

Inflammatory Markers and Genetic Polymorphisms in Workers Exposed to Flour Dust

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Objective: The aim of this study is to evaluate inflammatory markers and pro-inflammatory CD14 and Toll-like Receptor 4 (TLR4) polymorphisms in workers exposed to flour dust. **Methods:** Polymorphisms in TLR4 and CD14 were identified in our study population of 167 workers that included 63 healthy subjects (HS), 45 atopic subjects (A), and 59 subjects diagnosed clinically with occupational asthma/rhinitis (OAR). Endpoint measures in this study included fractional exhaled nitric oxide and serum concentrations of interleukin IL-6, IL-8, and tumor necrosis factor- α (TNF- α). **Results:** We identified a polymorphism in CD14 (rs2569190) that may be differentially expressed ($P=0.06$). IL-6 concentrations in the serum were significantly higher in the A and OAR groups ($P<0.01$) than in subjects in the HS group, while IL-8 concentrations were significantly elevated only in the OAR group ($P<0.01$). Interestingly, TNF- α concentrations in the OAR group were significantly reduced when compared with subjects in the HS group ($P<0.01$). **Conclusion:** Cytokines are likely a defensive response in atopic and healthy workers. A protective genotype is hypothesized for occupational asthma.

Baker asthma, one of the most frequently reported occupational respiratory diseases in western countries,¹ is characterized by rhinitis associated with other respiratory symptoms including airway obstruction and bronchial hyper-responsiveness. In Italy, 450,000 workers are employed in small bakeries, and the baking industry is ubiquitous worldwide (www.panifici.eu.) There has recently been a dramatic increase in the number of in-store franchise bakeries in Northern Europe and South Africa.^{2,3} Studies conducted among bakery workers have reported the prevalence of baker asthma to be 5 to 17%.⁴ Respiratory symptoms in bakery workers have commonly been attributed to the exposure to wheat flour proteins and the enzymes used to improve dough flavor.⁵

Accordingly, previous studies have shown that sensitization and respiratory symptoms in workers is positively correlated with levels of flour exposure.⁶ Atopy has also been found to be an important independent risk factor for the development of baker asthma,⁷ and at least one previous study has examined the prevalence of different asthma phenotypes in bakery workers.³

Baker asthma is associated with prolonged exposure to flour dust that includes a complex mixture of proteins and other organic materials, including the bacterial endotoxin, lipopolysaccharide (LPS).⁸ LPS stimulates an inflammatory response by ligating the Toll-like Receptor 4 (TLR4) receptor and its coreceptor CD14. This in turn activates the NF- κ B signaling pathway and downstream expression of pro-inflammatory cytokines including interleukin IL-8, which is a neutrophil chemoattractant, and IL-6, which is important in humoral immunity and the expression of acute phase proteins.^{9,10} Moreover, LPS can modify the allergic response in asthma.¹¹

We have previously shown that flour dust induces both nonallergic and allergic airway inflammation.¹² Polymorphisms in both TLR4 (c.896A>G, p.Asp299Gly) and CD14 (c.260C/T) in subjects occupationally exposed to organic dusts have been associated with elevated serum levels of IL-6 and IL-8 in both atopic and nonatopic workers.^{10,13,14} The capacity to mount an appropriate innate immune response to inflammatory stimuli is critical for host defense; therefore, defining the roles of TLR4 and CD14 in workers exposed to flour dust has been a recent focus in the field.^{15,16}

To address the role the innate immune response among subjects affected by rhinitis and baker asthma as well as in atopic and healthy (asymptomatic and nonatopic) subjects occupationally exposed to flour dust, we genotyped 167 study subjects for polymorphisms in TLR4 and CD14 and measured serum levels of cytokines and fractional exhaled nitric oxide (FeNO) to whether there is an association between these common innate immune polymorphisms and the development of allergic respiratory symptoms.

