



Pneumoproteins and markers of inflammation and platelet activation in the blood of grain dust exposed workers

Anne Straumfors, Wijnand Eduard, Kari K. Heldal, Marit Skogstad, Lars Barregård & Dag G. Ellingsen

To cite this article: Anne Straumfors, Wijnand Eduard, Kari K. Heldal, Marit Skogstad, Lars Barregård & Dag G. Ellingsen (2018) Pneumoproteins and markers of inflammation and platelet activation in the blood of grain dust exposed workers, *Biomarkers*, 23:8, 748-755, DOI: [10.1080/1354750X.2018.1485057](https://doi.org/10.1080/1354750X.2018.1485057)

To link to this article: <https://doi.org/10.1080/1354750X.2018.1485057>



© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.



Published online: 24 Jul 2018.



[Submit your article to this journal](#)



Article views: 1022



[View related articles](#)





[View Crossmark data](#)



Citing articles: 4 [View citing articles](#)

Pneumoproteins and markers of inflammation and platelet activation in the blood of grain dust exposed workers

Anne Straumfors^a , Wijnand Eduard^a, Kari K. Heldal^a, Marit Skogstad^a, Lars Barregård^b and Dag G. Ellingsen^a 

^aNational Institute of Occupational Health, Oslo, Norway; ^bSahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

ABSTRACT

Purpose: To investigate if blood biomarkers could indicate early signs of lung damage or cardiovascular risk due to exposure to grain dust.

Materials and methods: Pneumoproteins and markers of inflammation and platelet activation were analysed in blood samples of 102 grain elevator and compound feed mill workers. Differences between exposed ($n=67$) and controls ($n=35$), and associations with exposure measurements and respiratory health were investigated by multiple linear regression analyses.

Results: Concentrations of CC-16 and IL-6 were higher in exposed workers compared with controls ($p < 0.001$ for both), whereas fibrinogen was lower ($p = 0.005$). Concentrations of CRP, TNF- α , sCD40L and sP-selectin were similar in both groups. Serum CC-16 was significantly higher in workers with farm childhood, regardless of exposure. The impact of farm childhood on CC-16 interacted with smoking. None of the biomarkers were associated with exposure measurements or any of the tested respiratory health parameters.

Conclusion: Dust exposure induced inflammatory and anti-inflammatory reactions, but did not induce systemic inflammation and had no effect on platelet activation. No cause–effect relationship could be established in spite of relatively high exposure levels, particularly to endotoxin. Whether increased serum CC-16 is an early sign of lung damage or a reversible defense reaction remains unclear.

ARTICLE HISTORY

Received 27 March 2018

Revised 16 May 2018

Accepted 30 May 2018

KEYWORDS




Club cell protein 16; SP-D; bioaerosol exposure; grain elevator workers; compound feed mill workers; farm childhood

Introduction

Grain dust is generated during threshing and other handling of grain. It is a heterogeneous mixture of inorganic soil particles, plant fragments, insect and mite body parts and faeces, viable and non-viable microorganisms, and components such as endotoxins, β -1,3-glucans and mycotoxins (Smith 1989, Straumfors *et al.* 2015). Grain dust and its components may induce inflammation, and impair lung function of employees that are exposed to grain dust during work at grain elevators and compound feed mills (Huy *et al.* 1991, Chan-Yeung *et al.* 1992, Smid *et al.* 1994, Spurzem *et al.* 2002, Pahwa *et al.* 2008). An inconsistency in exposure–response relationships has been observed between studies that may be due to differential effects of individual grain dust components and differences between studies in grain dust composition (Straumfors *et al.* 2015, 2016, Halstensen *et al.* 2013). Although endotoxin has been proposed as a target for measurements of occupational health hazard related to grain dust exposure (Health Council of the Netherlands 2011), fungal spore exposure has been shown to be stronger associated with respiratory effects and fatigue than endotoxin (Straumfors *et al.* 2016). A possible reason for the diverging relationships in epidemiological studies is the healthy worker selection. As grain elevators and compound feed mills are

often located on the countryside, the healthy worker selection could possibly be related to the apparently protective effect of having grown up on a farm, that is, the ‘hygiene hypothesis’ (von Mutius 2007).

The pathological response to grain dust exposure suggests activation of inflammatory and allergic pathways, although exact mechanisms are not completely elucidated. The non-ciliated bronchial epithelial club cells play an important role in protecting the lung from inhaled toxicants. One of the major proteins secreted by the club cells is the anti-inflammatory club cell-16 protein (CC-16) (Dierynck *et al.* 1995, Lakind *et al.* 2007). Serum levels of CC-16 are mainly affected by its production rate by club cells into the alveolar lavage fluid, the rate of diffusion from alveolar fluid into the serum, which is affected by permeability of the pulmonary epithelial barrier, and renal clearance (Lakind *et al.* 2007). Inhalation of particles may affect this basic control, reducing the serum CC-16 by inhibiting, injuring or killing the club cells or increasing the concentration by inducing increased secretion and/or permeability. The CC-16 protein has been suggested to be a sensitive marker for detecting early permeability changes in the lung epithelial barrier and/or to evaluate the integrity of the club cells (Broeckaert and Bernard 2000). Reduced levels of serum CC-16 have been associated with cigarette smoking

CONTACT Anne Straumfors  anne.straumfors@stami.no  National Institute of Occupational Health, P.O. Box 5330, Majorstuen, 0304 Oslo, Norway
#Anne Straumfors and Dag G. Ellingsen were responsible for the statistical design and analyses.  anne.straumfors@stami.no (A.S.), dag.ellingsen@stami.no (D.G.E.).

