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ORIGINAL ARTICLE

Exposure to field vs. storage wheat dust: different consequences on respiratory symptoms and immune response among grain workers

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Abstract

Purpose The aim of this study was to understand the differential acute effects of two distinct wheat-related dusts, such as field or stored wheat dust handling, on workers' health and how those effects evolved at 6 month intervals.

Methods Exposure, work-related symptoms, changes in lung function, and blood samples of 81 workers handling wheat and 61 controls were collected during the high exposure season and 6 months after. Specific IgG, IgE, and precipitins against 12 fungi isolated from wheat dust were titrated by enzyme-linked immunosorbent assay, dissociation-enhanced lanthanide fluorescence immunoassay, and electrosyneresis. The level of fungi was determined in the workers' environment. Levels of exhaled fraction of nitrogen monoxide (F_ENO) and total IgE were obtained. Exposure response associations were investigated by mixed logistic and linear regression models.

Results The recent exposure to field wheat dust was associated with a higher prevalence for five of six self-reported airway symptoms and with a lower F_ENO than those in the control population. Exposure to stored wheat dust was only associated with cough. No acute impact of exposure on respiratory function was observed. Exposure to field wheat dust led to workers' sensitization against the three field fungi *Aureobasidum, Cryptococcus*, and *Phoma*, although exposure to storage wheat dust was associated with tolerance. The level of Ig remained stable 6 months after exposure.

Conclusion The clinical picture of workers exposed to field or storage wheat dust differed. The systematic characterization of the aerosol microbial profile may help to understand the reasons for those differences.

Keywords Grain workers · Cattle raisers · Respiratory symptoms · Occupational wheat dust exposure · Fungi-specific immunoglobulins

Introduction

Grain workers and cattle raisers are chronically exposed to the dust generated during wheat handling, which leads to airway symptoms and declining lung function (Dorribo et al. 2015). Operators handling grain or straw may complain of a chronic cough that can be accompanied in the short-term by a scratchy throat (Dorribo et al. 2015) or by a decline in lung function over the long term (Dorribo et al. 2015) and an increased incidence of chronic diseases, such as asthma (Rask-Andersen 2011; Kline et al. 2000), chronic bronchitis (Jouneau et al. 2012), or farmer's lung disease (Dalphin et al. 2009). To avoid the development of such pathologies, collective and personal protection has been greatly improved in developed countries to decrease the exposure level to grain dust by workers (Spankie and Cherrie 2012; Halstensen et al.

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2013). However, the increased risk of morbidity and mortality in this worker population continue to be a concern (Eduard et al. 2009; Dorribo et al. 2015). The prevalence of acute airway symptoms continues to increase with the level of exposure to dust during wheat handling even if its concentration seldom exceed 4 mg m^{-3}, which is the occupational exposure limit defined by the National Institute for Occupational Safety and Health (Dorribo et al. 2015; Halstensen et al. 2013).

Grain workers and cattle raisers are exposed to different levels of wheat dust throughout the year. Grain workers perform high exposure activities during summer when they handle freshly harvested grain in the field, at the grain terminal, or on the farms. The remainder of the year they are exposed to wheat dust during transfer of stored grain. In contrast, cattle raisers might be exposed to wheat dust year round during the handling of stored wheat straw bales and the spreading of straw as bedding for cows (Roussel et al. 2011b). The level of exposure to dust varies depending on the task involving grain or straw handling, use of mechanical assistance, the presence of collective protective equipment, and wearing of personal protective equipment (Dorribo et al. 2015; Halstensen et al. 2013).

Dust generated during wheat handling contains a large diversity of components capable of causing airway inflammation and an allergic response. These components could be derived from microorganisms, such as endotoxins, β-1,3-glucans, mycotoxins, species-specific allergens, or other biological materials (plant fragments, insect, mite and rodent body parts, pesticides, and soil particles) (Halstensen et al. 2013). Endotoxin exposure was the first etiological factor proposed to explain the biological effects caused by grain dust exposure (Jagielo et al. 1996). Since then, respiratory symptoms have been shown to have a stronger association with the air's fungal spore content than with endotoxin (Straumfors et al. 2016). Fungi act as irritants, toxins, aeroallergens, or pathogens that cause infection depending on underlying disease, species, and form (Wiszniewska et al. 2013; Vacher et al. 2015; Kuhn and Ghannoum 2003). Certain molds abundant in freshly harvested wheat are known to be allergenic, such as *Alternaria alternata* and *Cladosporium cladosporioides* (Pellissier et al. 2016; Madsen et al. 2015; Flannigan 1978; Gora et al. 2009; Swan and Crook 1998), or toxigenic, such as *Fusarium graminearum* and *Fusarium culmorum*, two mycotoxin-producing fungal species (Pellissier et al. 2016). The microflora of freshly harvested wheat differs from that of stored grain and straw which contains "storage fungi", such as *Penicillium brevicompactum*, and *Eurotium amstelodami* (Swan and Crook 1998). This variation in microbial flora between field and stored wheat might explain the contradictory results between studies on the health effects of grain dust (May et al. 2012). To test this hypothesis, we compared the health effects induced by handling freshly harvested wheat (called hereafter field wheat) to those induced by handling stored wheat. Despite the high microbial diversity of these dusts, only a few biological agents have been associated with the development of respiratory diseases in these occupational populations. We also chose a set of representative biological agents and researched how healthy wheat workers' immune systems respond to such a complex combination of microbial species and how long the response is maintained after exposure. Thus, grain workers and cattle raisers were visited at 6 month intervals to determine their health status, collect blood to screen for sensitization markers (specific IgE, IgG, and precipitins) to environmental fungi, and to sample settled dust looking for antigens.

