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**Inflammatory proteins in nasal lavage of workers exposed to occupational agents**

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**Abstract**

**Background:** Low-molecular-weight (LMW) and high-molecular-weight (HMW) agents have been recognized as causes of occupational rhinitis (OR). Immunological mechanisms underlying OR differ according to the type of exposure. While HMW agents act mainly through IgE-mediated mechanisms, LMW agents appear to act through both immunologic and non-immunologic mechanisms.

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**Objective:** The objective of this study was to identify potential differences in the upper airways inflammatory response after exposure to LMW and HMW agents by specific inhalation challenge test (SIC).

**Methods:** Nasal lavage (NL) samples from 20 subjects who were exposed to HMW (n=10, Group I) and LMW (n=10, Group II) at their workplaces were collected after SIC with control and specific occupational agents. These samples were analyzed for 47 inflammatory markers using multiplex bead technology.

**Results:** After exposure to specific agent, Group I exhibited higher concentrations of the following proteins compared to Group II: fibrinogen (median (interquartile range) Group I: 0.09 (0.00) µg/ml, Group II: 0.04 (0.05) µg/ml, p=0.05); haptoglobin (Group I: 0.86 (0.01) µg/ml, Group II: 0.14 (0.20) µg /ml, p=0.02); vascular cell adhesion molecule-1 (VCAM-1) (Group I: 0.34 (0.67) ng/ml, Group II: 0.11(0.11) ng/ml, p=0.01); vascular endothelial growth factor (VEGF) (Group I: 157.0 (154.0) pg/ml, Group II: 98.0 (20.25) pg/ml, p=0.01) and vitamin D (VDBP) (Group I: 0.06 (0.13) µg /ml, Group II: 0.03 (0.03) µg /ml, p=0.04). No statistically significant differences in proteins profiles were observed between the groups after exposure to control agent. Also, subjects exposed to HMW agents showed a significant increase in NL levels of C-reactive protein compared to control day exposure.

**Conclusions and clinical relevance:** Exposure to HMW and LMW agents by SIC induced a differential nasal airway response including acute-phase reactants proteins (fibrinogen, haptoglobin and CRP), cell adhesion molecules (VCAM-1), endothelial growth factors (VEGF) and VDBP.

## Introduction

Exposure to high-molecular-weight (HMW) and low-molecular-weight (LMW) agents at the workplace are known risk factors that may exacerbate or aggravate pre-existing rhinitis or induce occupational rhinitis (OR). [1] The mechanism by which HMW agents induce OR is thought to be IgE-mediated similar to that known to cause allergic rhinitis in the general population whereas the pathogenesis of OR caused by LMW agents remains poorly understood but may include both immunologic and non-immunologic mechanisms.[2;3]

Specific inhalation challenge (SIC) with HMW or LMW agents can induce changes in airways inflammatory response. [4] SIC in subjects with OR and occupational asthma (OA) induces increases in eosinophils and neutrophils in nasal lavage (NL) and induced sputum samples regardless the type of exposure[5;6] as well as expression of proinflammatory cytokines that exert effects on neutrophils and eosinophils.[7]

There is a paucity of research regarding pathogenic mechanisms of OR depending on the type of agent, i.e. HMW vs LMW. Nasal inflammatory responses to SIC with HMW and LMW agents have not been studied thoroughly. NL is a known, non-invasive technique to assess upper airways inflammation. [8] Emerging multiplex bead technology allows accurate and sensitive detection of multiple analytes in human fluids.[9] This technology is being used more often in research for biomarker identification and validation; measurement of changes

in protein levels in serum and human fluids, and analysis of pathogenic pathways in physiological and disease states.[10] However, limited data is available using this technology for the analysis of inflammatory markers in NL samples.

The objective of this study was to use a proteomics approach to identify potential differences in the nasal inflammatory response regarding cytokines, chemokines and acute-phase reactants after exposure to HMW and LMW occupational agents by SIC. Understanding these differences can provide important insights on pathogenic mechanisms of OR that may be also applicable to allergic and non-allergic rhinitis in the general population.