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

(Bernard *et al.* 1994, Robin *et al.* 2002). In contrast, acute or repeated exposures to bioaerosols in wastewater workers (Heldal *et al.* 2013) (Steiner *et al.* 2005) have been associated with increased serum CC-16 levels.

Detection of specific proteins in peripheral blood of exposed workers may give valuable information of inflammation and early processes in disease development. Changes in serum levels of pneumoproteins, such as CC-16 or surfactant protein A and D (SP-A and SP-D) may indicate lung injury following inhaled exposure to dust (Lakind *et al.* 2007, Broeckaert and Bernard, 2000), whereas interleukin 6 (IL-6), tumour necrosis factor- α (TNF- α), fibrinogen and C-reactive protein (CRP) are parts of the acute phase response to systemic inflammation.

By direct action or through oxidative stress and activation of inflammatory pathways, dust exposure may furthermore increase the risk of cardiovascular disease (Du *et al.* 2016). Exposure to particulate matter from various sources has indeed been associated with cardiovascular effects, such as increased blood coagulation, atherosclerosis and heart disease (Fang *et al.* 2010, Du *et al.* 2016). Soluble CD40 ligand (sCD40L) is contained in platelet granules, and thus, its presence in the blood is a marker of platelet activation. By interacting with CD40, which is found on endothelial and smooth muscle cells, sCD40L may trigger the release of inflammatory mediators, leading to increased activity of matrix metalloproteinases and activating of the coagulation cascade. CD40 Ligand dysregulation contributes to immune pathology in atherosclerosis, atherothrombosis, and restenosis (Aukrust *et al.* 1999, Schonbeck *et al.* 2001, Heeschen *et al.* 2003). Soluble P-Selectin (sP-Selectin) is released from platelets and the endothelial cell surface and has pro-thrombotic and pro-inflammatory properties (Fijnheer *et al.* 1997, Ridker *et al.* 2001, Ferroni *et al.* 2008). Grain dust exposure may have the potential to induce markers of platelet activation, but has to our knowledge not been studied.

The primary objectives of this study were to investigate whether workers exposed to grain dust and its components had (1) increased serum pneumoprotein levels compared to controls, as a sign of exposure-induced increase in secretion from club cells, or increased permeability of the lung epithelium barrier and (2) increased levels of acute phase reaction markers of inflammation or markers of activated platelets, as indicators of increased risk of cardiovascular disease. Secondary objectives were to investigate how the serum biomarkers were associated to (3) respiratory health effects measured by lung function tests and self-reported symptoms, and (4) exposure to dust from grain elevators and compound feed mills, including bacteria, endotoxin, fungal spore and 1,3- β -glucans (bioaerosol exposure), published previously from the same study (Halstensen *et al.* 2013, Straumfors *et al.* 2016).

Clinical significance

- Increased levels of biomarkers in blood can potentially predict increased risk for future pulmonary and cardiovascular diseases, but this was not confirmed in this study.
- Associations between grain dust exposure and proteins involved in inflammation and activation of platelets may

provide the basis for the establishment of early biomarkers for disease progression or increased risks in exposed workers.

- Early life exposures to farm dust during farm childhood may influence later responses of exposure to organic dust and microbial components.

Methods

Study population

Details of recruitment and description of the study population, participating companies and work tasks carried out by the workers have been published previously (Straumfors *et al.* 2015, 2016). Twenty companies of grain elevators and compound feed mills in the Norwegian grain industry geographically distributed throughout 20 municipalities in nine counties in central and south-eastern Norway were included in this study. The study population consisted of all employees working on relevant shifts; 67 grain dust exposed workers and 36 assumedly unexposed administrative workers as controls (Figure 1). The exposed workers included 28 workers employed in the grain elevator department, 17 workers in the compound feed mill department, 6 working both in grain elevator and compound feed mill departments, and five transport workers. Characteristics of the study population are presented in Table 1.

The Regional Ethical Committee of South-East Norway and the Norwegian Data Inspectorate approved the study. All participants gave their written informed consent upon participation in the study. All but one of the workers that received the written information agreed to participate. The one that refused medical examinations participated in the exposure measurements only.

Study design

The field team rigged the equipment at the work site and informed the participating workers on a Monday, and performed the exposure measurements and health examinations on a Tuesday. For two companies, Wednesday was the day of exposure measurements and health examinations. The employees were examined before work, defined as baseline, and after work in a cross-shift design. The examinations included spirometry, gas diffusion tests and acoustic rhinometry, and the results have been published previously (Straumfors *et al.* 2016). The employees were asked to abstain from smoking at least one hour before the health examinations. Blood samples were collected after shift for atopy testing, biomarkers and haemoglobin measurements. After work the employees filled in a questionnaire reporting smoking and work history, as well as possible work related health complaints on that day or in the past week.

Bioaerosol exposure measurements and analyses

Full shift (6–8 hrs) personal inhalable samples ($n = 130$) were collected two consecutive days with PAS-6 samplers (van der Wal, 1983) and portable pumps (PS101; National Institute of