Materials and methods

Study design

A longitudinal study was conducted on two different wheat working populations: a population of grain workers handling large volumes of field wheat and a population of cattle raisers handling large volumes of stored wheat. The acute effects of inhaling wheat dust on the respiratory health of those two worker populations were determined by comparison to a reference population with no occupational exposure to wheat dust during the study period. Each population was seen twice between August 2012 and June 2013; the first time during the period when the largest quantities of wheat grain or straw are handled (field or storage), and the second time 6 months later. All participants were examined at work. Detailed information on occupational exposure and work-related acute symptoms were obtained by questionnaire. The examinations included spirometry and a measure of the fraction of exhaled nitric oxide (F_ENO), which is a non-invasive marker for early detection of airway inflammation caused by exposure to organic dust (Sundblad et al. 2002; Moen et al. 2016). Blood was systematically sampled to determine sensitization markers (specific IgE, IgG, and precipitins) to specific biological agents in field or stored wheat. The presence of the respective fungal species in the workers' environment was researched in settled dust by high-throughput sequencing and those results were described in detail elsewhere (Pellissier et al. 2016).

Subjects

The enrollment and cross-sectional survey of the populations handling field or stored wheat have been described previously (Dorribo et al. 2015). Exclusion criteria were an ongoing corticosteroid or immunosuppressant treatment, obesity (BMI > 40 kg m^{-2}), difficulty in understanding the

questionnaire, or current inclusion in another study protocol. From the 149 volunteers recruited in 2012 from the Vaud region, Switzerland, 142 accepted to participate in the overall protocol, including 32 grain workers handling large quantities of field wheat, 42 handling large quantities of stored straw, and 68 workers not occupationally exposed to wheat dust during the study period (61 subjects employed at different hospital facilities of the university hospital of Lausanne and seven grain farmers). The characteristics of the study population are given in Table 1. The Human Research Ethics Committee from Vaud, Switzerland approved this study (Protocol 130/12). Written informed consent was obtained from all participants.

Collection of work-related symptoms

The participants' interview was done by a trained nurse following a questionnaire adapted from the European Coal and Steel Community questionnaire (Minette 1989) that included questions on smoking habits, symptoms of the airways, eyes, and skin, as well as gastrointestinal and systemic symptoms experienced during or after work. Self-declaration of cough, wheezing, dyspnea, runny/stuffy nose, sneezing and scratchy throat was considered acute respiratory symptoms. Systemic symptoms were defined as the presence of any of the following: headache, fatigue, muscles aches, or fever. Chronic symptoms were defined as the presence of cough or phlegm symptoms for at least 3 months during the last 2 years. Usual respiratory problems were defined as the presence of any usual subjective, non-spontaneously reversible respiratory troubles reported by the subject. Work-related symptoms were defined as any symptoms present during work that improved or disappeared at night, during the weekend, or on holidays.

Clinical atopy was obtained from the questionnaire as the presence of any allergic symptoms or disease during childhood (asthma, eczema, or rash). Total IgE was quantifiably assessed for all non-specific acute immune reactions.

Table 1 Characteristics of the study population

	Controls	Exposed to wheat dust in V1 or/and V2	Exposed to field wheat in V1		Exposed to stored wheat in V1	
			But not exposed in V2	And exposed to stored wheat in V2	But not exposed in V2	And exposed to stored wheat in V2
Volunteer number	68	74	12	20	10	32
Age, mean (SD)	40.6 (10.4)	41.4 (12.3)	42.3 (13.8)	39.5 (12.8)	44.8 (9.6)	41.2 (12.5)
Male gender, *n* (%)	68 (100)	73 (99)	12 (100)	19 (95)	10 (100)	32 (100)
BMI, mean (SD)	24.6 (4.0)	26.0 (3.0)	26.2 (3.6)	26.7 (2.7)	25.0 (3.0)	25.8 (3.1)
Smoking status at V1						
Smokers, *n* (%)	11 (16)	24 (32)	3 (25)	12 (60)	2 (20)	7 (22)
Cigarettes/day, median (IQR)	2.0 (12.0)	12.6 (14.8)	10.0 (9.3)	20.0 (10.0)	10.3 (19.4)	10.0 (7.4)
Ex-smokers, *n* (%)	23 (34)	13 (18)	3 (25)	2 (10)	2 (20)	6 (19)
Smoking status at V2						
Smokers, *n* (%)	17 (25)	27 (36)	4 (33)	12 (60)	4 (40)	7 (22)
Cigarettes/day, median (IQR)	4.3 (14.0)	10.0 (15.0)	8.5 (9.0)	17.5 (10.0)	5.7 (14.3)	5.0 (9.9)
Ex-smokers, *n* (%)	19 (28)	11 (15)	3 (25)	2 (10)	0 (0)	6 (19)
Duration (in hours) of wheat-related tasks in the 6 weeks preceding						
V1, mean (SD)	0.0 (0.0)	83.4 (102.2)	165.9 (108.7)	151.0 (119.0)	18.4 (11.7)	30.4 (43.0)
V2, mean (SD)	0.0 (0.0)	18.2 (30.8)	0.0 (0.0)	38.4 (41.0)	0.0 (0.0)	18.2 (26.5)
Number of subjects exposed to more than 4 mg m^{-3}						
Before V1, *n* (%)	0 (0)	8 (11)	2 (17)	2 (10)	0 (0)	4 (13)
Before V2, *n* (%)	0 (0)	8 (11)	0 (0)	4 (20)	0 (0)	4 (13)
Clinical atopy, *n* (%)	4 (6)	3 (4)	0 (0)	2 (10)	1 (10)	0 (0)
Total IgE > 100 UI/ml serum, *n* (%)	18 (13)	20 (14)	5 (21)	8 (20)	3 (15)	4 (6)

V1 first medical examination, *V2* second medical examination, *BMI* body mass index

Lung function

Spirometry was performed using the EasyOne® device, (NDD, Zürich, Switzerland) following the 2005 American Thoracic Society/European Respiratory Society (ERS) guidelines. Three reproducible measurements for the following parameters were recorded: forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), peak expiratory flow, and the Tiffeneau index (FEV1/FVC%). According to the ERS guidelines, these parameters are expressed as the percentage between observed values and predicted values by sex, age, and height. All study participants were Caucasian, so that no adjustment on race was necessary.