METHODS

Subjects and Clinical Investigation

A total of 167 bakery workers of artisanal bakeries, including 161 males and 6 females, with a mean age of 41.0 ± 11.0 years were examined. These workers had been exposed to flour dust for an average period of 19.4 years (range, 2 to 52 years). Periodic, clinical, and functional examinations were performed over a 3-year period according to a voluntary health surveillance program. The clinical assessment included skin prick tests (SPTs) (Lofarma Allergeni, Milan, Italy) and measurements of allergen-specific IgE (Phadia ImmunoCAP System, Uppsala, Sweden) for environmental allergens such as house dust mites, grass pollen, birch pollen, cockroach, cat, dog, molds, *Parietaria officinalis*, and *Ambrosia eliator*, and also for occupational allergens such as wheat, rye, oat grain, barley grain, soya, and fungal α -amylase (Stallergenes, Antony, France). The diagnosis of occupational rhinitis/asthma was made on the basis of an integrated evaluation of work-related symptoms and positive clinical tests (ie, SPT, specific IgE, spirometry, aspecific, and specific challenges) according to previously published criteria.^{17,18} Atopy was established on the basis of at least one positive allergen in the environmental panel (mean wheal >3 mm) and/or a specific increase in IgE levels more than 0.35 kUA/L. All subjects donated a venous blood sample for the

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measurement of cytokine levels and the detection of genetic polymorphisms. All of the subjects were also evaluated for fractional exhaled nitric oxide (FeNO). Clinical examinations were conducted in accordance with the recommendations outlined in the Declaration of Helsinki.¹⁹ All the patients signed informed consent.

Detection of Serum Levels of Cytokines IL-6, IL-8, and TNF- α

Serum levels of IL-6, IL-8, and tumor necrosis factor- α (TNF- α) were detected by a quantitative enzyme-linked immunoassay (Quantikine ELISA; R&D Systems, Minneapolis, MN). Briefly, 100 μ L of serum samples were added to wells pre-coated with monoclonal antibodies. Then, cytokine-specific enzyme-linked polyclonal antibodies and the substrate solution were added, after 2-hour incubation at room temperature (RT) each. Finally, the enzymatic reaction, confirmed by color development, was stopped after 20 minutes and plates were read by spectrophotometer at 450 nm.

The minimum cytokine levels that were detected were 0.70, 1.6, and 3.5 pg/mL for IL-6, TNF- α , and IL-8, respectively.

Analysis of TLR4 and CD14 Variants by Sanger Sequencing and Real-time PCR

Genomic DNA was extracted from peripheral blood leukocyte (500 μ L) using Maxwell 16 DNA Purification Kits (Promega Italia SRL, Milan, Italy) according to the manufacturer's instructions. Genomic DNA was amplified by PCR using a specific set of primers for the loci containing the polymorphisms rs4986790 in the *TLR4* gene (c.896A>G p.Asp299Gly, NCBI reference sequence NM_138554.3) and rs2569190 in the *CD14* gene (c.-260T>C, NCBI reference sequence NM_000591.3): 5'-TGTATT-CAAGGTCTGGCTGG-3' (forward) and 5'-TCCCACCTTTGTTGGAAGTG-3' (reverse) and 5'-TAAGG-CACTGAGGATCATCC-3' (forward) and 5'-TGTCATT-CAGTTCCTCCTC-3' (reverse), respectively. Sanger sequencing of amplified fragments, performed with the BigDye Terminator v3.1 Cycle Sequencing Kit and a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA), allowed to determine samples haplotype and to validate the allele discrimination analysis performed by predesigned TaqMan real-time PCR assays (C_11722238_20 for rs4986790 and C_16043997_10 for rs2569190, LifeTechnologies) and the SDS 2.3 software (7900 Fast;

Applied Biosystems). Moreover, the assay for the C-reactive protein gene was used as an internal amplification control. For each subsequent series of real-time PCR runs, a positive and a negative DNA control obtained from individuals bearing different genotypes at *TLR4* and *CD14* polymorphisms, previously tested by DNA sequencing, was included. Then, the three groups of workers were categorized according to the detected rs4986790 and rs2569190 genotypes: AA and TT wild type, AG and TC heterozygous, and GG and CC homozygous, respectively.

FeNO Measurement

FeNO was measured using a chemiluminescence analyzer (NIOX Nitric Oxide Monitoring System, version 2.0; Aerocrine AB, Solna, Sweden) before spirometry measurements according to the recommendations of the American Thoracic Society.²⁰ Values less than 35 parts-per-billion (ppb) were considered normal.