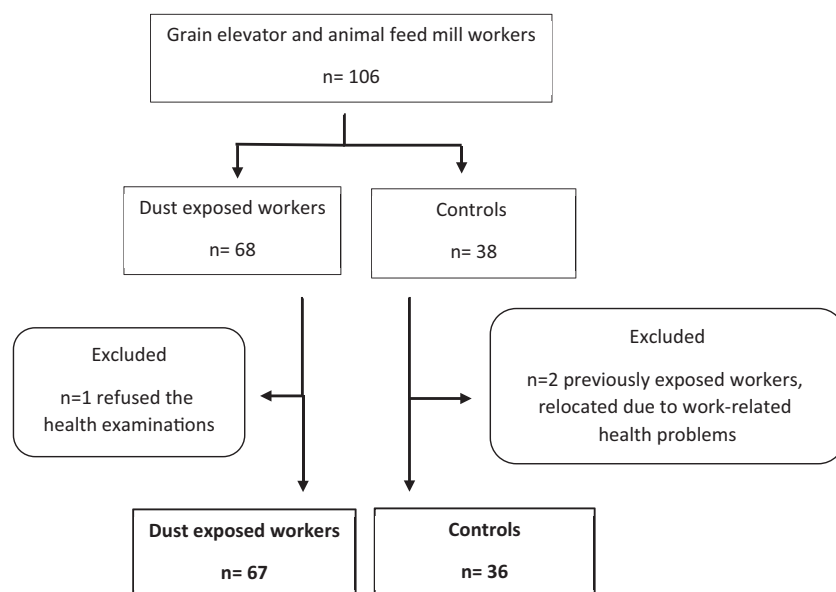


Figure 1. Flow chart of the number and selection of individuals in the study population.

Table 1. Characteristics of the study population of grain and animal feed industry workers in Norway.

	Exposed workers (n = 67)	Controls (n = 36)
Age (year) ^a	42 (16–59)	50 (21–60)
Height (cm) ^a	179 (162–196)	177 (156–191)
Weight (kg) ^a	89 (63–123)	86 (55–109)
Body mass index (kg/m ²) ^a	28 (21–38)	27 (21–38)
% body fat ^a	26 (12–43)	27 (14–43)
Never-smoker (in %)	43	44
Current smoker (in %)	27	17
Ex-smoker (in %)	30	39
Male/Female (in %)	94/6	83/17
Atopy ^b (in %)	22	22
Physician-diagnosed asthma (in %) ^c	5	6
Having a cold (in %)	19	9
Farm childhood (in %) ^d	36	42
Living at a farm (in %)	16	28
RPE use (in %)	16	0
FVC % of predicted at baseline ^a	95 (66–133)	99 (80–122)
FEV ₁ % of predicted at baseline ^a	92 (68–127)	93 (51–120)

^aMedian (min-max).

^bPositive serum reaction towards inhalation panel.

^cSelf-reported.

^dIncluded one exposed worker that moved to a farm in his youth.

RPE: respiratory protective equipment; FVC: forced vital capacity; FEV₁: Forced expired volume in the first second.

Occupational Health, Oslo, Norway) using a flow rate of 2 L min⁻¹. Samples were analyzed for grain dust, endotoxins, bacteria, fungal spores and β -1,3-glucans as previously described (Halstensen et al. 2013). In brief, dust was weighed, bacteria were stained with acridine orange and counted by epifluorescence microscopy, fungal spores were counted by scanning electron microscopy, endotoxins were analyzed by Limulus amoebocyte lysate assay, and β -1,3-glucans were analyzed by enzyme-immuno-assay. The job groups and exposure variability have been described previously (Halstensen et al. 2013, Straumfors et al. 2015).

Questionnaire

After work-shift, the participants filled out a questionnaire based on the ATS recommended respiratory disease

questionnaire for epidemiological research (Medical Research Council, 1960) that included questions on respiratory protective equipment (RPE) use, smoking habits, work history, symptoms of the airways, eyes, skin, and general illness experienced during or after work on the day of the examinations. Questions about farm childhood or living at a farm (having farm/livestock at home) were also included as part of the relevant exposure history.

Skinfold test

To determine the subcutaneous fat layer thickness, a pinch of skin was measured to the nearest mm at the biceps, triceps, subcapsular and supra-iliac areas on the right side of all workers standing in a relaxed position using a skinfold caliper (GPM Instruments, Zürich, Switzerland). The log of the sum of skin fold measurements was used to calculate body density with age-dependent prediction equations (Durnin and Womersley 1974). The predicted density values were then converted to percent body fat using the Siri Equation (Siri 1961): % body fat = (495/body density) – 450.

Blood sampling and analyses of biomarkers

Blood samples were collected after work between 1 and 3 PM. Blood for fibrinogen analyses was collected in vacutainers containing citrate (BD Vacutainer, Franklin Lakes, NJ, US), and the tubes were immediately inverted 8–10 times. Vacutainers without additives (BD Vacutainer) were used to separate serum from blood for analysis of inflammatory markers and atopy. The blood samples were left at room temperature for 30 to 90 min. Serum was separated in tubes without additives by 10 min centrifugation at 3500 rpm followed by careful pipetting of serum into new tubes. Plasma from citrates tubes was centrifuged as above followed by careful pipetting of plasma into new tubes. Samples for C-reactive protein (CRP) and atopy testing were sent directly to a commercial routine laboratory for analysis, whereas the

rest of the plasma and serum samples were immediately frozen to -20°C , replaced to -80°C upon arrival at the laboratory, where they were kept until analysis.