Exhaled nitric oxide

F_ENO was used as a surrogate marker for eosinophilic airway inflammation (Dweik et al. 2011). F_ENO was measured by an electrochemical analyzer (NIOX MINO device; Aerocrine®, Stockholm, Sweden), as described previously (Dorribo et al. 2015).

Assessment of exposure to wheat dust

A detailed occupational history, including job title, workplace, start and stop dates, technological changes during their career, tasks undertaken in the previous 6 months with their duration and frequency, the collective and personal respiratory protective equipment used, and plants handled was obtained by face-to-face questionnaire with each participant during the first visit after the medical examination (V1). At the second visit (V2), only the recent occupational exposure, including tasks undertaken within the last 6 months with their duration and frequency, plants handled, and the collective and personal protective devices used was questioned. The workplace was systematically visited on V1 and V2 by an occupational hygiene specialist who estimated the exposure level of each participant to wheat dust during each wheat-related task in the previous 6 weeks based on a task-exposure matrix established in a previous study (Dorribo et al. 2015).

The following formulas have been applied for recent exposure to wheat dust (E_{6w}) and cumulative chronic exposure over a career (E_{tot}):

$$E_{6w} = \left[\left(h \times d_{6w}\right)_{task1} \times l_{task1} + \left[\left(h \times d_{6w}\right)_{task2} \times l_{task2} + [\ldots + [\left(h \times d_{6w}\right)_{taskn} \times l_{taskn}$$

$$E_{tot} = \left[\left(h \times d_y \times y_{tot}\right)_{task1} \times l_{task1} + \left[\left(h \times d_y \times y_{tot}\right)_{task2} \times l_{task2} + [\ldots + [\left(h \times d_y \times y_{tot}\right)_{taskn} \times l_{taskn}$$

where h is the number of exposed hours per day, d_{6w} is the number of days in which the task was performed in the 6 weeks before the medical examination, l_{taskn} is the level of exposure to wheat dust during the task "n" estimated by Dorribo et al. (2015), d_y is the number of days in which the task was performed per year and y_{tot} is the number of years in which the task was performed over a career.

To estimate possible confounding exposure to dust generated during handling of other plants, such as hay, we computed separate cumulative exposure indicators under the assumption that the level of those dusts was similar to that of wheat dust when similar tasks were accomplished.

Assessment of exposure to microbes

Settled dust was collected in the environment of each participant with the electrostatic dust collector (EDC) described by Noss et al. (2008) and validated for the efficiency of microbe quantification by molecular biology as described by Scherer et al. (2014). The EDC was exposed to the air on a horizontal surface between 1.20 and 1.60 m above the floor at the workplace or in an occupied room. Dust was allowed to settle over a 6-week period starting with each medical examination. Participants returned the EDC by mail at the end of the sampling period.

An EDC washing step was performed as described previously (Scherer et al. 2014), as well as DNA extraction, generation of internal transcribed spacer 1 (ITS1) amplicons, and their high-throughput sequencing (Pellissier et al. 2016). Briefly, each wipe contained in the EDC was washed with a 0.1% Tween 80 solution for 10 min in a Stomacher™ (AES®, Combourg, France). The collected liquid was centrifuged for 30 min at 8500×g. The pellet was mechanically disrupted with a Tissue Lyser (Qiagen, Hilden, Germany) in the first buffer of a FastDNA Spin Kit for Soil (MP Biomedical, Zurich, Switzerland). Then, total DNA was extracted according to the manufacturer's instructions. The ITS1 region was amplified using the forward primer ITS1F and the reverse primer ITS2 and paired-end sequenced on a GS FLX instrument with the FLX Titanium reagents at Microsynth (Balgach, Switzerland). The sequenced paired-end reads were demultiplexed and quality filtered using in-house scripts. The filtered reads were clustered into operational taxonomic units (OTUs) at the 97% similarity threshold using QIIME v.1.7.0 (Caporaso et al. 2010). The OTUs that fit at 100% to the following IDs AM161136, AJ853759, AJ244269, AM286197, LK022839, AJ269841, AJ853761, AJ491291, AJ491293, AJ608949, AJ493582, and AJ853460 were identified with the CLUSTALW alignment program and considered to correspond to the respective fungal species: *Acremonium strictum, Alternaria alternata, Aureobasidium pullulans, Cladosporium cladosporioides, Cryptococcus victoriae, Epicoccum nigrum, Eurotium amstelodami,*

Fusarium culmorum, Fusarium graminearum, Penicillium chrysogenum, Phoma sp., and *Sporobolomyces roseus*. The number of reads of each OTU per sample was considered an indicator of the corresponding species abundance.

Antigen extract preparation

Strains of 11 of the 12 targeted species were successfully isolated by culture from wheat dust on one of the following media: DG18, Malt-agar, salt malt, PDA or Chromagar candida at 20 or 37 °C, Sabouraud at 12 °C, R8 at 52 °C, and *Actinomycetes* Difco at 30 °C. The only *Cryptococcus* isolated from the environmental samples was *Cryptococcus albidus*. This strain was used to prepare the antigen. The crude-extracted antigens were produced as described previously by Reboux et al. (2007) and the protein extract was purified as described by Roussel et al. (2011a).

Blood sampling

Blood was collected at the workplace in lithium-heparin plasma separator tubes and left at room temperature for 30 min before a 10-min centrifugation at 3500 rpm. In the lab, the plasma was gently transferred into new tubes without additives and maintained at − 80 °C until analysis. The present allergic status was assessed by quantifying total serum IgE using the Immuno-Cap-100 System (Phadia, Uppsala, Sweden). An ImmunoCAP value ≥ 0.35 kU_A/L was considered positive. Total IgE levels > 100 kU_A/L were considered elevated (Wiszniewska et al. 2013).