## **Materials and methods**

### *Study population*

Study population consisted of 20 subjects referred to Hôpital du Sacré-Coeur de Montréal with a history of work-related rhinitis and asthma symptoms due to exposure to HMW (Group I; n=10) and LMW (Group II; n=10). Subjects underwent a comprehensive investigation including SIC and collection of NL samples for analysis of inflammatory cells. Multi-analyte analysis of NL samples was not part of the initial investigative SIC protocol. Accordingly, study subjects were not consecutive patients. Inclusion criteria were: i) history of workplace exposure to either HMW or LMW agents, ii) history of work-related rhinitis and asthma symptoms, iii) complete investigation of work-related rhinitis and asthma symptoms by SIC and iv) availability of NL supernatant collected and stored during SIC investigation. All selected subjects were referred specifically to rule-out OA and a diagnosis of OR confirmed by SIC was incidental in all cases.

The study was approved by the research ethics committee of the Hôpital du Sacré-Coeur de Montréal (2009-12-100) and written and informed consent was obtained from all participants.

### *Specific inhalation challenge (SIC) and nasal lavage*

All subjects underwent standardized SIC with monitoring of nasal responses on two separate days. As previously described [11], subjects were exposed to a nonspecific agent (lactose, methyl-ethyl ketone) on the first day. On the following day, they were exposed gradually to the suspected specific agent by recreating working conditions in an isolated challenge chamber (N=10) or using a generator of particles (for flour, n=5)[12] or isocyanates (n=5)[13;14]. Exposure was stopped when FEV1 had dropped by at least 20% compared to baseline or after a maximum of 2 hours of exposure. In the case of high molecular weight (HMW) agents, exposure was carried out on a single day because they usually induce immediate or dual reactions. For low molecular weight (LMW) agents, the exposure was progressively increased from day to day (usually for total durations of 4, 30 and 120 minutes) because of the possibility of late reactions that are difficult to predict. Nasal patency was measured by acoustic rhinometry (AR) before challenge and serially for 6 hours after challenge. An acoustic rhinometer (Hoods Laboratories, Pembroke, MA) was used to measure the nasal volume between 2 and 5 cm into the nose (Vol2–5) and the minimal cross-sectional area (MCA) according to international recommendations. The criteria for scoring a positive SIC for OA and OR was based on objective physiologic changes in nasal and bronchial responses namely, a decrease in FEV1 of >20% after exposure for OA and a

decrease of >30% in Vol2–5 after exposure for OR in the absence of a positive reaction during the control day.

The NL samples available for analysis were collected on two consecutive days namely, 6 hours after challenge with the control and the specific agents. NL was performed using a modified 10-F standard silicone Foley catheter (Teleflex Medical GmbH, Ruesch, Germany). [15] The tip of the catheter was cut distal to the balloon to avoid touching the septum and the anterior tip of the inferior turbinate. Then, the catheter was introduced about 1.0 cm into the vestibulum and inflated to form a seal. Ten milliliters of warm normal saline solution (0.9%) was instilled into the nose via a 10-mL needleless syringe connected to the catheter and then aspirated after 30 second. The entire procedure involved 3 instillation/aspiration cycles. The balloon was then deflated and the procedure was repeated on the other nostril. The NL fluid from both nasal cavities was pooled in the same plastic container and kept on ice until processed. [16]

Within 2 hours the sample was measured and centrifuged at 3300 revolutions per minute (rpm) (1705 g) for 8 minutes at 4°C. The pellet was resuspended in 0.5mL phosphate-buffered saline (PBS) containing 0.1% wt/vol bovine serum albumin. Cytocentrifuge preparations were made by using 100  $\mu$ L of the remaining resuspended cell suspension. The preparation was centrifuged at 450 rpm (32 g) and slides were stained with Wright-Giemsa for differential cell counting

#### *Multi-analyte profile*

The supernatant obtained from NL samples centrifugation was apportioned into 0.5 ml aliquots and stored at –80°C. Samples were analyzed using the Human Inflammation Multi-Analyte Profile (MAP), version 1.0 and a Luminex xMAP technology platform (Myriad Rule Based Medicine (RBM), Austin, TX).