Atopy was defined by the presence of specific serum immunoglobulin E antibodies towards one or more allergens in a panel of typical outdoor airway allergens (birch, timothy, mugwort, *Alternaria tenuis*, *Cladosporium herbarum*) determined by Phadiatop screening (Fürst laboratories, Oslo, Norway). CC-16 (Human club Cell Protein ELISA kit, BioVendor Laboratory Medicine, Inc., Brno, Czech Republic), SP-D (SP-D ELISA kit, BioVendor), IL-6 (Quantikine HS-Human IL-6 immunoassay kit, R&D Systems Inc. Minneapolis, MN), sP-selectin (Human sP-selectin/CD62P, R&D) and TNF- α (Quantikine HS-Human TNF- α /TNFSF1A, R&D), fibrinogen (ICL Inc., Portland, OR, US) and sCD40L (Human CD40 Ligand/TNFSF5 Quantikine ELISA Kit, R&D) were analysed by ELISA according to the manufacturer's instructions. SP-A was analysed by sandwich ELISA as described in detail previously (Ellingsen et al. 2017). In short, the primary antibody was AB3422 (Millipore, Billerica, MA); the secondary antibody was HYB 238-04 (Antibody Shop, Gentofte, Denmark). Optical density were read at 450 nm adjusted for background signal read at 540 for kits with HRP and 490 nm adjusted for background signal read at 650 nm for kits with AP in a Spectramax i3 spectrophotometer (Molecular Devices LLC., Sunnyvale, CA, US) or a BioTek microtiter spectrophotometer (BioTek Instruments, Inc. Winooski, USA).

Statistical analyses

The concentrations of bioaerosols and biomarkers were ln-transformed to achieve normal distribution and homoscedasticity, and are presented as geometric mean (GM) and geometric standard deviation (GSD). Differences in the GM

Table 2. Geometric mean (GM) exposure in the Norwegian grain and compound feed industry.

Exposure parameter	<i>n</i>	GM	GSD ^a
Grain dust (mg/m ³)	130	1.1	3.32
Endotoxin (EU/m ³)	130	777	5.53
β -1 \rightarrow 3-glucan ($\mu\text{g}/\text{m}^3$)	130	9.4	5.21
Bacteria (counts/m ³)	130	35×10^4	4.90
Fungal spores counts/m ³)	130	4×10^4	3.51

^aGSD = geometric standard deviation.

between exposed workers and controls were tested by independent sample *t*-test, and a *p* value ≤ 0.05 was regarded statistically significant. Multiple linear regression models were used to investigate associations between blood biomarkers and exposure. Categorical exposure variables (exposure versus controls) were tested with all workers, whereas linear exposure variables (ln-transformed values of the personal exposure of dust, endotoxin, bacteria, fungal spores and β -1,3-glucans) were tested with exposed workers only. Confounding effects of age, gender, % body fat, smoking, atopy, farm childhood, living at a farm and having a cold were tested by stepwise backward procedure and adjusted for at a *p*-value < 0.1 and when the regression coefficient changed by $> 20\%$. General linear models (GLM) were built to calculate estimates of adjusted biomarker concentrations and to explain the variance of the serum biomarker concentration. The IBM software package SPSS version 25.0 was used for the statistical analyses (IBM Corp, Armonk, NY).

Results

The distribution of age, body size parameters, atopy, asthma in the exposed workers and controls was similar (Table 1). The study population included mainly men, with only 6% and 17% females, respectively, in the two groups. The prevalence of current smokers and current cold were somewhat higher in the exposed group, whereas the prevalence of farm childhood and currently living at a farm were somewhat higher among controls. Only 16% of the exposed workers reported to use RPE.

Bioaerosol exposure

The high endotoxin exposure of GM 662 EU/m³ exceeded the health-based recommended occupational exposure limit of 90 EU/m³ by more than 7-fold (Table 2). The mean exposures of the other components were moderate, but high GSDs reflected large variances between workers.

Blood biomarkers in exposed workers and controls

The concentrations of pneumoproteins and markers of inflammation and platelet activation in blood of exposed

Table 3. Concentrations of pneumoproteins and markers of inflammation and platelet activation in blood of grain dust exposed workers as observed and adjusted for confounders.

	Exposed workers (<i>n</i> = 67)		Controls (<i>n</i> = 35)		Group differences	
	GM (GSD)	GM _{adj} (GSE)	GM (GSD)	GM _{adj} (GSE)	<i>p</i> value	<i>p</i> value _{adj}
CC-16 (ng/mL)	4.9 (1.5)	4.9 (1.05)	3.6 (1.4)	3.6 (1.07)	<0.001	<0.001
SP-D (ng/mL)	102 (1.7)	106 (1.07)	93 (1.9)	100 (1.09)	0.4	0.8
SP-A ($\mu\text{g}/\text{mL}$)	22 (12.2)	–	30 (7.5)	–	0.5	–
IL-6 (pg/mL)	1.9 (1.9)	2.5 (1.11)	1.3 (1.7)	1.5 (1.11)	0.04	<0.001
TNF- α (pg/mL)	1.1 (1.3)	–	1.1 (1.5)	–	0.6	–
Fibrinogen (mg/mL)	3.3 (1.3)	3.6 (1.05)	3.9 (1.3)	4.2 (1.06)	0.001	0.005
CRP (mg/L)	1.7 (2.9)	2.4 (1.22)	1.3 (2.7)	1.7 (1.22)	0.3	0.1
sCD40L (ng/mL)	1.7 (2.7)	1.6 (1.11)	1.6 (3.0)	1.4 (1.22)	0.5	0.5
sP-selectin (ng/mL)	50 (1.6)	40 (1.11)	54 (1.6)	43 (1.11)	0.5	0.6

GM: geometric mean; GSD: geometric standard deviation; GM_{adj}: geometric mean adjusted for confounders; GSE: standard error of GM_{adj}; adjustments: CC-16: smoking and farm childhood; SP-D: % body fat and farm childhood; IL-6: % body fat, age, smoking, living at a farm and having a cold; fibrinogen: age, gender and smoking; CRP: % body fat, smoking and having a cold; sCD40L: atopy; sP-selectin: atopy, age, gender and % body fat.

Table 4. Influence of confounders and covariates on ln-transformed biomarker concentrations (β and p-value in multiple linear regression models).