Precipitin analysis

Serum precipitins (precipitating antibody–antigen complex visualized by Coomassie Blue staining) were investigated by electrosyneresis on cellulose acetate with the crude extract antigens from each of the species of interest as described previously (Reboux et al. 2006). All results were read blindly by two operators. Two arcs of precipitins were chosen as the positive cut-off point.

Antigen-specific immunoglobulin analysis

Fungi-specific immunoglobulin (Ig) G antibodies to the purified protein extract from each of the species of interest were measured using an enzyme-linked immunosorbent assay (ELISA) protocol described previously (Roussel et al. 2011a). Fungi-specific IgE antibodies to the purified protein extract from the 12 different species were measured in sera of participants by dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA®), as described previously (Barrera et al. 2016). All experiments were performed in triplicate. Results are expressed as an optical density value for the ELISA antibody measurement and as Europium counts for fluorometric measurement by DELFIA® and were normalized to the value of a reference serum. The ELISA reference serum was composed of five sera from patients with Farmer's lung disease confirmed by clinicians. The DELFIA® reference serum was composed of sera of patients with multiple allergies confirmed by clinicians. Patient sensitivity to one particular Ig was defined as negative, positive, or borderline depending on the sample to reference ratio falling below 0.95, above 1.1 or between these intervals, respectively.

Statistical analysis

Four types of outcome categories are reported as a function of exposure in the statistical analysis. The first was the reported work-related symptoms, including cough, wheezing, dyspnea, lower airway symptoms (at least one of the three preceding), sneezing, scratchy throat, runny or stuffy nose, upper airway symptoms (at least one of the three preceding), and systemic symptoms (any non-specific symptom related to work except the former, headache, and fever). These symptoms, recorded at both visits, were analyzed using a two-level logistic model with the subject ID as a random effect adjusted for smoking, season, and recruitment type (hospital-based controls vs. non-exposed grain farmers). Four statistical models were considered. The first model (model 1) included recent occupational exposure to field wheat dust or stored wheat dust (yes vs. no), the second model (model 2) included the maximal level of recent occupational exposure to field or stored wheat dust coded as 1: no recent exposure, 2: exposed but to < 4 mg m^{-3} of personal inspirable dust, 3: exposed to > 4 mg m^{-3}. The third model (model 3) considered the duration of wheat-related tasks in hours with a distinction between those handling field or stored wheat in the 6 weeks prior to the medical examination. The fourth model (model 4) included E_{6w}, which was the overall recent occupational exposure to wheat field or stored dust. The second type of outcome considered was the humoral response to a recent exposure to specific fungal antigens. A positive response was considered when the specific Ig concentrations were higher than the internal reference by 10% and when at least two precipitin arcs were observed. These outcomes were again analyzed using a two-level logistic model with subject ID as a random effect adjusted for smoking and season following the same models as those used for the symptoms. The third category of outcomes was log-transformed F_ENO, which was analyzed using a linear mixed model with subject ID as a random effect adjusted for smoking and atopy following the same occupational exposure models. The fourth outcome was FEV_1, which was expressed as the difference between its value in L and the predicted value by age, sex, and height

according to the ERS reference values. This was analyzed using a linear mixed model with subject ID as a random effect adjusted for smoking category and pack-years. The same models as before were applied. A supplementary model was fitted for the last two outcomes by considering estimated lifelong cumulative exposure (E_{tot}) as an independent variable. All analyses were carried using the Stata 14 statistical software (StataCorp LP, TX USA).

Results

Exposure assessment

A major difference was observed between the occupational exposure profile to wheat dust of grain workers and cattle raisers. Overall, the grain workers handled field grain intensively during the short period of wheat harvesting of about 10.5 ± 3.5 h during 15 days. Six months later, 62.5% performed stored wheat, grain or straw tasks. In contrast, cattle raisers were exposed to wheat dust during handling of stored straw which was a regular activity over the entire year for 76.2% of them (Table 1). In general, the duration of wheat-related tasks drastically decreased between the first (V1) and second visit (Table 1), particularly for the workers handling field wheat at V1. The level of exposure was not dependent on the type of wheat handled but on the type of task and the presence of collective protective equipment (Table 1). The most exposing activities (i.e., > 4 mg m^{-3}) were machines/infrastructure cleaning and direct contact with wheat during harvesting or unloading (absence of collective protective equipment). However, 90% of the volunteers accomplished wheat-related tasks that exposed them to < 4 mg m^{-3}.

The frequencies of the antigens in the volunteers' environment were significantly different among dust types only for *Eurotium amstelodami* ($p = 0.025$), which was less frequent in field wheat dust than in stored wheat dust or house dust. However, the abundance of most antigens differed significantly among dusts. *Aureobasidium pullulans, Cryptococcus victoriae*, and *Fusarium* species were more abundant in the field wheat dust, although *E. amstelodami* was more abundant in the stored wheat dust (Table 2). Three of the four antigens with a very low mean number of reads corresponded to antigens with a low frequency in the samples (< 12.5% of samples). Notably, the most abundant antigens among those described here (*Alternaria alternata, Cladosporium cladosporioides*, and *Epicoccum nigrum*) were also less abundant in stored wheat dust than in house dust (Table 2).