Statistical Analysis

Standard descriptive statistics were used to describe each group's characteristics and levels of inflammatory indicators. To evaluate differences between the groups, χ^2 (Fisher exact test), Wilcoxon, and Kruskal-Wallis tests were applied. Rough associations between variables were evaluated with Pearson correlation index and Spearman rank test. Multivariate linear regression models were used to account for the effects of all study variables on the responses of interest. All tests were two-sided and an alpha level of less than 0.05 was considered significant. All analyses were performed with SAS 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

A total of 167 subjects were analyzed, including workers suffering from baker asthma and rhinitis (OAR group) ($n = 59$, 35.5%), workers classified as atopic (manifesting light seasonal rhinitis or asymptomatic and sensitized to environmental allergens) (A group) ($n = 45$, 27.0%), and asymptomatic healthy working subjects who had negative SPT and specific IgE results (Table 1). Table 1 lists the main characteristics of the three groups, including age, presence of familial allergies, smoking status, and *CD14* and *TLR4* genotypes. Workers in the HS group were older than workers in the other groups. Workers in the OAR group reported more frequent familial allergies, while there was no difference in smoking habits across all three groups. Only subjects

TABLE 1. Characteristics of the Three Worker Groups Evaluated

Cohort Characteristics		OAR Group	A Group	HS Group	P *
Age (yrs), mean $\pm$ SD		40.8 $\pm$ 12.7	37.0 $\pm$ 9.4	43.7 $\pm$ 10.6	<0.01
Smoking status, n (%)	Nonsmoker	34 (57.6)	22 (48.9)	39 (61.9)	
	Ex-smoker	7 (11.9)	8 (17.8)	4 (6.4)	
	Current smoker	18 (30.5)	15 (33.3)	20 (31.7)	0.42
No. of cigarettes smoked/d		3.1 $\pm$ 5.6	3.9 $\pm$ 6.4	4.6 $\pm$ 8.4	0.53
Antihistamine or steroid use, n (%)	Yes	32 (54.2)	0 (0)	0 (0)	
	No	27 (45.8)	45 (100)	63 (100)	<0.01
Familial allergy, n (%) [†]	Yes	18 (30.5)	8 (17.8)	3 (4.8)	
	No	41 (69.5)	37 (82.2)	60 (95.2)	<0.01
<i>TLR4</i> polymorphism, n (%) [†]	AA	49 (84.5)	35 (85.4)	53 (86.9)	
	GG	0 (0)	0 (0)	1 (1.64)	
	AG	9 (15.5)	6 (14.6)	7 (11.5)	0.88
	TT	24 (41.4)	11 (26.8)	14 (23.7)	
<i>CD14</i> polymorphism n (%) [†]	CC	10 (17.2)	8 (19.5)	21 (35.6)	
	CT	24 (41.4)	22 (53.7)	24 (40.7)	0.06

A group, atopic subjects; HS group, healthy subjects; OAR group, occupational asthma/rhinitis subjects.

*P values were calculated using Student *t*-test and Fisher exact test for continuous and categorical variables, respectively.

[†]A missing value due to undetectable sample.

TABLE 2. Levels of Inflammatory Parameters by Group

Inflammatory Parameters	OAR Group				A Group				HS Group			
	N	25%	Median	75%	N	25%	Median	75%	N	25%	Median	75%
Interleukin-6 (pg/mL)	22	0.0500	6.96	11.9	27	0.100	11.1	16.2	51	0	1.46	11.1
Interleukin-8 (pg/mL)*	59	1.8	50	53	45	1.8	1.8	48	63	1.8	1.8	1.8
TNF- α (pg/mL)	59	0.800	7.38	9.68	45	0.800	8.26	12.5	63	6.60	9.68	12.3
FeNO (ppb)*	47	8	49	82	31	16	21	47	40	15	19	26

A group, atopic subjects; HS group, healthy subjects; OAR group, occupational asthma/rhinitis subjects; TNF- α , tumor necrosis factor-alpha.

*A statistically significant difference was observed among the three groups for IL-8 and FeNO ($P < 0.01$).

in the OAR group reported taking antihistamines and/or inhaled steroids (54.2%).