The inflammation MAP v.1.0 is a commercial validated platform, which measures a battery of 47 inflammatory proteins including cytokines, chemokines and acute-phase reactants. Supplemental Table 1 shows the full list of markers, unit of measurement and the assay's sensitivity in terms of the least detectable dose (LDD) (mean  $\pm$  3 standard deviations of 20 blank readings). The analysis of NL samples was fully automated according to established standards. [10]

#### *Statistical analysis*

NL cell counts were expressed as means  $\pm$  SD or median and interquartile range (IQR). Protein concentrations were calculated as medians with IQR. Between-group comparisons of NL analytes levels were computed with Mann–Whitney *U*-tests. Spearman's rank correlations were used to calculate data correlation. Data was conducted with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

## Results

### *Study subjects*

Twenty subjects (mean age  $43.8 \pm 11.0$  years) were investigated for suspected OR and OA. Baseline characteristics of subjects based on type of occupational exposure to HMW (group I) vs LMW (group II) are summarized in Table 1. Most subjects were men (6 in Group I and 8 in Group II), atopic (8 in Group I and 4 in Group II) and non-smokers (8 never or ex-smokers in both groups) at the time of investigation. Groups did not significantly differ in terms of age, baseline nasal patency measured by AR and rhinitis symptoms severity measured by a quality of life questionnaire. [17]

Ten patients were exposed to HMW agents (Group I): wheat flour (7), cat allergen (1), rat allergen (2) and 10 patients were exposed to LMW agents (Group II): isocyanates (6), acrylates (3), formaldehyde (1). After SIC, a diagnosis of OR with OA was confirmed in 8 of 20 subjects; a diagnosis of OR without OA was confirmed in 4 subjects and a diagnosis of OA without OR was confirmed in 1 subject. The SIC was negative for both OR and OA in 7 subjects.

### *Cellular characteristics of nasal lavage samples*

Table 2 shows that in both the control and specific challenge day there were not significant differences in cellular characteristics of NL between Groups I and II.

There was a significant increase in the percentage of eosinophils in Group I ( $p=0.02$ ) and macrophages in Group II ( $p=0.05$ ) comparing values from the control and active challenge days. A non-significant increase in the percentage of neutrophils was observed in both groups (Table 2).

### *Between-group comparisons of nasal lavage analytes*

Twenty proteins either with values below the LDD or that could not be detected were not included in the analysis (Supplementary Table 1). In total, 27 out of 47 proteins were above the limit of detection for most subjects and included in the analysis. Regardless of occupational agent, these proteins were detected in samples collected after control and specific agent exposure (Table 3). The percentage of NL samples with detectable protein levels is shown as % detectable samples for each protein (Table 3).

After exposure to control agent, no difference in NL levels of proteins was observed between subjects exposed to HMW and LMW agents (Table 3). After exposure to the specific agent, NL fibrinogen ( $p=0.05$ ), haptoglobin ( $p=0.01$ ), vascular-cell-adhesion-molecule-1 (VCAM-1) ( $p=0.01$ ), vascular-endothelial-growth-factor (VEGF) ( $p=0.01$ ) and vitamin D (VDBP) ( $p=0.04$ ) levels were significantly elevated in subjects exposed to HMW agents (Group I) compared to LMW agents (Group II) (Table 3). In the control day, NL levels of these

proteins showed the same pattern but differences between the groups were not statistically significant.