Test variable	CC-16	SP-D	SP-A	IL-6	TNF- α	Fibrinogen	CRP	sCD40L	sP-selectin
Constant	1.96	5.51	3.18	1.45	0.07	0.80	1.16	-0.19	3.66
Exposed/referent	0.32***	0.05		0.51***		-0.17**			
Farm childhood yes/no	0.14*	0.24**							
Living at a farm yes/no				0.26*					
Smoker yes/no	-0.10			0.23*		0.10*	0.44*		
Atopy yes/no								0.41*	0.17*
Gender female/male						-0.16*			-0.40**
Age (year)				0.01*		0.005**			-0.01**
% body fat		-0.02**		0.03**			0.03**		0.03**
Having a cold yes/no				0.18**			0.71**		

* $p < 0.1$; ** $p < 0.05$; *** $p < 0.001$.

Table 5. General linear model of CC-16.

Model	B	SE	p-value	LCI	UCI
Ln CC16 ($r^2_{adj}=0.20$)					
Intercept	1.28	0.07		1.14	1.43
Exposed	0.34	0.08	<0.001	0.18	0.49
Controls	0				
Current smoker Yes	-0.28	0.11	0.01	-0.49	-0.07
No	0				
Farm childhood Yes	0.03	0.09	0.76	-0.14	0.20
No	0				
Farm childhood Yes*Current smoker Yes	0.51	0.18	0.006	0.15	0.87
Farm childhood No*Current smoker Yes	0				

LCI: lower limit of the 95% confidence interval; UCI: upper limit of the 95% confidence interval.

workers versus controls are shown in Table 3. The concentration of CC-16 was significantly higher in exposed workers than in controls, whereas the concentration of the other pneumoproteins, SP-D and SP-A, were similar in the two groups. The pro-inflammatory cytokine IL-6 level was significantly higher in exposed workers than in controls. However, simultaneous increases in TNF- α , CRP and fibrinogen in exposed workers were not observed. On the contrary, fibrinogen was significantly lower in exposed workers compared to controls. The concentrations of the platelet activation markers sCD40L and sP-selectin were similar in the two groups.

The strength and significance of influential confounders and covariates for the biomarkers are shown in Table 4. Biomarker concentrations were adjusted for significant confounders by multiple regression (Table 3), whereas the effects of significant covariates were explored further. This showed that exposed workers with farm childhood had higher serum concentrations of CC-16 ($p = 0.07$, adjusted for smoking) and SP-D ($p = 0.03$, adjusted for % body fat). Furthermore, although smoking was no influential covariate in the basic CC-16 model, the effect of farm childhood interacted strongly with smoking ($p = 0.006$) (Table 5). Serum CC-16 was significantly higher in current smokers with farm childhood compared with other current smokers. There were no effect of farm childhood on CC-16 in never- and former smokers. The same pattern was observed in both exposed workers and controls, although CC-16 was significantly higher in exposed workers compared with controls in all groups (Figure 2). The effect of current smoking in reducing CC-16 became significant in the model when this interaction term was included ($p = 0.01$) (Table 5).



Figure 2. Estimated marginal mean (95% confidence interval) of transformed values of serum CC-16 in exposed workers and controls by farm childhood and smoking status. Solid symbols represents current smokers, whereas hollow symbols represent never and ex-smokers. The EMMs are adjusted for interaction between smoking and farm childhood.

Farm childhood and living at a farm had otherwise no significant impact on any of the other biomarker concentrations.

Biomarker associations with exposure measurements and respiratory health effects. None of the biomarkers in blood were significantly correlated with the personal exposure measurements of dust or any of the other bioaerosol components. None of the biomarkers were associated with differences in respiratory health effects measured by spirometry and gas diffusion test or by self-reported questionnaire.

Discussion

The present study describes the concentrations of pneumoproteins, markers of systemic inflammation and platelet

activation in the blood of healthy workers in the grain industry. Workers exposed to grain dust had significantly higher levels of CC-16 and IL-6 compared with controls, and significantly lower fibrinogen concentrations. A strong influence from farm childhood on CC-16 and SP-D was observed. Farm childhood interacted with current smoking by counteracting the smoking-associated reduction of CC-16, whereas this effect was not present in workers without such childhood exposure. This interaction was stronger than the effect of dust exposure or any other exposure measurement. Although both CC-16, IL-6 and fibrinogen were significantly different between exposed and controls, none of the markers were associated with dust exposure or any other exposure component of the dust measured among exposed workers. This was in spite of exposures to potentially health-impairing bioaerosol concentrations. There were also no association with respiratory health outcomes observed.

Increased levels of inflammatory cytokines, such as TNF- α , IL-1- β , IL-6 and IL-8, in bronchoalveolar lavage fluid have been associated with reduced lung function after grain dust exposure (Jagiello *et al.* 1996, Blaski *et al.* 1996). TNF- α and IL-6 regulate the hepatic production of CRP and fibrinogen during the acute phase response of inflammation (Moshage 1997) and are considered relatively stable markers of inflammation. There were, however, no signs of systemic inflammation in the present study, as none of these additional markers were increased, although a significantly higher serum IL-6 concentration was observed in exposed workers compared with controls. The observed IL-6 increase could be a snapshot of the start of a pro-inflammatory change that did not progress to systemic inflammation, or it could also be due to chance. Another possible explanation is that dust exposure has induced tolerance, as seen in pig farmers (PalMBERG *et al.* 2002). The significantly lower fibrinogen concentration in exposed workers is difficult to relate to dust-exposure, but as physical activity is associated with reduced plasma fibrinogen (Gomez-Marcos *et al.* 2014), a possible explanation could be that exposed workers have higher physical activity than controls. Both study groups were within the reference area for fibrinogen of 1.5–4 mg/mL.

