-D), a collectin with a structure similar to MBL, binds LPS and microorganisms and mediates their clearance from the lung. In animal models, SP-D levels increased in response to tracheal instillation of LPS [9].

Acute systemic and pulmonary responses occur in naïve subjects after high level exposure [4, 7, 17, 18, 27, 46]. Whilst this is illustrative of the concept, it does not reflect the ordinary working situation. Results from cross-sectional studies are biased because of the cumulative exposure, that may down regulate the response [11, 15, 16, 30, 37]. In order to unveil the acute inflammatory response in an ordinary work situation, we exposed non-naïve subjects, who previously worked in SCB, but had not been exposed for at least 3 years, to moderate levels of organic dust in a SCB. Cases and controls were selected to comprise 2 groups of persons who were either known or not known to develop lower airway symptoms (cough, wheeze, tightness of chest) when exposed to this environment. Here, we report the complement and acute phase response of cases and controls during exposure in SCB with moderate levels of LPS and dust.

METHODS

Participants selected from a study of respiratory health of farming apprentices [38] who either developed lower airway symptoms or not were exposed for 3 hours in an SCB selected to contain dust and endotoxin levels at the lower end of the exposure range of Danish and European farms (Tab. 1) [32]. Inclusion criteria were having participated in the SUS study (a follow-up study of Danish agricultural apprentices initiated in 1992), and not having worked in a swine confinement building for at least 3 months. The time since participants had worked in a SCB ranged from three to nine years. Cases had an anamnetic record of chronic cough, wheeze or tightness of chest associated with work in an SCB, and had stopped such work because of these symptoms. Controls had not developed symptoms while working in an SCB, and had stopped such work for personal reasons. Exclusion criteria were asthma, rhinitis or any other pulmonary or other disease, and having worked in an SCB within the 3 months preceding participation. Atopy, defined as a positive prick test to a panel of 12 common and 8 work-related allergens (milk, soya, prok, rye, oat, wheat, barley, pork bristles), was scored *post hoc*. By these criteria, 4 cases and 2 controls were atopic, reacting with cat (3 persons reacted), birch (2), mites (1), cockroach (1), grass (3), dog (2), oat (1). Participants paired as case-control couples matched for smoking status were exposed to a light work situation, consisting in cycling for the first 10 minutes every hour for 3 hours in an SCB. Exposure was assessed by collecting inhalable dust with person-borne collectors, and analysing the dust for LPS content by the LAL method (BioWhittaker, Walkersville, USA) [10] and particle size distribution. One week before and 1 and 2

Table 1. Demographic data and exposure levels (mean ± standard deviation).

	Cases	Controls
Demographic data		
Number of males	7	7
Age, years	26	27
Number of smokers	4	4
Work exposure under SUS, years	2.32 ± 1.5	1.56 ± 1.2
Time since last exposure, years	5.18 ± 1.7	6.56 ± 2.6
Exposure data		
Dust, inhalable (mg/m^3)	4.10 ± 1.13	4.51 ± 1.42
Dust respirable (mg/m^3)	0.14 ± 0.10	0.14 ± 0.05
Endotoxin, inhalable (ng/m^3)	3.77 ± 6.12	11.12 ± 18.37
Endotoxin, respirable (ng/m^3)	0.245 ± 0.339	0.818 ± 1.528

Table 2. Soluble mediator levels (mean ± standard deviation) of cases and controls at baseline and 24 hours after exposure.

Component	Class	BAL				Blood			
		Cases		Controls		Cases		Controls	
		baseline	exposure	baseline	exposure	baseline	exposure	baseline	exposure
SP-D, mg/l		3.28 ± 1.5	3.17 ± 0.9	5.45 ± 3.0	5.14 ± 2.8	0.68 ± 0.2	0.72 ± 0.2	0.89 ± 0.3	0.88 ± 0.3
MBL, mg/l		<dl	2/8	1/8	1/8	1.29 ± 1.2	1.20 ± 1.2	0.99 ± 0.9	0.96 ± 1,0
C3, µmol/l	I	<dl	<dl	<dl	<dl	4.61 ± 0.9	4.77 ± 0.5	4.97 ± 0.5	4.74 ± 0.9
C3d, mU/l		Not done	Not done	Not done	Not done	29.3 ± 6.8	28 ± 6.9	29.6 ± 7.7	28.5 ± 5.5
CRP, nmol/l	I	<dl	<dl	<dl	<dl	23.5 ± 22.8	8.0 ± 5.0	13.6 ± 19.4	13.7 ± 11.5
Fibrinogen, g/l	II	<dl	<dl	<dl	<dl	4.0 ± 0.7	3.7 ± 0.5	3.8 ± 0.4	3.7 ± 0.4
α_1-AT, µmol/l	II	<dl	<dl	<dl	<dl	24.3 ± 4.0	21.1 ± 3.7	21.1 ± 3.2	24.5 ± 3.9
α_1-AG, µmol/l		<dl	<dl	<dl	<dl	17.6 ± 3.4	15.6 ± 3.1	16.2 ± 3.5	17.9 ± 4.4
α_2-M, µg/ml		62.1 ± 85.6	59.3 ± 74.2	34.8 ± 20.4	90.1 ± 85.6	2450 ± 800	2430 ± 700	2360 ± 600	2190 ± 600
Fibronectin, mg/ml		159 ± 122	154 ± 104	179 ± 116	287 ± 320	Not done	Not done	Not done	Not done