When comparing the change in NL proteins levels from control and specific challenge days, a significant increase in C-reactive protein ( $p=0.02$ ) and a borderline significant increase in haptoglobin ( $p=0.07$ ) was observed in the HMW group. In the LMW group, a non-significant decrease in NL levels of the same proteins was observed (Table 3). The comparison of NL proteins levels from control and specific challenge days in the LMW group showed a significant decrease in NL levels of ferritin ( $p=0.03$ ) and (VCAM-1) ( $p=0.04$ ). In the HMW group, a non-significant decrease in ferritin and a non-significant increase in VCAM-1 levels were observed (Table 3).

A further comparison of changes in NL protein levels from control and specific challenge days showed a significant increase in C-reactive protein ( $p=0.02$ ) and IL-18 ( $p=0.04$ ) in subjects with SIC-confirmed diagnosis of OR. No significant increases were observed in subjects with a negative SIC.

#### *Correlations between inflammatory proteins*

Table 4 shows correlations between inflammatory proteins showing a significantly different pattern of expression in subjects exposed to HMW and LMW agents. Statistically significant correlations were only observed in subjects exposed to HMW agents during the specific challenge day.

#### **Discussion**

To date, only a few studies have investigated the nasal inflammatory response induced by occupational agents. In the present study, we observed differences in the levels of NL inflammatory proteins after exposure to HMW and LMW agents using the Luminex® xMAP® technology. After specific inhalation challenge, subjects exposed to HMW agents exhibited significantly elevated NL levels of the acute-phase reactants fibrinogen and haptoglobin as well as of VCAM-1, VEGF and VDBP compared to subjects exposed to LMW agents. Also, subjects exposed to HMW agents showed a significant increase in NL levels of C-reactive protein compared to control day exposure.

Exposure to HMW and LMW agents also induced a differential response in terms of inflammatory cells. The percentage of eosinophils in NL increased in subjects exposed to HMW and LMW agents when comparing values from the control and specific challenge days but the observed increase was statistically significant only in those exposed to HMW agents. This cellular pattern confirms findings from our previous studies showing that exposure to HMW and LMW agents can induce an influx of eosinophils in nasal secretions.[5;7] Also, significant increases in the number of sputum eosinophils compared to baseline have been demonstrated in patients with confirmed OA after SIC with HMW and LMW agents. [18] Besides, we observed a non-significant increase in the number of neutrophils in both groups

regardless of exposure agent. This inflammatory response pattern has been observed in previous OR and OA challenge studies. [7;19]

The acute-phase response is an important regulatory phenomenon contributing to body homeostasis by modulating immune responses in response to injury.[20] Acute-phase proteins (APP) are postulated to play a beneficial role in inflammation and healing after exposure to noxious stimulus.[20] However, a persistent acute-phase response may contribute to chronic inflammatory state as well as tissue damage and disease.[21] No research has been conducted assessing the potential role of APP in immunological mechanisms of work-related rhinitis and asthma.

Haptoglobin, an APP, has been previously linked to airway inflammation. For example, serum level of haptoglobin has been found to correlate with bronchial hyperresponsiveness [22] and increased [23] and decreased [24] serum haptoglobin levels have been reported in asthma. In addition, serum levels of haptoglobin were increased 24 h after allergen challenge in children.[25] In this study exposure to HMW agents induced significantly higher NL haptoglobin levels compared to LMW agents. Also, subjects exposed to HMW agents exhibited increased levels of NL haptoglobin compared to control day levels, however, although the increase was not statistically significant. Interestingly, in a recent nasal mucus proteomic study, significantly more abundant haptoglobin levels were reported in allergic rhinitis patients compared to controls.[26] However, the mechanism by which exposure to HMW agents may induce higher levels of haptoglobin in NL compared to LMW agents is not known. As expected, in this study, eosinophil counts in NL increased in subjects exposed to HMW compared to LMW agents. As discussed above, eosinophils are the major effector cells in the nasal secretions of rhinitis patients during IgE-mediated allergic response. Haptoglobin has been reported to be produced not only in alveolar macrophages but also in eosinophils suggesting a potential implication in respiratory allergy.[27] We speculate that this protein may play a more important role in allergic IgE-mediated immunologic mechanisms as those involved in rhinitis and asthma caused by HMW agents than in non-allergic mechanisms as those thought to be involved in cases caused by LMW agents.