According to what is known about CC-16 control, the increased serum levels observed in exposed workers may reflect an increase in CC-16 secretion from the club cells and/or increased permeability of the lung epithelium barrier (Lakind *et al.* 2007) as a response to an inflammatory reaction elicited by exposure. However, in spite of relatively high exposures during work, no dose-response with grain dust or microbial components were observed. It is possible that other components than the measured aerosol components among the exposed workers induced this difference, but this could not be identified in the present study. Regardless of the toxic mechanism for increased serum CC-16, the results indicate ongoing anti-inflammatory reaction in exposed workers. However, since we could not relate the CC-16 increase to impaired lung function or any respiratory symptoms, it is possible that homeostasis is restored and the inflammatory response do not lead further to disease. The heterogeneous exposure, with all the different biological components, may induce inflammatory as well as anti-

inflammatory reactions in a pattern that collectively contribute to prevent further disease progression. It has been shown that inhalation of LPS results in a dose-dependent increase in blood CC-16 that peaks at 6 hours and normalizes at 24 hours (Michel *et al.* 2005). A parallel decrease in the bronchoalveolar lavage fluid has been observed in animals, in the absence of a cytotoxic effect of LPS on club cells (Arsalane *et al.* 2000). Heldal and coworkers also showed a dose dependent association between increased serum CC-16 and bacterial exposure ($GM\ 22 \times 10^3$ bacteria/m³) among wastewater workers (Heldal *et al.* 2013). The arithmetic mean serum CC-16 concentration was, however, lower in exposed workers than in controls. The bacterial exposure was almost 10-fold lower than in the present study, but most likely not similar species with similar inflammatory potential.

The hygiene hypothesis proposes that the rising prevalence of allergic diseases is causally related to a reduced exposure to environmental microbes and harmless infections (von Mutius 2007). One of the most important observations that supports the hygiene hypothesis is that children grown up on traditional farms are less prone to allergic diseases than children living in the countryside, but not on a farm (Jatzlauk *et al.* 2017). The protection seems to be associated with the exposure to a wide range of microbes provided by the farm environment (Ege, Mayer, Normand, Genuneit, Cookson, Braun-Fahrlander, Heederik, Piarroux, von Mutius and Grp, 2011, Haahtela *et al.* 2015, Stein *et al.* 2016). Early life contact to complex microbial communities is a main driver for the development of a balanced immune system (Torow *et al.* 2017) and human microbiota (Hanski *et al.* 2012). The hygiene hypothesis has consequently been refined and replaced by the biodiversity hypothesis (von Hertzen *et al.* 2011). Although the mechanisms behind the association between microbial diversity and allergy protection are still unknown, the impact of farm childhood on SP-D and CC-16 could be due to an improved tuning of several parts of the immune system, including the cell responses to inhaled pneumotoxicants. However, exposed workers are physically more active during the day, and we cannot exclude that this may be the reason for the increased CC-16 in exposed workers compared with controls that were office workers. CC-16 has indeed been shown to be higher after exercise and heavy work (Nanson *et al.* 2001), but lower in elite athletes (Kurowski *et al.* 2014). Helleday and colleagues showed that serum CC-16 concentrations in healthy nonsmoking subjects drops from 7 am to 1 pm, stabilizes somewhat, and then starts to increase again at 7 pm, following a U-shaped curve (Helleday *et al.* 2006). The time window for blood sampling in our study corresponded to the time when the smallest changes in the concentrations were observed in the study by Helleday and coworkers, and the relatively short time window makes the influence of diurnal variation unlikely.

High plasma sP-selectin (83.2 ng/mL) and sCD40L (2.89 ng/mL) concentration have been associated with increased cardiovascular risk in apparently healthy women (Schonbeck *et al.* 2001, Ridker *et al.* 2001). The sP-selectin and sCD40L concentrations in the present study were, however, lower than those levels. No significant difference in serum levels of sCD40L or sP-selectin were observed between exposed

workers and controls indicating that neither grain dust nor any other exposure in the study population had any significant role in platelet activation as indicators of increased risks of cardiovascular effects. The lack of increased CRP or fibrinogen that also is found in atherosclerosis supports this conclusion. Of note, the platelet function was not assessed, as no platelet function tests were performed. However, both exposed workers and controls in the grain elevator and compound industry had GM serum levels of sCD40L and sP-selectin 10 and 2-fold higher than plasma levels of tunnel construction workers that had slightly reduced platelet activation after high exposure to particulate matter (Ellingsen *et al.* 2017). The sP-selectin level was also higher than observed in plasma of healthy volunteers, who 24 hrs after diesel exhaust inhalation had a significantly higher sP-selectin level (36.5 ng/mL) compared with persons that inhaled clean air (33.7 ng/mL) (Tornqvist *et al.* 2007). Serum values of sP-selectin and sCD40L for healthy individuals are known to be higher than plasma values (mean 82 ng/mL, range 51–113 versus mean 29 ng/mL, range 18–40 for sP-selectin and 5.4 ng/mL, range 0–11 versus 0.09 ng/mL, range 0–0.14 for sCD40L, R&D), probably explaining the concentration differences observed between the present and other studies. Despite the differences, serum and plasma sP-selectin levels have been shown to be significantly correlated, whereas this is not the case for sCD40L (Thom *et al.* 2004). According to the reference values, the levels in the present study were in the lower range.