Population characteristics and self-reported symptoms

The participants were on average 42 years old at the first medical examination. None of the participants had a history of chronic obstructive pulmonary disease (COPD), asthma, or other chronic disease and none had suffered a respiratory tract infection during the 6 weeks prior to each medical examination. Among the workers recently exposed to wheat, 31% were current smokers, and 27% had elevated

Table 2 Frequency and abundance of antigens in different types of dusts

	Presence, expressed in %			Abundance, expressed by the mean number of reads ± SD		
	House dust	Field wheat dust	Stored wheat dust	House dust	Field wheat dust	Stored wheat dust
Ag more abundant in field wheat dust						
Aureobasidium pullulans	95.4	100.0	93.6	19.69 ± 16.91	52.36 ± 53.46	19.09 ± 25.12
Cryptococcus victoriae	97.7	100.0	95.2	26.19 ± 50.99	115.9 ± 71.83	50.79 ± 61.46
Fusarium culmorum/Fusarium graminearum	79.1	100.0	87.1	8.51 ± 16.31	51.95 ± 47.70	21.60 ± 46.25
Ag more abundant in stored wheat dust						
Eurotium amstelodami	39.5	18.8	54.8	0.66 ± 1.17	0.34 ± 1.01	1.58 ± 2.67
Ag less abundant in stored wheat dust						
Alternaria alternata	97.7	100.0	96.8	112.62 ± 105.90	77.18 ± 43.13	34.29 ± 42.74
Cladosporium cladosporioides	100.0	100.0	100.0	302.98 ± 175.30	293.40 ± 152.38	170.35 ± 222.09
Epicoccum nigrum	100.0	100.0	96.8	220.08 ± 185.11	217.15 ± 142.52	135.31 ± 149.36
Ag with a low level in all tested dusts						
Acremonium strictum	11.6	12.5	11.3	0.24 ± 0.87	0.27 ± 0.99	0.86 ± 7.03
Penicillium brevicompactum	2.3	0.0	0.0	0.05 ± 0.30	0	0
Phoma sp.	58.1	37.5	48.4	2.57 ± 5.26	0.50 ± 0.80	0.94 ± 1.67
Sporobolomyces roseus	7.0	0.0	8.1	0.16 ± 0.61	0	0.15 ± 0.90

total IgE (> 100 UI/ml serum), although among controls 16% were current smokers and 21% had elevated total IgE (Table 1). Work-related respiratory symptoms due to wheat dust exposure were more common than ocular and cutaneous symptoms. Distinct significant associations between the type of dust generated by handling of field or stored wheat and the prevalence of the different respiratory symptoms were observed (Table 3). The prevalence of all declared lower airway symptoms, including cough, wheezing and dyspnea, as well as those of two upper airway symptoms, such as runny/stuffy nose and scratchy throat, and the systemic symptoms increased significantly with exposure level to the aerosols generated during handling of the field wheat, although only the prevalence of cough increased with the level of exposure to the aerosols generated during the handling of stored wheat. Interestingly, dyspnea also increased with the duration of the tasks related to field wheat. Significant increases in the prevalence of most of those respiratory symptoms, except cough and nose congestion was also associated with the abundance of *A. pullulans* and *C. victoriae* in the settled dust (data not shown).

Effects on lung function and acute airway inflammation

FEV_1 measurements were not significantly different between workers handling wheat during the present study and controls and were not associated with recent exposure to wheat dust, regardless of whether the subjects handled field or stored wheat. Moreover, they were remarkably stable at the 6-month interval, even if exposure to organic aerosols decreased or stopped between the first and second medical examinations. Nevertheless, chronic exposure to wheat dust was significantly associated with a decline of FEV1 in wheat workers (Table 4).

An increase in recent exposure to field wheat dust was associated with a decrease in F_ENO concentration, even if the effect of smoking on F_ENO was considered. However, no association was found between recent exposure to stored wheat dust and the F_ENO values (Table 4).

Table 3 Multiple two-level logistic regression models of declared symptoms with the subject ID as a random effect according to four models with different exposure indices to wheat dust

Outcome	Exposure to field wheat dust [OR (95% CI)]				Exposure to stored wheat dust [OR (95% CI)]			
	Yes/No[Model 1]	Level[Model 2]	Duration in the previous 6 weeks[Model 3]	E_{6w} [Model 4]	Yes/No[Model 1]	Level[Model 2]	Duration in the previous 6 weeks [Model 3]	E_{6w} [Model 4]
Symptoms								
Lower airway respiratory symptoms	9.76 [2.54–37.49]***	5.22 [1.79–15.20]**	1.04 [1.00-1.08]	2.39 [0.77–7.41]	2.82 [1.03–7.70]*	1.75 [0.87–3.53]	0.99 [0.89–1.09]	0.63 [0.19–2.10]
Cough	6.88 [1.43–33.27]*	3.91 [1.22–12.53]*	1.00 [0.96–1.05]	1.13 [0.72–1.77]	5.10 [1.30-20.01]*	3.08 [1.17–8.10]*	0.97 [0.87–1.09]	0.74 [0.20–2.73]
Wheezing	5.44 [0.91–32.50]	4.11 [1.06–15.98]*	1.043 [0.993–1.096]	1.60 [0.83–3.06]	1.14 [0.27–4.88]	1.01 [0.33–3.05]	0.97 [0.83–1.12]	0.77 [0.15–4.08]
Dyspnea	12.96 [1.65–101.88]*	6.32 [1.55–25.86]**	1.09 [1.01–1.16]*	1.96 [0.94–4.12]	1.34 [0.28–6.37]	0.89 [0.29–2.72]	0.88 [0.69–1.12]	0.05 [0.00–7.84]
Systemic symptoms	21.23 [1.64–275.23]*	6.24 [1.48–26.24]*	1.02 [0.97–1.08]	1.51 [0.92–2.47]	4.68 [0.50-43.84]	1.68 [0.55–5.15]	0.99 [0.85–1.16]	0.63 [0.07–5.57]
Upper airway respiratory symptoms	2.74 [0.78–9.66]	2.46 [0.89–6.81]	1.01 [0.97–1.05]	1.72 [0.72–4.13]	1.38 [0.47–4.03]	1.33 [0.60–2.96]	1.03 [0.92–1.16]	1.10 [0.27–4.46]
Runny/stuffy nose	5.59 [0.97–32.17]	4.22 [1.07–16.66]*	1.00 [0.95–1.06]	2.21 [0.74–6.59]	3.31 [0.73–14.99]	2.14 [0.71–6.45]	1.10 [0.96–1.26]	2.06 [0.42–10.18]
Sneezing	3.01 [0.72–12.67]	2.73 [0.93–8.06]	1.04 [0.99–1.08]	2.45 [0.87–6.91]	0.80 [0.22–2.98]	0.69 [0.24–1.97]	1.05 [0.93–1.19]	1.10 [0.22–5.53]
Scratchy throat	8.43 [1.19–59.20]*	7.61 [1.71–33.82]**	1.03 [0.98–1.09]	3.08 [0.89–10.61]	2.79 [0.45–17.49]	2.75 [0.77–9.82]	0.96 [0.79–1.17]	0.55 [0.02–12.18]