Tissue repair is a pathophysiologic phenomenon observed in respiratory allergy particularly in asthma. Haptoglobin and fibrinogen are both involved in wound healing.[21] Similar to haptoglobin, we observed significantly higher levels of fibrinogen in subjects exposed to HMW agents as well as a strong correlation between these APPs. Their involvement in tissue repair mechanisms has been suggested because haptoglobin can stimulate angiogenesis and fibrinogen is involved in endothelial cell adhesion, spreading and proliferation.[21] Another potential linkage between these two APP in the pathophysiology of tissue repair in airway inflammation is suggested by their biological effect on fibroblast cell differentiation and activation. Elevated levels of haptoglobin expression in bronchoalveolar fluid was observed in patients with mild asthma as compared to nonasthmatic control subjects and this increase was associated with differentiation of fibrocytes into fibroblast cells.[28] On the other hand, fibrinogen has been shown to stimulate the proliferation of fibroblasts in a dose-dependent manner in renal fibrosis.[29] Interestingly, fibrinogen is also a marker of plasma exudation during airway inflammation. Studies have shown that fibrinogen is a leakage marker

associated with asthma and increased levels of fibrinogen in sputum of asthmatics have been observed compared to controls.[30] In rhinitis, plasma exudation from the nasal mucosa microvasculature is a known phenomenon contributing to rhinitis symptoms particularly, nasal discharge. Nasal provocation with capsaicin induces significant increases of fibrinogen levels in NL providing evidence of plasma exudation.[31]

As discussed above, higher levels of haptoglobin and fibrinogen in subjects exposed to HMW agents in our study suggests that these APPs may be more implicated in IgE-mediated immunologic pathways. However, this assumption requires confirmation and further investigation by similar challenge studies to provide more insight into the role of these APPs in IgE-mediated rhinitis and asthma.

VEGF is postulated to contribute to the pathogenesis of allergic airway disease. Increased vascular permeability is a feature of allergic rhinitis in which VEGF plays an important role.[32;33] VEGF facilitates plasma proteins to leak into extravascular spaces inducing an edematous nasal airway wall manifested by nasal congestion.[32;33] We observed significantly higher NL levels of VEGF in subjects exposed to HMW agents during the active challenge day compared to levels in subjects exposed to LMW agents. Studies have shown that allergic rhinitis patients have increased levels of VEGF in their nasal mucosa compared to controls.[34] Also, increased VEGF levels have been demonstrated in asthma patients and these levels were even higher during acute asthma exacerbations.[35] Besides, VEGF has proinflammatory properties in chronic inflammation by increasing endothelial cell permeability and promoting ICAM-1 and VCAM-1 expression [36] which may be relevant to the pathophysiology of rhinitis and asthma.

Eosinophilia in nasal mucosa is a characteristic of allergic rhinitis. VCAM-1 is involved in the trafficking of eosinophils into the airways during inflammation and adhesion of eosinophils in endothelial cells.[37] The involvement of this protein during the allergic response has been observed in in-vitro and in-vivo studies.[38] VCAM-1 was significantly upregulated by allergen challenge in allergic rhinitis patients compared to control subjects.[39] We also observed significant higher levels of VCAM-1 in subjects exposed to HMW agents in contrast to subjects exposed to LMW agents. Besides, there is a growing evidence supporting direct participation of soluble adhesion molecules in the angiogenic process.[40] Although our results are not surprising, in this context, further studies are required to confirm these results.