<dl; all samples below detection level. For BAL MBL, the fraction of samples that could be measured is listed. Where classified APP types are listed [25].

Table 3. Pulmonary function parameters (mean ± standard deviation).

	SUS [37]				Present study			
	Inclusion		Final		Baseline		Exposure	
Parameter	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
TLC, L	Not done	Not done	Not done	Not done	6.4 ± 0.7	7.5 ± 1.2	6.7 ± 0.6	7.4 ± 1.4
F2575	Not done	Not done	Not done	Not done	4.6 ± 1.1	3.7 ± 1.0	4.6 ± 1.0	3.6 ± 1.1
FEV_1, L	4.57 ± 0.7	4.47 ± 0.4	4.49 ± 0.8	4.57 ± 0.4	4.48 ± 0.9	4.43 ± 0.5	4.48 ± 0.9	4.31 ± 0.5
FVC, L	5.12 ± 0.8	5.52 ± 0.5	5.16 ± 0.9	5.83 ± 0.3	5.56 ± 1.0	6.08 ± 0.5	5.60 ± 1.1	5.93 ± 0.4
FEV_1/FVC %	89.6 ± 4.5*	81.0 ± 5.7	86.9 ± 2.4*	78.4 ± 4.8	81.0 ± 3.1*	73.3 ± 3.1	81.3 ± 3.6*	72.1 ± 3.1

* - significant difference between case and control FEV_1/FVC.

weeks after exposure, baseline and follow up visits were performed. Participants were bronchoscopied at baseline visit and 24 hours past exposure (hpe) as detailed in the exposure plan (Fig. 1) and 7 blood samples were taken on the day of exposure. Bronchoalveolar lavage (BAL) was performed under local anaesthesia with 3 aliquots of 60 ml saline as described previously [30]. For plasma preparation, blood was taken into heparin and EDTA tubes. For serum preparation, the blood sampling procedure was optimised for secretion of ECP [33].

C3, α_1-AT, CRP, α_1-acid glycoprotein (α_1-AG) and fibronectin were determined by turbidimetric methods. C3d was determined by double zone rocket immuno electrophoresis [3, 6]. SP-D and MBL were determined by ELISA [19] and TRIFMA [39], respectively. Levels of α_2-Macroglobulin (α_2-M) in serum samples were measured by a sandwich ELISA. α_2-M polyclonal antibodies (A033, DAKO) were used for capture, and an α_2-M monoclonal antibody (ICG4) followed by peroxidase conjugated anti(mouse IgG) (P260, DAKO) for detection. Standard curves were established using purified human α_2-M. Fibrinogen was measured by RIA. Intra- and inter-assay coefficients of variation were 10% and 15%, respectively. FEV_1, FVC, TLC and F_{25-75} were determined with a Vitalograph S-20600 according to published guidelines [31].

Normality of distributions was determined with the Kolmogorov-Smirnov and Shapiro-Wilk tests. Data not normally distributed was log transformed and retested. Normally distributed data was analysed by *t*-test, otherwise the Wilcoxon and Mann-Whitney tests were used. The area under the curve (AUC) for reactants was calculated for total values of measurements at 0, 1, 2, 3, 4 and 6 hpe. Each series of measurements was investigated first for a change in response to exposure, and next for differences between cases and controls, atopics and non atopics and smokers and non smokers. Correlations between biologically relevant parameters was investigated for all participants, and then for sub groups. $P < 0.050$ was considered significant. Spearmanns correlation was used to investigate the relationship between different data sets. Data was analysed with SPSS v10.