"-" Lack of convergence due to the small sample size; odds ratio (OR) derived using the category of grain workers not exposed in the previous 6 weeks adjusted for age, smoking status, and season; E_{6w} corresponds to the indicator of recent exposure to wheat dust in the last 6 weeks

*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$

Table 4 Results of multiple linear mixed model of lung function parameter FEV1 and inflammatory marker F_ENO with the subject ID as a random effect according to four models with different exposure indices to wheat dust

Variable	FEV1[a]		F_ENO[b]	
	Regression coefficient	*p* value	Regression coefficient	*p* value
Exposure to field wheat dust				
Yes/No[Model 1]	−0.037	0.531	−0.432	< 0.001
Level[Model 2]	−0.003	0.952	−0.244	0.002
Duration in the last 6 weeks[Model 3]	0.000	0.580	−0.001	0.004
E_{6w}[c,Model 4]	0.009	0.713	0.009	0.713
Exposure to stored dust				
Yes/No[Model 1]	−0.012	0.841	−0.031	0.721
Level[Model 2]	−0.008	0.865	0.055	0.420
Duration in the last 6 weeks[Model 3]	0.001	0.193	0.002	0.219
E_{6w}[d,Model 4]	0.029	0.658	0.029	0.170
E_{tot}	−0.005	0.006	0.002	0.217

[a]FEV1 is forced expiratory volume in 1 s expressed as observed–predicted in L; confounders are smoking categories (current, former, and non-smokers) as well as cumulative smoking in pack-years, season and recruitment type

[b]F_ENO is the exhaled fraction of nitrogen monoxide expressed in ppb and is log transformed; confounders include season, recruitment type, smoking category, cumulative smoking, and atopy (IgE > 100 UI mL^{-1})

[c]E_{6w} corresponds to the indicator of recent exposure to wheat dust in the last 6 weeks

[d]E_{tot} corresponds to the indicator of cumulative chronic exposure over a career

Immune response to fungal aerosols generated by handling wheat

The sensitization of the wheat-exposed population and controls to 12 different fungal antigens associated with wheat was tested at a 6-month interval (V1 and V2). No decrease in the level of different specific immunoglobulins quantified in sera (specific IgG, specific IgE, and precipitins) was observed between V1 and V2 (data not shown). However, positivity to one type of specific immunoglobulin was dependent on the type of dust and the level of exposure (Table 5, see details in online Supplementary Tables I–III). Thus, the prevalence of positivity for *Cryptococcus albidus* IgE increased with the level of exposure to field wheat dust where this antigen has been found significantly more abundant than in other dusts. Notice the increased prevalence of *A. pullulans, C. albidus*, and *Phoma* sp. IgE with increased duration of field wheat handling.

In contrast, the level of exposure to stored wheat dust was not associated to an increase in the prevalence of sensitization against *E. amstelodami*, which was the only antigen found more abundantly in stored wheat dust, but to a lower prevalence of precipitins produced against this antigen. Workers exposed to stored wheat dust also had a lower prevalence of positivity for the *A. alternata*-specific IgG.

The proportion of subjects positive for at least one specific IgG was higher in the population never exposed to or not recently exposed to wheat dust (67%) than in the population that handled recently freshly harvested or stored wheat (respectively 35 and 42%) (Kruskal–Wallis, $p = 0.01$).

Discussion

This study showed difference in the effects of exposure to dust on the respiratory health and immune system depending on whether the aerosols were generated from handling of field or stored wheat. Thus, a significant dose–response relationship was found between levels of exposure to field wheat dust and most declared work-related acute symptoms (cough, wheezing, dyspnea, runny/stuffy nose, scratchy throat, and systemic symptoms), while the level of exposure to stored wheat dust was associated only with an increased prevalence of cough. Nevertheless, the level of exposure to both types of dust did not seem to be high enough to visibly impact the lung function parameters, but it was still sufficient, considering the level of field wheat dust to decrease the F_ENO concentration. Nitric oxide (NO) is produced by various cells including airway epithelial cells and inflammatory cells under the action of inducible NO synthase enzyme which converts L-arginine to L-citrulline. Some components in field wheat dust, which remain to be identified, seemed to repress NO production by those cells in the lung even if we considered volunteer's age and tobacco exposure, two factors known to influence F_ENO concentration (Xu et al. 2016). Indeed, such an effect has been already described after tobacco exposure, however, the mechanism is still

Table 5 Multiple two-level logistic regression models of sensitization outcomes with the subject ID as a random effect according to four models with different exposure indices to wheat dust