C-reactive protein (CRP) is an APP known as a sensitive marker of inflammation, infection and tissue damage.[21] The relevance of CRP as a marker of inflammation in rhinitis has not been studied. However, there is evidence showing the association between high CRP levels and asthma. A recent study showed that serum CRP levels were significantly increased in steroid-naïve patients compared with controls and were associated with airflow obstruction and inflammation.[41] Also, another study concluded that raised CRP levels were associated with respiratory symptoms and non-allergic asthma.[42] In this study, we did not observe differences in NL levels of CRP between subjects exposed to HMW and LMW agents during control and after specific inhalation challenge. But interestingly, CRP was the sole APP

showing a significant increase in NL samples when comparing levels from control and specific challenge day. Besides, the observed increase was limited to subjects who showed a positive reaction to challenge and exposed to HMW agents. These findings require further investigation in order to confirm results and assess the relevance of CRP as a key protein in IgE-mediated nasal inflammatory response to allergens.

The potential role of vitamin D deficiency in respiratory allergy, including allergic rhinitis, has received significant attention in recent years. Vitamin D may have immunomodulatory effects in respiratory allergy by regulating mechanisms and pathways that reduce the airways inflammatory response and influence immune cell differentiation.[43] In our study NL levels of vitamin D were higher in patients exposed to HMW agents compared to LMW agents which suggest a more predominant role of vitamin D in IgE-mediated nasal inflammatory responses.

Our study had limitations including small sample size due to the relative rarity of OR and the absence of a control group comprised of healthy subjects since it is ethically challenging to expose healthy subjects to noxious substances. NL samples were collected in the context of real-world clinical investigations requested by a worker's compensation board agency. However, the subjects had rigorous phenotypic characterization with specific inhalation challenge to occupational HMW and LMW agents that increased the discriminatory power between the groups. In addition, novel multiplex bead array technology allowed the detection and quantification of multiple inflammatory markers in a small volume of NL samples.

In summary this study showed that exposure to HMW and LMW agents using challenge test induced a differential nasal airway inflammatory response which included acute-phase reactants (fibrinogen, haptoglobin and CRP), cell adhesion molecules (VCAM-1), endothelial growth factors (VEGF) and VDBP. A larger validation study is required to confirm the findings of this study and provide more insights on mechanistic differences in nasal inflammatory response caused by HMW and LMW agents.

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Table 1. Clinical characteristics of subjects

| Description                | Group 1: HMW    | Group 2: LMW    | P Value |
|----------------------------|-----------------|-----------------|---------|
| Number of patients         | 10              | 10              |         |
| Age, yr                    | 39.7            | 47.8            | 0.1     |
| Sex, male/female           | 6/4             | 8/2             |         |
| Smoking, n (%)             |                 |                 |         |
| Current                    | 2               | 2               |         |
| Never                      | 6               | 6               |         |
| Ex-smokers                 | 2               | 2               |         |
| Atopy, yes                 | 8               | 4               |         |
| AR-vol2-5, mean $\pm$ (SD) | 3.26 $\pm$ 1.3  | 3.2 $\pm$ 0.7   | 0.9     |
| AR-MCA, mean $\pm$ (SD)    | 0.58 $\pm$ 0.27 | 0.59 $\pm$ 0.09 | 0.8     |
| OR <sup>&amp;</sup>        | 9               | 3               |         |
| QoL score                  | 84.4 $\pm$ 34.6 | 76.8 $\pm$ 24.5 | 0.6     |
| Nasal lavage* median (IQR) |                 |                 |         |
| Neutrophils, %             | 21.8 (71.3)     | 66.0 (55.1)     | 0.3     |
| Eosinophils, %             | 0.0 (2.8)       | 0.7 (1.5)       | 0.7     |
| Macrophages, %             | 12.6 (15.9)     | 4.3 (7.8)       | 0.1     |

Abbreviations: HMW: high molecular weight; LMW: low molecular weight; AR-Vol2-5: volume between 2-5 cm into the nose measured by acoustic rhinometry; AR-MCA: Minimal nasal crosssectional area measured by acoustic rhinometry; QoL: quality of life; OR: occupational rhinitis; IQR: interquartile range.