RESULTS

The systemic response to exposure. C3d was measured before start of exposure, and at 1 and 4 hours, as well as 1 day after exposure start. C3d correlated with respirable dust at all 3 times it was measured after exposure start, ($r > 0.543$, $p < 0.030$, Fig. 2a), but not at baseline. C3d did not correlate with endotoxin. The amount of C3d was higher in atopics (33.2 mU/l) than in non atopics (26.0 mU/l)

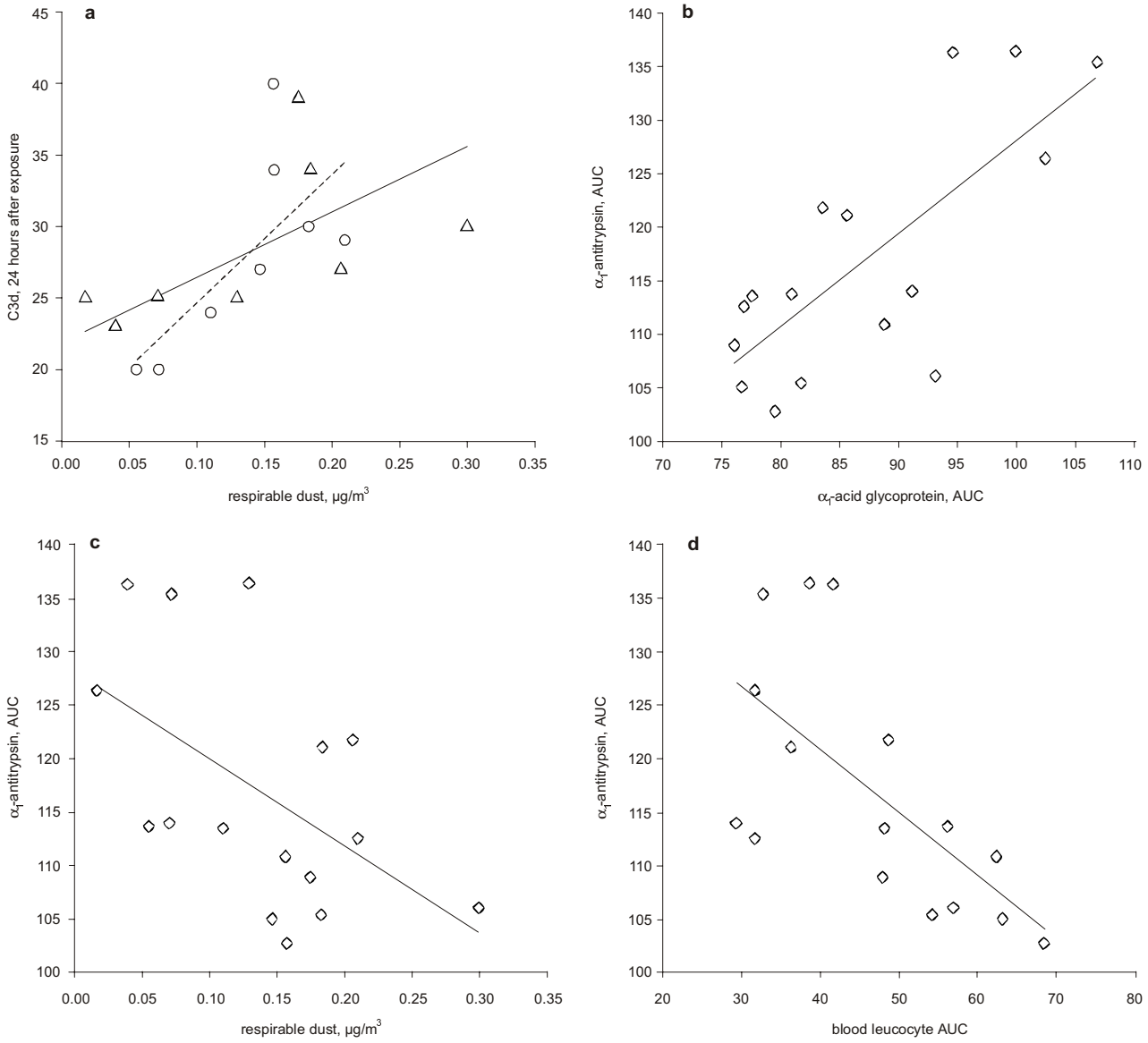

Figure 2. Correlation of acute phase protein concentrations with exposure and cell numbers. (a) The level of C3d of all participants (solid line) 24 h after exposure correlated with dust exposure, significantly for cases (circles, dotted line), but not for controls (triangles). (b) α_1-AT AUC and α_1-AG AUC correlate positively during exposure. (c) Plasma α_1-AT AUC correlated negatively with respirable dust levels and (d) leukocyte concentration AUC.

at 4 hpe ($p = 0.043$, Fig. 3a). There was a significant increase in plasma C3 of atopic participants from baseline (4.39 µmol/l) to 2 hpe (4.93 µmol/L, $p = 0.010$).