Outcome	Exposure to field wheat dust, OR [95% CI]				Exposure to stored wheat dust, OR [95% CI]			
	Yes/No[Model 1]	Level [Model 2]	Duration in the previous 6 weeks[Model 3]	E_{6w} [Model 4]	Yes/No[Model 1]	Level[Model 2]	Duration in the previous 6 weeks[Model 3]	E_{6w} [Model 4]
Positive for specific IgE								
Ag more abundant in field wheat dust								
Aureobasidium pullulans	4.28 [0.82–22.44]	2.00 [0.57–7.03]	1.06 [1.01–1.11]*	1.05 [0.54–2.03]	1.14 [0.28–4.66]	0.74 [0.26–2.14]	1.08 [0.95–1.23]	1.09 [0.22–5.32]
Cryptococcus albidus[a]	4.58 [0.79–26.63]	5.19 [1.28–21.01]*	1.07 [1.01–1.13]*	3.82 [0.96–15.23]	0.95 [0.20–4.48]	0.98 [0.29–3.28]	0.93 [0.73–1.18]	0.30 [0.01–15.02]
Ag with a low level in all tested dusts								
Penicillium brevicompactum	0.16 [0.03–0.90]*	0.16 [0.04–0.73]*	0.97 [0.92–1.02]	0.83 [0.44–1.56]	0.88 [0.21–3.68]	0.68 [0.22–2.13]	1.03 [0.88–1.20]	0.46 [0.08–2.67]
Phoma sp	3.14 [0.82–12.02]	2.18 [0.75–6.30]	1.06 [1.01–1.12]*	2.25 [0.79–6.45]	2.20 [0.72–6.79]	1.56 [0.67–3.61]	1.02 [0.91–1.15]	0.57 [0.14–2.30]
Positive for specific IgG								
Ag less abundant in storage wheat dust								
Alternaria alternata	2.94 [0.27–31.54]	0.84 [0.13–5.29]	1.02 [0.93–1.11]	0.23 [0.03–1.79]	0.26 [0.02–2.79]	0.14 [0.02–0.96]*	0.76 [0.55–1.06]	0.00 [0.00-62.69]
Positive for precipitins ($n \geq 2$ arcs)								
Ag more abundant in storage wheat dust								
Eurotium amstelodami	1.13 [0.27–4.76]	1.27 [0.41–3.98]	1.03 [0.97–1.08]	1.44 [0.80–2.59]	0.17 [0.05–0.58]**	0.28 [0.11–0.72]**	0.99 [0.86–1.14]	0.67 [0.15–3.01]

"–" Odds ratio (OR) not calculable; OR derived using the category of grain workers not exposed in the previous 6 weeks adjusted for age, smoking status, and season

*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$

[a]*Cryptococcus albidus* was used instead of *C. victoriae* for immunological tests

unknown. This association was significant despite taking into account that exposure to field wheat dust was also associated with an increased prevalence of sensitization to 3 of the 12 environmental antigens tested, including *A. pullulans, C. albidus*, and *Phoma* sp., while exposure to stored wheat dust was associated with a lower prevalence of positivity for IgG or precipitins (composed essentially of IgG) to *A. alternata* and for *E. amstelodami*, respectively.

One important finding of the present study is the major difference in the frequency of self-declared symptoms between the population-handling field wheat and the one handling stored wheat. This difference observed between healthy populations suggests that distinct mechanisms might lead to the respiratory effects observed in grain workers or cattle raiser patients. Indeed, asthma-like syndrome was mostly described in grain elevator operators, although COPD has been reported in multiple cattle raisers (May et al. 2012). Different effects on inhalation of distinct grain dust components have been suggested previously in healthy grain worker populations (Straumfors et al. 2016). In this recent study, the self-reported airway symptoms were related to the individual microbial components in a complex manner. In particular, cough was equally associated with grain dust and fungal spores, although wheezing, chest tightness, and dyspnea were mostly associated with grain dust and nose congestion with different microbial components. In our study, the prevalence of all of those symptoms correlated with the level of exposure to field wheat dust. Interestingly, the abundance of two field fungi, *Aureobasidium* and *Cryptococcus* (Le Bars et al. 1973), has also been associated with an increased prevalence of most of those respiratory symptoms. However, although the prevalence of cough also increased with the level of exposure to stored dust, no association has been found between *Eurotium amstelodami* abundance, a storage fungus, and the prevalence of this symptom. Furthermore, such a difference in the clinical picture of workers exposed to field or stored wheat dust might explain the difference in the health effects described among studies (Smid et al. 1994;

Dorribo et al. 2015; Straumfors et al. 2015). However, the frequent reporting of cough among workers handling wheat remains a constant between studies on healthy populations. Another common feature between our study and previous studies is a baseline decline in lung function with chronic exposure to wheat dust. Decreases in FVC among grain handlers have been significantly correlated with increasing grain dust exposure (doPico et al. 1983). Cross-shift lung function changes have also been observed among grain workers (doPico et al. 1983) and wheat harvest workers (Viet et al. 2001), but not in the most recent studies (Straumfors et al. 2016). Our results are consistent with those published by Straumfors et al. (2016) and support a normal decline in lung function at a 6-month interval in grain workers and cattle raisers. Taken together, those results suggest that decreasing the level of exposure was not strong enough to affect lung function, but was still sufficient to induce acute symptoms during wheat harvesting. The question that arises now is whether an allergic, irritative, or toxic mechanism leads to this difference in the reaction to field or stored wheat dust.

To explore the allergic mechanism hypothesis, we investigated the immune responses of healthy grain workers and cattle raisers to different antigens present in their environment and compared the results to those of the general population. It was remarkable to find such a stable immune response at the 6 month interval. The immune system of grain workers reacted with an IgE response to an increase in *Cryptococcus* antigen abundance in their environment, but also to the duration of exposure to this antigen as well as to *Aureobasidium* and *Phoma*. Interestingly, the immune system of cattle raisers had an opposite response to increased exposure to this antigen. Thus, positivity for the precipitins against *E. amstelodami* in this population decreased with the exposure level to this antigen. This finding supports the hypothesis of clinical tolerance to environmental fungi in cattle raisers.