Concentrations of IL-6, IL-8, and TNF- α were determined in serum samples collected from the three groups and are presented in Table 2. IL-6 levels were significantly higher in both the OAR and the A groups when compared with the HS group. The OAR group also had higher serum concentrations of IL-8 than the HS group (Fig. 1). However, the HS group had the highest serum concentrations of TNF- α , while the levels of TNF- α differed significantly between the OAR and the HS groups. Both age and smoking status were associated with increased concentrations of TNF- α (Table 3). Subjects in the OAR group reported the use of antihistamines and inhaled steroids (54.2%); however, neither atopic workers (A) nor asymptomatic workers (HS) reported use of these medications. Moreover, in the OAR group, medication use was negatively associated with TNF- α levels (Table 3).

The FeNO values also varied significantly between the three groups (Table 2), with the highest values detected in the OAR group (Fig. 1). A negative association between FeNO levels and the daily number of cigarettes was also observed (Table 4). A statistically significant correlation was identified between the log values of FeNO and IL-8 (Pearson test, $r = 0.36$, $P < 0.01$).

Although the common TLR4 polymorphism did not segregate by group (Table 1), the CD14 polymorphism was found to segregate between the groups ($P = 0.06$). No association was found

between the CD14 polymorphism and IL-6 or IL-8 (data not shown); however, the CC/CT alleles in the A and HS groups (Table 1) were significantly associated with higher serum TNF- α levels. Not surprisingly, the TT CD14 allele was more commonly associated with the OAR group (Fig. 2).

DISCUSSION

Since the work of Bernardino Ramazzini in the 17th century,²¹ it has been common knowledge that exposure to flour dust can cause respiratory symptoms that lead to baker asthma, which can develop into chronic bronchial obstruction.²² In the present study, workers in the OAR group reported familial allergies, whereas subjects in the HS group did not. These results suggest that familial allergies, in addition to atopic status (which is currently considered to be a risk factor for baker asthma), should be considered as risk factors for the development of occupational asthma. Workers exposed to flour dust generally exhibit an increase in cytokine levels that may be a consequence of exposure to pro-inflammatory components flour dust as part of a normal response in atopic and healthy workers. Hence, these cytokines have been found to be useful as markers of exposure to organic dust.¹⁰ Moreover, for the 10 nonatopic males (mean age 42.0 ± 9.8 years) in the present study, their inflammatory cytokine serum concentrations were below the detection levels of the assays performed (data not shown).

FIGURE 1. Evaluation of cytokine levels and FeNO in the three observed groups. The bars represent the median values. Elevated levels of IL-6 were detected in the OAR and A groups, and the levels in the A group were statistically higher than those of the HS group (A). A statistically significant increase in IL-8 levels was observed in the OAR group compared with both the A and HS groups (B). The HS group had the highest levels of TNF- α , and exhibited a statistically significant difference compared with the OAR group (C). The OAR group had the highest levels of FeNO (D). A, atopic subjects; HS, healthy subjects; OAR, occupational asthma/rhinitis subjects.