In Figure 3, acute phase proteins are grouped by response; C3, α_1-AG and fibrinogen respond maximally after 1 and 6 hours, α_1-AT and MBL after 2 hours, and CRP and α_2-M even later. SP-D was reduced during and after exposure. There was an inverse relationship between α_1-AT AUC and respirable dust ($r = -0.526$; $p = 0.037$, Fig. 2c) and between α_1-AT AUC and blood leucocytes AUC ($r = -0.651$, $p = 0.006$) (Fig. 2d) and C3 AUC ($r = 0.567$, $p = 0.022$). α_1-AG AUC correlated significantly with α_1-AT AUC ($r = 0.749$, $p = 0.001$, Fig. 2b), more so for non-smokers, non-atopics and cases. Plasma α_2-M of cases was significantly up regulated at 4–6 hpe (day 1 1.36 µmol/l, 4 hpe 1.50 µmol/l, $p = 0.012$, 6 hpe 1.40 µmol/l, $p < 0.004$). The variation in the amount of plasma CRP did not reach significance, and no dependence on any of the discriminators was measured. The large increase and variation of CRP at 12 h after exposure was due to samples from atopic cases.

Due to the large interpersonal variation, increases in MBL during exposure were not significant (Fig. 3f). MBL was significantly reduced at 24 hpe (1.08 µg/ml, $p = 0.049$) and 1 week after exposure (1.06 µg/ml, $p = 0.039$) compared to baseline (1.14 µg/ml, Fig. 2a). There was no difference in MBL between the groups investigated. The level of plasma SP-D decreased from baseline (0.79 µg/ml) to 4 hpe (0.69 µg/ml, $p = 0.003$) and 6 hpe (0.68 µg/ml, $p = 0.005$), but had returned to normal after 24 hours (Fig. 3c). There was no significant difference between the groups investigated.

Acute pulmonary response assessed in BAL. Fibronectin, α_2-M and SP-D were detected in BAL, but no differences