The immune response against *Cryptococcus* discriminated not only the grain workers from cattle raisers but also the overall workers recently exposed to wheat dust from the controls. *Cryptococcus* is an understudied yeast genus with regard to allergic disease due to difficulties with culturing (Simon-Nobbe et al. 2008). We also encountered this difficulty in the present study when screening for *C. albidus* sensitivity in the target population. Nevertheless, comparing the level of exposure to wheat dust helped link exposure data to health effects in a healthy worker population. Exposure to *C. albidus* has been described previously to induce an immune response in patient populations diagnosed with summer-type hypersensitivity pneumonitis (Miyagawa et al. 2000). Moreover, the presence of several *Cryptococcus* spp. in the asthmatic environment has been associated with increased or decreased asthma severity depending on the species (Dannemiller et al 2016). Too few data are available to estimate the importance of exposure to environmental *Cryptococcus* and the development of respiratory pathology. However, frequent exposure of grain workers to such species makes them an interesting population to follow in further studies for a better understanding of the mechanism. Molecular methods are preferred to identify and quantify *Cryptococcus* in aerosols to resolve the role of exposure to this microbial agent in workers (Pitkaranta et al. 2008). Nevertheless, the role of other biological agents that differ in abundance between field and stored wheat dust cannot be excluded. An overall characterization of the field and storage microbial communities, with high-throughput sequencing tools, might be needed to answer this question.

Similarly, the causes of clinical tolerance to *E. amstelodami* by cattle raisers need to be further explored. Indeed, cattle raisers are exposed to multiple types of organic dust, such as hay dust, animal feed, and manure, which might be an important source of endotoxins. Endotoxins have already been proposed to have a protective effect on allergic sensitization (Portengen et al. 2005; Smit et al. 2008; Basinas et al. 2012). The tolerance phenomenon to acute proinflammatory agents other than endotoxins has also been suggested in pig farmers as an attenuation of clinical, physiological, and inflammatory airway responses (Sundblad et al. 2009). The clinical tolerance to repeated exposure to organic dust seems to be expressed by a decrease in the level of the IgE-allergen complex that binds to B cells and an increase in the levels of specific IgG and IgG4 (Jones et al. 2014), but also by a decrease in the acute inflammatory response (May et al. 2012). In our case, the precipitins test was not designed to make such a distinction between Ig types. Specific experiments must be conducted to test this hypothesis.

Finally, the last hypothesis compatible with the results is that the occupational respiratory effects observed in the farmers and grain workers are not mediated by allergic mechanisms, but instead by an irritative or toxic reaction (Wiszniewska et al. 2013). This hypothesis is supported by the previous results of Schachter et al. (2004) who showed that a wheat dust extract induces in vitro constriction of tracheal smooth muscle, which could be responsible for the respiratory symptoms declared by workers exposed to it (Schachter et al. 2004). Respiratory symptoms mainly due to an irritant effect of the dust rather than an allergic effect to grain dust exposure have also been proposed in a grain terminal operator population (Lucas et al. 2013). The dose–response relationship found in our study between exposure to field grain dust and respiratory symptoms support the findings of these previous studies and suggest that an irritative mechanism (Lucas et al. 2013) and/or a mechanical mechanism (Schachter et al. 2004) might mediate the respiratory pathologies developed by the grain workers.

The toxic effect of wheat dust has been suggested by the frequent presence in field wheat dust of the *Fusarium*

mycotoxin deoxynivalenol (DON) (Niculita-Hirzel et al. 2016), a secondary fungal metabolite known to have a different toxic effect on human alveolar cells depending on its combination with summer dust (PM10 fraction) or winter dust, at least in vitro (Camatini et al. 2012; Gualtieri et al. 2012; Capasso et al. 2015), but it does not increase the allergic response to allergens, at least in mice (Instanes and Hetland 2004). Thus, the presence/absence of this mycotoxin or its combination with dust of different compositions might explain the differential effect observed on the respiratory health of workers handling field wheat and those handling stored wheat. In addition to this differential toxic effect, in vitro studies suggest that DON might also induce different effects on the immune system depending on its dose. Thus, at high doses, DON might lead to immunosuppression, while at low doses it can stimulate cytokine production and immune function of human T lymphocytes and macrophages (Moon and Pestka 2002, 2003; Katika et al. 2012; Kankkunen et al. 2009). This hypothesis is in accordance with the lack of immune and inflammatory responses in wheat workers observed in the present study.

The results of the present study must be interpreted in light of its strengths and weaknesses. First, our population size was relatively small, so we cannot exclude that some relationships between exposure and health effects might have gone undetected because of the lack of power. Nevertheless, our study provides a comprehensive view on how the immune system responds to a complex mixture of fungi by screening for specific IgG, IgE, and precipitins against a representative selection of environmental antigens. Moreover, a main strength of this study is the choice of worker populations with clear exposure patterns, as exposure to field wheat dust is possible only during the summer. Thus, intra-individual comparisons were done between the immune response to field wheat dust at V1 and storage dust at V2, which gave more power to the results obtained from our study. Finally, the study was designed to follow-up individual health with the level of exposure, between a season where the exposure is at its maximum and another where the exposure might decrease. Consequently, the changes in stored wheat dust effects on workers' health might be hidden by a similar level of exposure at V1 and V2 by cattle farmers.

In conclusion, the major finding of our study was that grain workers and cattle raisers presented distinct dose–response relationships between the self-declared respiratory symptoms and the level of exposure to field or stored wheat dust. However, although grain workers might develop a sensitization against the most abundant antigens present in field wheat dust, cattle raisers seemed to be protected from an immune response. This difference in the clinical picture might be due to a distinct immunosuppressive effect of mycotoxins depending on the other components present in the field and stored wheat dust. Nevertheless, an irritative response cannot be excluded. Wheat workers must protect themselves from grain dust during the most exposing activities and avoid direct handling of grain or straw wheat as much as possible.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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