The effect of farm childhood on CC-16 was robust in the present study, and the interaction with smoking seemed plausible. However, as the number of current smokers were only 5 of 18 in the exposed worker group with farm childhood and 3 of 6 in the corresponding referent group these results should be interpreted with caution.

Although the clotting time in this study was kept within 30–90 minutes for all blood samples, it is possible that this time variation has resulted in variable extent of *ex vivo* release of sP-selectin and sCD40L from platelets. Such a variable influence on the *in vivo* circulating levels of soluble platelet activation markers may have obscured possible group differences.

Conclusions

Work in grain elevators and compound feed mills induced inflammatory and anti-inflammatory reactions, as shown by the significantly higher serum concentration of IL-6 and CC-16 in exposed workers compared with controls. These reactions were not associated with bioaerosol exposure or to the respiratory health effects previously measured. We could therefore not establish any cause-effect relationship with the investigated parameters. As no significant increase in the serum levels of other pneumoproteins than CC-16 were observed, the difference was most likely not due to increased permeability of the epithelial lung barrier, but could rather be due to increased secretion from club cells. Whether this is an early sign of lung disease or a reversible immunological response is still not clear. The serum CC-16 levels were

higher in workers with farm childhood, regardless of exposure, and this effect counteracted the smoking-induced reduction of serum CC-16. Grain dust exposure did not induce systemic inflammation, as no increases in CRP or fibrinogen were observed, although IL-6 was increased in exposed workers. Neither were any exposure-induced platelet activation observed, as exposed workers had similar levels of sCD40L and sP-selectin as controls, providing no support for increased risk of cardiovascular disease in this industry.

Acknowledgements

Thanks are due to Camilla Husvik and Lena Anderson for technical assistance with the ELISAs.

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethics approval

The study was approved by The Regional Ethical Committee of South-East Norway.

Funding

This study received support from the Confederation of Norwegian Enterprise (S-2585).

ORCID

Anne Straumfors  <http://orcid.org/0000-0003-1142-1671>

References

- Arsalane, K., *et al.*, 2000. Clara cell specific protein (CC16) expression after acute lung inflammation induced by intratracheal lipopolysaccharide administration. *American Journal of respiratory and critical care medicine*, 161 (5), 1624–1630.
- Aukrust, P., *et al.*, 1999. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation*, 100 (6), 614–620.
- Bernard, A.M., *et al.*, 1994. Serum Clara cell protein: an indicator of bronchial cell dysfunction caused by tobacco smoking. *Environmental research*, 66 (1), 96–104.
- Blaski, C.A., *et al.*, 1996. The role of atopy in grain dust-induced airway disease. *American journal of respiratory and critical care medicine*, 154 (2 Pt 1), 334–340.
- Broeckeaert, F., and Bernard, A., 2000. Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker. *Clinical and experimental allergy: journal of the British society for allergy and clinical immunology*, 30 (4), 469–475.
- Chan-Yeung, M., *et al.*, 1992. Five cross-sectional studies of grain elevator workers. *American journal of epidemiology*, 136 (10), 1269–1279.
- Dierynck, I., *et al.*, 1995. Potent inhibition of both human interferon-gamma production and biologic activity by the Clara cell protein CC16. *American journal of respiratory cell and molecular biology*, 12 (2), 205–210.
- Dopico, G.A., *et al.*, 1983. Acute effects of grain dust exposure during a work shift. *The American review of respiratory disease*, 128 (3), 399–404.