<sup>&</sup> Positive challenge confirming diagnosis of OR

\* Nasal lavage measurement performed before exposure to control agent (baseline)

Table 2. Inflammatory cells after challenge with control and specific agent

|                           | Group 1: HMW      | Group 2: LMW      | P Value |
|---------------------------|-------------------|-------------------|---------|
| <b>Control challenge</b>  |                   |                   |         |
| Neutrophils               | 40.0 (13.9, 65.7) | 29.1 (5.2, 82.2)  | 0.9     |
| Eosinophils               | 0.0 (0.0, 3.9)    | 0.0 (0.0, 0.4)    | 0.9     |
| Macrophages               | 13.0 (5.8, 33.2)  | 7.2 (0.0, 12.4)   | 0.06    |
| <b>Specific challenge</b> |                   |                   |         |
| Neutrophils, %            | 65.3 (41.4, 82.6) | 45.1 (27.0, 73.8) | 0.1     |
| Eosinophils, %            | 3.3 (0.0, 22.1)*  | 0.0 (0.0, 4.2)    | 0.2     |
| Macrophages, %            | 5.3 (3.0, 8.9)    | 9.0 (0.8, 22.5)*  | 0.6     |

Abbreviations: HMW: high molecular weight; LMW: low molecular weight.

Data are expressed as median (IQR)

\*  $P < .05$  comparing values from control and specific challenge days

Table 3. Nasal concentrations of inflammatory proteins after challenge with control and specific agent