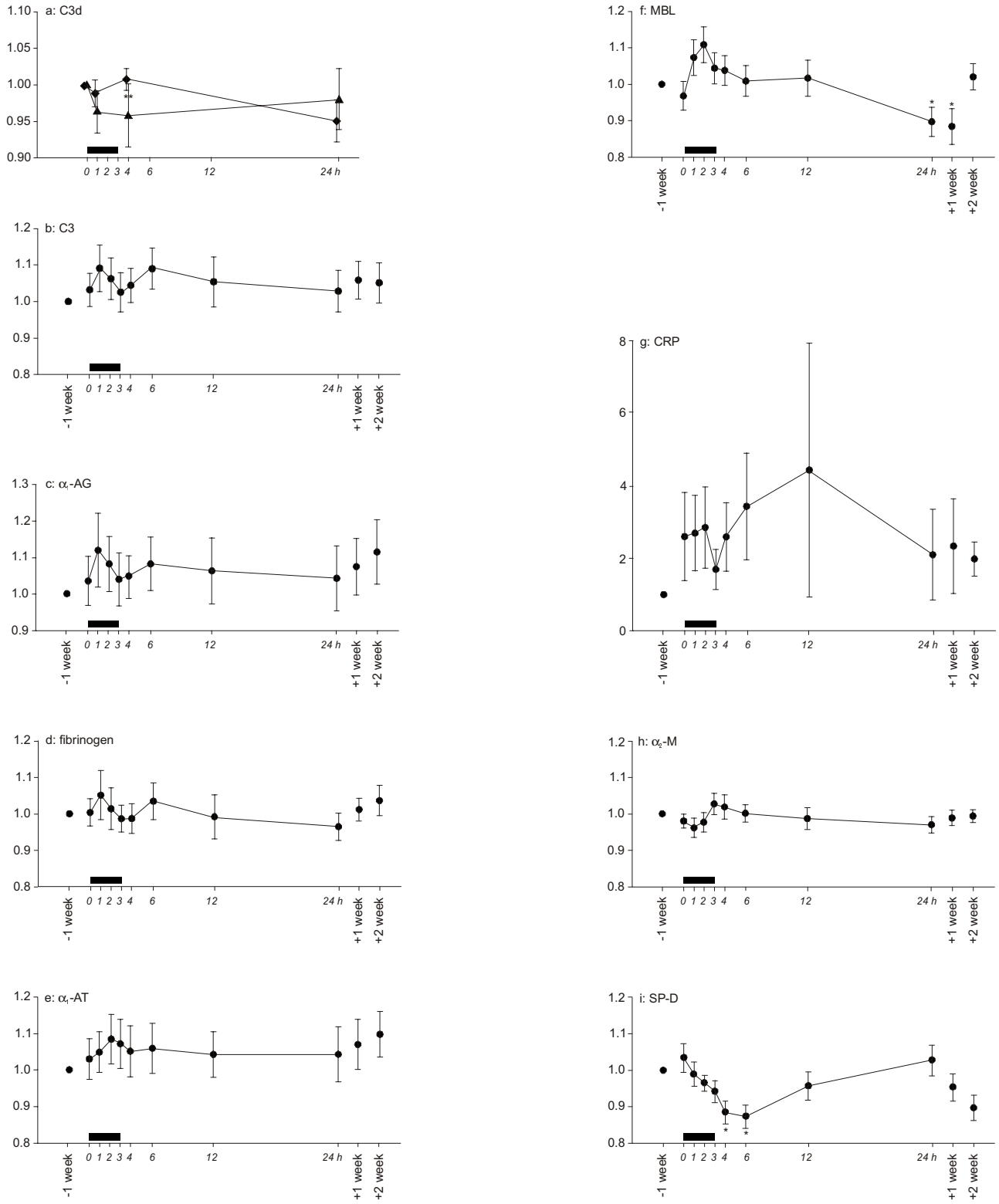

Figure 3. Acute phase and complement protein concentrations following exposure in an SCB, expressed with baseline (day 1) values set to 1. Asterisks indicate significant change from baseline. Repeated measurement on day of exposure are linked by a line, and baseline and follow up measurements 1 week before and 1 and 2 weeks after exposure are represented by unlinked dots. A bar marks the 3 h exposure. (a) C3d of atopics (rhombi) is higher than that of non-atopics (triangles). The difference is significant at 4 h after exposure. (b-d) C3, α-AG and fibrinogen concentration peaked at 1 and 6 h after start of exposure. (e, f) Concentration of α_1-AT and MBL peaked at 2 hours after exposure start. MBL was significantly reduced on days 8 and 15 with respect to baseline and day 22. (g) The concentration of CRP peaked at 2 and 12 h after exposure. Atopic cases account for the large variation at 12 h after exposure. (h) The concentration of α_2-M was reduced during the first hour, but peaked at 4 h after exposure start. The difference between the baseline value on exposure day and 4 h after exposure was significant. (i) The concentration of SP-D was significantly reduced at 4 and 6 h after start of exposure.

were detected between baseline and exposure measurements of the group as a whole, or when considering the discriminators lower airway symptoms, smoking or atopy. MBL could be detected in 1 of 16 baseline BAL, and in 3 of 16 BAL after exposure. Other soluble mediators could not be detected in lavage fluid (Tab. 2).

Physiological pulmonary parameters. FEV_1, FVC, TLC and F_{25-75} were measured in the present study, and FEV_1 and FVC had also been recorded during the previous SUS study [38], from which the participants had been recruited (Tab. 3). There were no significant differences during exposure of any of these parameters.

DISCUSSION

Exposure to organic dust as occurs in an SCB is thought to activate the innate cellular and humoral immune system. Here we have examined the effect of an exposure comparable to normal work on soluble markers selected amongst complement components and acute phase proteins that had previously been associated with the acute response to very high levels exposure [24, 28, 43]. Cytokine levels, cell numbers and cell surface markers will be reported elsewhere. In contrast to cross-sectional studies of chronically exposed workers [11, 15, 16, 30, 37] and to naïve persons exposed once to an SCB [4, 7, 17, 18, 27, 46] the selection of non-naïve, presently non-exposed persons makes the study unique. Persons known to tolerate work or acquire lower airway symptoms during work in SCBs were exposed to normal work-related levels of dust and endotoxin [32] rather than to the very high levels used in some of the earlier studies [15, 17, 18, 21]. Although symptoms were not recorded, resting breathing and heart rates during the exposure remained constant, suggesting that the participants did not develop clinical symptoms.