- Du, Y.X., et al., 2016. Air particulate matter and cardiovascular disease: the epidemiological, biomedical and clinical evidence. *Journal of thoracic disease*, 8 (1), E8–E19.
- Durnin, J.V., and Womersley, J., 1974. Body Fat Assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 years. *The British journal of nutrition*, 32 (1), 77–97.
- Ege, M.J., et al., 2011. Exposure to environmental microorganisms and childhood asthma. *New England journal of medicine*, 364 (8), 701–709.
- Ellingsen, D.G., et al., 2017. Biomarkers of endothelial activation and thrombosis in tunnel construction workers exposed to airborne contaminants. *International archives of occupational and environment health*, 90 (4), 309–317.
- Fang, S.C., Cassidy, A., and Christiani, D.C., 2010. A systematic review of occupational exposure to particulate matter and cardiovascular disease. *International journal of environmental research and public health*, 7 (4), 1773–1806.
- Ferroni, P., et al., 2008. In vivo platelet activation is responsible for enhanced vascular endothelial growth factor levels in hypertensive patients. *Clin chim acta*, 388 (1–2), 33–37.
- Fijnheer, R., et al., 1997. The origin of P-selectin as a circulating plasma protein. *Thromb haemostasis*, 77, 1081–1085.
- Gomez-Marcos, M.A., et al., 2014. Relationship between physical activity and plasma fibrinogen concentrations in adults without chronic diseases. *PLoS one*, 9 (2), e87954.
- Haahela, T., et al., 2015. Hunt for the origin of allergy - comparing the Finnish and Russian Karelia. *Clinical and experimental allergy*, 45 (5), 891–901.
- Halstensen, A.S., et al., 2013. Exposure to grain dust and microbial components in the Norwegian grain and compound feed industry. *The annals of occupational hygiene*, 57 (9), 1105–1114.
- Hanski, I., et al., 2012. Environmental biodiversity, human microbiota, and allergy are interrelated. *Proceedings of the national academy of sciences of the United States of America*, 109 (21), 8334–8339.
- Health Council of the Netherlands 2011. Grain dust. Health-based recommended occupational exposure limit. *The Hague: Health council of the Netherlands* publication no. 2011/13.
- Heeschen, C., et al., 2003. Soluble CD40 ligand in acute coronary syndromes. *The new England journal of medicine*, 348 (12), 1104–1111.
- Heldal, K.K., et al., 2013. Pneumoproteins in sewage workers exposed to sewage dust. *International archives of occupational and environmental health*, 86 (1), 65–70.
- Helleday, R., et al., 2006. Exploring the time dependence of serum Clara cell protein as a biomarker of pulmonary injury in humans. *Chest*, 130 (3), 672–675.
- Huy, T., et al., 1991. Grain dust and lung function. Dose-response relationships. *The American review of respiratory disease*, 144 (6), 1314–1321.
- Jagiello, P.J., et al., 1996. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. *Chest*, 110 (1), 263–270.
- Jatzlauk, G., et al., 2017. Influences of environmental bacteria and their metabolites on allergies, asthma, and host microbiota. *Allergy*, 72 (12), 1859–1867.
- Kuroski, M., et al., 2014. Association of serum Clara cell protein CC16 with respiratory infections and immune response to respiratory pathogens in elite athletes. *Respiratory research*, 15, 45.
- Lakind, J.S., et al., 2007. A critical review of the use of Clara cell secretory protein (CC16) as a biomarker of acute or chronic pulmonary effects. *Biomarkers*, 12 (5), 445–467.
- Medical, R.C., 1960. Standardized questionnaires on respiratory symptoms: medical research council's committee on the aetiology of chronic bronchitis. *British medical Journal*, 2, 1665.
- Michel, O., Murdoch, R., and Bernard, A., 2005. Inhaled LPS induces blood release of Clara cell specific protein (CC16) in human beings. *The journal of allergy and clinical immunology*, 115 (6), 1143–1147.
- Moshage, H., 1997. Cytokines and the hepatic acute phase response. *The journal of pathology*, 181 (3), 257–266.
- Nanson, C.J., et al., 2001. Exercise alters serum pneumoprotein concentrations. *Respiratory physiology*, 127 (2–3), 259–265.
- Pahwa, P., Dosman, J.A., and McDuffie, H.H., 2008. Effects of grain dust on lungs prior to and following dust remediation. *Journal of occupational and environmental medicine*, 50 (12), 1394–1400.
- Palmberg, L., et al., 2002. Airway responses of healthy farmers and non-farmers to exposure in a swine confinement building. *Scandinavian journal of work, environment and health*, 28 (4), 256–263.
- Ridker, P.M., Buring, J.E., and Rifai, N., 2001. Soluble P-selectin and the risk of future cardiovascular events. *Circulation*, 103 (4), 491–495.
- Robin, M., et al., 2002. Serum levels of CC16, SP-A and SP-B reflect tobacco-smoke exposure in asymptomatic subjects. *The European respiratory journal*, 20 (5), 1152–1161.
- Schonbeck, U., et al., 2001. Soluble CD40L and cardiovascular risk in women. *Circulation*, 104 (19), 2266–2268.
- Siri, W., 1961. Body composition from fluid space and density. In: Brozek, J. & Hanschel, A. (eds) *Techniques for measuring body composition*. Washington DC: National Academy of Science, 223–244.
- Smid, T., et al., 1994. Dust-related and endotoxin-related acute lung-function changes and work-related symptoms in workers in the animal feed-industry. *American journal of industrial medicine*, 25 (6), 877–888.
- Smith, J. D., 1989. Some biological components of grain and forage dusts. In: Dosman, J.A., Cockroft, D. W. eds. *Principles of health and safety in agriculture*. Boca Raton, FL: CRC Press.
- Spurzem, J.R., Romberger, D.J., and Von Essen, S.G., 2002. Agricultural lung disease. *Clin chest med*, 23 (4), 795–810.
- Stein, M.M., et al., 2016. Innate immunity and asthma risk in Amish and Hutterite farm children. *The New England journal of medicine*, 375 (5), 411–421.
- Steiner, D., et al., 2005. Clara cell protein and surfactant protein B in garbage collectors and in wastewater workers exposed to bioaerosols. *International archives of occupational and environmental health*, 78 (3), 189–197.
- Straumfors, A., et al., 2015. Mycotoxins and other fungal metabolites in grain dust from Norwegian grain elevators and compound feed mills. *World mycotoxin J*, 8 (3), 361–373.
- Straumfors, A., et al., 2015. Work tasks as determinants of grain dust and microbial exposure in the norwegian grain and compound feed industry. *The annals of occupational hygiene*, 59 (6), 724–736.
- Straumfors, A., et al., 2016. Cross-shift study of exposure-response relationships between bioaerosol exposure and respiratory effects in the Norwegian grain and animal feed production industry. *Occupational and environmental medicine*, 73 (10), 685–693.
- Thom, J., et al., 2004. Measurement of soluble P-selectin and soluble CD40 ligand in serum and plasma. *Journal of thrombosis and haemostasis: JTH*, 2 (11), 2067–2069.
- Tornqvist, H., et al., 2007. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *American journal of respiratory and critical care medicine*, 176 (4), 395–400.
- Torow, N., et al., 2017. Neonatal mucosal immunology. *Mucosal immunology*, 10 (1), 5–17.
- van, d., and Wal, A. 1983. Comparative measurements of the total dust concentration at the work place with different samplers—part 1. *Staub-reinhalt luft*, 43, 292–294.
- von Hertzen, L., Hanski, I., and Haahela, T., 2011. Natural immunity. Biodiversity loss and inflammatory diseases are two global megatrends that might be related. *EMBO reports*, 12 (11), 1089–1093.
- von Mutius, E., 2007. Allergies, infections and the hygiene hypothesis—the epidemiological evidence. *Immunobiology*, 212 (6), 433–439.