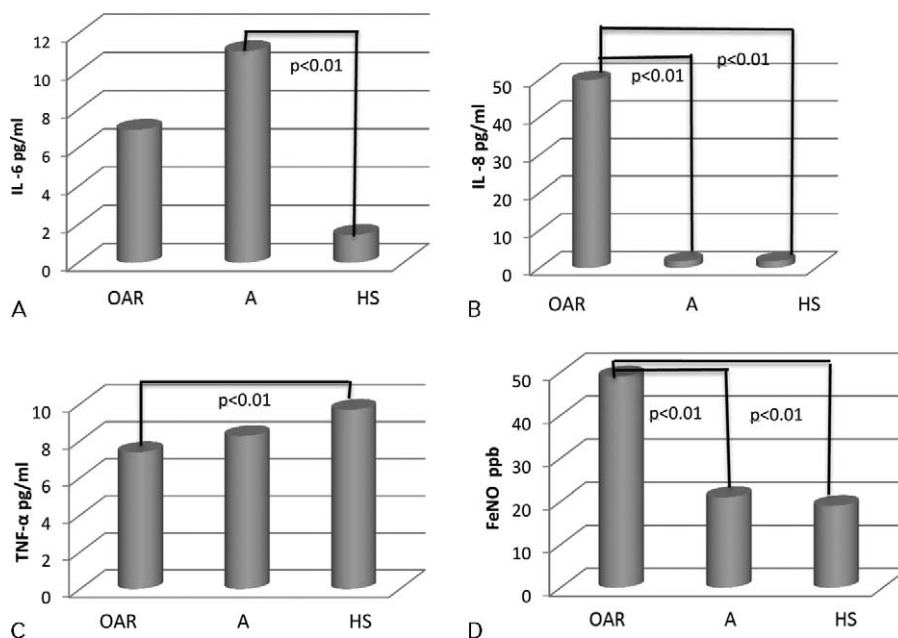


TABLE 3. Increase in TNF- α Levels According to Relevant Variables*

Variables		β (95% CI)	P
Age		0.09 (0.02–0.15)	<0.01
Smoking status	Nonsmoker	Ref.	—
	Ex-smoker	3.1 (0.70–5.50)	0.01
	Current smoker	1.48 (–0.20 to 3.16)	0.08
CD14 polymorphism	TT	Ref.	—
	CT	1.56 (–0.18 to 3.29)	0.08
	CC	2.33 (0.33–4.32)	0.02
Drug therapy	Yes	–2.83 (0.98–4.68)	<0.01
	No	Ref.	—

A group, atopic subjects; CI, confidence interval; HS group, healthy subjects; OAR group, occupational asthma/rhinitis subjects.

*Two subjects analyzed in a subgroup to control their excess of leverage in the computation of regression estimates.

Our results further support the use of FeNO as a marker of inflammation, as FeNO levels correlate with levels of IL-8, which is responsible for neutrophilic recruitment into the lung.²³ The HS group had the lowest FeNO values, and these were neither associated with atopic status nor smoking habits, as previously reported.¹⁹ However, FeNO was associated with occupational allergy status ($P < 0.01$). Therefore, determination of FeNO values may represent an easy and practical method for monitoring occupational asthma and evaluating the efficacy of drug therapies, immunotherapies, and preventive measures.

In both the A and HS groups, the levels of TNF- α were found to increase progressively. These results may be evidence of a switch in cell-mediated responses from Th2 to Th1 and suggest a possible protective role of prolonged exposure to flour dust, a phenomenon named *healthy worker survival effect*,²⁴ particularly for workers who did not receive treatment for rhinitis and asthma (none of the A workers were on medication). Levels of TNF- α were also associated with smoking habits, and this has previously been observed in experimental studies in rats.²⁵ Intriguingly, evidence from recent human studies also suggests that environmental tobacco smoke is not associated with IgE sensitization.²⁶ Thus, additional studies on the biological mechanisms by which components of tobacco smoke influence the immune response are still needed.

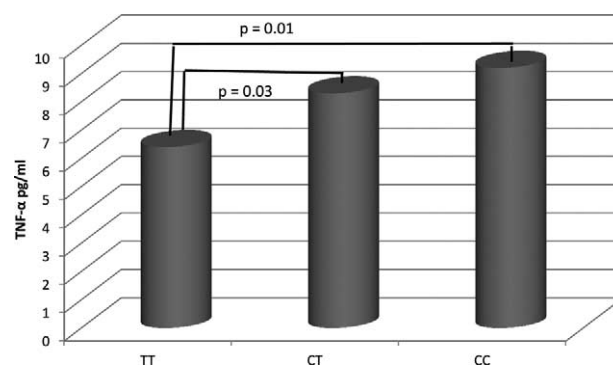
IL-6 and IL-8 levels differed between the groups. Levels of IL-6 increased in both the OAR and A groups compared with the HS group; IL-6 has been associated with atopy and asthma involving plasma cells and IgE antibody production.²⁷ IL-8 concentrations were also higher in the OAR group than in the HS group. A

TABLE 4. Increase in FeNO Levels According to Relevant Variables

Variables		β (95% CI)	P
Worker group	OAR group	0.74 (0.40–1.07)	<0.01
	A group	0.33 (–0.01 to 0.66)	0.06
	HS group	Ref.	—
Age		–0.01 (–0.02 to 0.01)	0.35
No. of cigarettes smoked/day		–0.04 (–0.06 to –0.02)	<0.01
Interleukin-8*		0.1 (0.02–0.18)	0.02
Familial allergy	Yes	–0.22 (–0.54 to 0.10)	0.18
	No	Ref.	—

A group, atopic subjects; CI, confidence interval; HS group, healthy subjects; OAR group, occupational asthma/rhinitis subjects; Ref, reference value.