The plasma level of the complement factor C3 split product, C3d, a marker of complement activation, was highly correlated to the amount of respirable dust, significantly for cases but not for controls. The lack of upregulation of C3d reflects the low, work related, level of exposure. The finding that respirable dust, but not endotoxin, correlates with C3d is consistent with the finding that the element magnesium, found in large quantities in swine confinement building dust, activates complement [1]. Here the conversion to C3d is measured, which is a more stable marker than C3b. Plasma levels of C3 increased significantly only on the exposure day. Complement activation was significantly related to the amount of respirable dust.

The acute phase proteins have been shown to increase in experimental exposures of naïve subjects [7, 13, 18] with significantly higher levels of exposure. Similar results were obtained when studying persons exposed to LPS [24, 28]. In cross-sectional studies, SCB workers had slightly elevated levels of acute phase proteins compared to controls [15, 16]. Similar results were obtained in cross-sectional studies of workers in the paper [34] and grain handling industry [4, 5]. Here, non-naïve subjects (in which the adaptive immune system may attenuate the response) showed a slight, insignificant increase in α_1-AT, α_1-AG, C3, fibrinogen, MBL and CRP during or after a work-related exposure (Fig. 3). α_2-M and SP-D concentrations were reduced during exposure, suggesting that these factors are consumed during exposure.

Interpersonal MBL levels varied considerably, but there was no exposure-related variation [14]. This makes MBL insufficiency an unlikely cause for the lower airway symptoms [40]. The effect on MBL was more delayed, there was a significant reduction 1 day after exposure, which persisted for 1 week but returned to baseline 2 weeks after exposure.

Serum SP-D levels were reduced during exposure by 10%, but the level of SP-D did not differ significantly between the 2 groups. It has previously been shown that the serum SP-D concentration is significantly lower in patients with acute lobar pneumonia at the day of admission to hospital compared to healthy subjects. On day 5 after hospitalization, the SP-D concentration increased on average 3 times the concentration on admission and then slowly declined towards normal levels, however 22-fold increases were observed in individual patients [20].

Only SP-D, α_2-M and fibronectin were detected in BAL, and did not increase significantly. Increases in BAL α_2-M after exposure to high levels of dust and LPS have previously been ascribed to plasma exudation [44]. As alveolar macrophages secrete α_2-M [29], the elevated levels of α_2-M in BAL are more likely locally produced. Possible markers for plasma exudation are CRP, fibrinogen, α_1-AT and α_1-AG (all primarily of hepatic origin) that were measured in plasma, but had been below detection limit in BAL at rest, and after the low level of exposure in this study.

Plasma α_1-AT [47], α_1-AG [41, 47] and fibrinogen and BAL fibronectin [35] increased during the onset of exposure, but sharply decreased in smokers, whereas in non smokers plasma levels increased, suggesting that smokers were more susceptible to effects of exposure. α_1-AT and α_1-AG can protect fibroblasts in vitro against toxic effects of LPS [48], suggesting a function of these proteins in inflammation.

Pulmonary function of the participants had been followed for between 6–9 years, culminating in the measurements flanking the experimental exposure. There was no difference in the parameters measured. The FEV_1/FVC of the participants recruited here was better than that of a cohort of grain workers [4]. During exposure with grain dust containing more than 30 mg endotoxin per exposure, the ratio of FEV_1/FVC was significantly reduced for more than 5 hours [2]. The exposure level in the present study is more than 3 orders of magnitude lower, and does not lead to acute obstruction.

In contrast to exposure with high levels of organic dust, there was a weak acute phase protein response including complement consumption. There was no decrease of pulmonary function, and concentrations of acute phase proteins, MBL and SP-D did not vary significantly. During

exposure with work-related levels of organic dust correlation of reactants with exposure is a more sensitive measure than determination of a significant increase. Here, complement system activation correlated with respirable dust, and was contained when the environment was left and no persistent infection was detected.

Repeated attempts have been made to find a threshold for exposure in SCB [8, 25, 43]. The findings presented here with work-related levels of organic dust in an SCB argue that there is no lower threshold. Instead, there is a continual response to substances that signal danger to the immune system, and the evaluation of varying ability of the individuals' immune system to respond to these signals [13, 24, 36] and cope with the environment is a more robust approach to dealing with exposure to organic dust.

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