*Expressed in log value.

**FIGURE 2.** Serum levels of TNF- α according to the polymorphisms CD14. The bars represent the median values. The CC and CT polymorphisms were more frequent in the A and HS groups, while lower concentrations of TNF- α were associated with the TT polymorphism. Statistically higher significant concentrations of TNF- α were detected in CT and TT polymorphisms.

neutrophilic response has been observed in cases of moderate to severe asthma; therefore, the observed increase in IL-8 levels may represent an occupational rhinitis/asthma phenotype due to chronic exposure to flour dust.⁹ Furthermore, an IL-8 response has been detected in experimental mouse studies in which bakery flour dust was found to not only cause nonallergic inflammation in the lung, but it also enhanced allergic airway inflammation.¹² Previously, an increase in IL-8 levels was associated with neutrophil number and was found to be related to LPS-contaminated dust in healthy subjects.²³ These results suggest a correlation between LPS exposure and respiratory symptoms/disease. However, in agreement with Arbour et al¹³ and Rylander and Michel,¹⁰ we did not find an association between atopy and asthma due to LPS exposure. Furthermore, the data from the present study suggest that the LPS content in dust does not contribute to the response to flour dust that was observed in the asymptomatic workers, and that TLR4-dependent mechanisms can be excluded in humans because the distribution of TLR4 rs4986790 polymorphism did not differ in our population.¹² This is in contrast with the results of other studies of atopic and healthy subjects who were exposed to organic dust and showed an association between low IL-6 and IL-8 levels and the TLR4 rs4986790 AG and GG alleles.^{10,13} This difference may be due to the inclusion of both exposed workers and subjects with occupational rhinitis/asthma. Previously, the AG TLR4 polymorphism has been associated with a low inflammatory cell response, cytokine production, and bronchial reactivity to an LPS stimulus.²⁸ However, in the present study, no difference in the distribution of this specific polymorphism among the three groups examined was observed. In contrast, differences in the CD14 rs2569190 polymorphisms in exposed workers were observed. The TT genotype was overrepresented, particularly in workers who were affected by rhinitis and baker asthma. Similarly, other authors reported an association between the T allele and nonatopic asthma, food allergies, eczema, and atopy.^{29–31} In addition, Leynaert et al³² suggested that in individuals exposed to a farming environment early in life, the CD14 TT polymorphism protects against the development of atopic sensitization and allergic rhinitis in childhood. For adults, our results suggest that a CD14 CC polymorphism may be protective against the development of baker asthma. Furthermore, bakery workers with the CT or CC genotypes had significantly higher serum TNF- α concentrations than workers with the TT genotype. In addition, the CC genotype has previously been related to IgE production and the development of atopic sensitization.^{33,34} Taken

together, these results suggest that the CC polymorphism mediates a protective role in the development of occupational respiratory symptoms, and this will need to be confirmed in future studies. Because of the limited number of study subjects, it is possible that the associations observed were attributable to chance. However, the homogeneous occupational exposure and the selection of the groups of bakery workers through a health surveillance program provide a good setting for evaluating these mechanistic questions and limit bias and chance findings. Elucidating the causes of baker asthma is difficult due to the contributions made by both environmental and genetic components. In the present study, we show that CD14 polymorphism and TNF- α levels play a protective role against the development of baker asthma. Higher levels of IL-6 and IL-8 were also found to correlate with atopy and occupational asthma, respectively, and, in combination with FeNO quantification, these may serve as biomarkers of exposure to flour dust in workers at a higher risk. These putative markers may also facilitate the development and adoption of measures to prevent baker asthma in the workplace. Given the observation that the prolonged use of drugs in workers with OAR resulted in an increase in FeNO, the present results further suggest that new immunotherapy strategies or biological therapies involving flour allergens should be developed. Further studies with a larger sample size are warranted to confirm the findings of the present investigation.

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