| Protein           | After control exposure |                 |                 |         | After specific exposure |                 |               |             |
|-------------------|------------------------|-----------------|-----------------|---------|-------------------------|-----------------|---------------|-------------|
|                   | % DS                   | HMW             | LMW             | P-Value | % DS                    | HMW             | LMW           | p-Value     |
| AAT µg/ml         | 100                    | 0.31 (0.43)     | 0.25 (0.83)     | 0.85    | 100                     | 0.30 (1.80)     | 0.25 (0.56)   | 0.43        |
| A2 Macro µg/ml    | 100                    | 0.37 (0.65)     | 0.38 (0.63)     | 0.85    | 100                     | 0.37 (2.20)     | 0.37 (0.39)   | 0.35        |
| B2M µg/ml         | 100                    | 0.14 (0.13)     | 0.22 (0.16)     | 0.43    | 85                      | 0.17 (0.19)     | 0.165 (0.19)  | 0.97        |
| CPR ng/ml         | 90                     | 0.02 (0.26)     | 0.18 (0.46)     | 0.69    | 85                      | 0.41 (1.70)     | 0.2 (0.90)    | 0.67        |
| C3 µg/m           | 90                     | 0.12 (0.27)     | 0.21 (0.34)     | 0.43    | 65                      | 0.90 (1.20)     | 0.13 (0.17)   | 0.63        |
| Factor VII ng/ml  | 70                     | 0.44 (0.38)     | 0.86 (0.42)     | 0.36    | 65                      | 0.78 (0.76)     | 0.44 (0.42)   | 0.23        |
| FRTN ng/ml        | 70                     | 0.14 (0.12)     | 0.17 (0.13)     | 0.35    | 100                     | 0.12 (0.08)     | 0.08 (0.03)   | 0.43        |
| Fibrinogen µg/ml  | 100                    | 0.07 (0.15)     | 0.06 (0.08)     | 0.68    | 100                     | 0.09 (0.00)     | 0.04 (0.05)   | <b>0.05</b> |
| Haptoglobin µg/ml | 100                    | 0.23 (0.34)     | 0.16 (0.23)     | 0.31    | 60                      | 0.86 (0.01)     | 0.14 (0.20)   | <b>0.02</b> |
| ICAM-1 ng/ml      | 65                     | 0.48 (0.10)     | 0.26 (0.22)     | 0.07    | 60                      | 0.26 (0.51)     | 0.26 (0.18)   | 0.26        |
| IL-1 α ng/ml      | 65                     | 0.08 (0.01)     | 0.10 (0.01)     | 0.86    | 100                     | 0.06 (0.01)     | 0.01 (0.01)   | 0.97        |
| IL-1ra pg/ml      | 100                    | 425.50 (878.70) | 733.00 (938.70) | 0.57    | 100                     | 361.50 (835.00) | 431.0 (299.0) | 0.79        |
| IL-8 pg/ml        | 100                    | 63.50 (221.20)  | 93.50 (91.20)   | 0.79    | 45                      | 182.00 (266.20) | 39.0 (45.0)   | 0.16        |
| IL-10 pg/ml       | 40                     | 0.53 (0.48)     | 0.56 (0.08)     | 0.76    | 45                      | 0.56 (0.43)     | 0.56 (0.24)   | 0.73        |
| IL-15 ng/ml       | 40                     | 0.14 (0.10)     | 0.14 (0.20)     | 0.68    | 45                      | 0.09 (0.12)     | 0.08 (0.10)   | 0.41        |
| IL-17 pg/ml       | 65                     | 0.46 (0.48)     | 0.27 (0.81)     | 0.60    | 65                      | 0.27 (0.49)     | 0.27 (0.38)   | 0.94        |
| IL-18 pg/ml       | 65                     | 14.50 (13.40)   | 9.40 (4.90)     | 0.80    | 70                      | 6.40 (4.45)     | 4.60 (6.40)   | 0.49        |
| MIP-1 α pg/ml     | 55                     | 2.70 (2.10)     | 2.70 (0.32)     | 0.53    | 50                      | 2.70 (0.65)     | 2.70 (0.80)   | 0.69        |
| MMP-3 ng/ml       | 55                     | 0.10 (0.08)     | 0.06 (0.07)     | 0.97    | 100                     | 0.09 (0.20)     | 0.05 (0.60)   | 0.24        |
| MMP-9 ng/ml       | 65                     | 9.90 (14.80)    | 5.60 (8.42)     | 0.30    | 55                      | 7.60 (12.00)    | 4.00 (6.37)   | 0.16        |
| MCP-1 pg/ml       | 65                     | 15.00 (10.70)   | 13.00 (11.15)   | 0.73    | 100                     | 16.0 (17.90)    | 10.00 (9.27)  | 0.10        |
| SCF pg/ml         | 90                     | 7.70 (9.80)     | 14.00 (9.80)    | 0.54    | 50                      | 14.0 (6.30)     | 7.70 (6.0)    | 0.15        |
| TIMP-1 ng/ml      | 90                     | 10.00 (10.60)   | 10.00 (8.15)    | 0.52    | 100                     | 11.85 (13.00)   | 9.25 (6.65)   | 0.57        |
| TNFR2 ng/ml       | 90                     | 0.05 (0.08)     | 0.02 (0.03)     | 0.43    | 95                      | 0.03 (0.06)     | 0.01 (0.02)   | 0.07        |
| VCAM-1 ng/ml      | 90                     | 0.29 (0.38)     | 0.17 (0.21)     | 0.16    | 100                     | 0.34 (0.67)     | 0.11 (0.11)   | <b>0.01</b> |
| VEGF pg/ml        | 100                    | 148.00 (116.50) | 139.00 (47.00)  | 0.52    | 100                     | 157.0 (154.0)   | 98.00 (20.25) | <b>0.01</b> |
| VDBP µg/ml        | 100                    | 0.06 (0.09)     | 0.03 (0.03)     | 0.48    | 100                     | 0.06 (0.13)     | 0.03 (0.03)   | <b>0.04</b> |

Abbreviations: AAT: Alpha-1-Antitrypsin; A2 Macro: Alpha 2 Macroglobulin; B2M: Beta-2-Macroglobulin; CPR: C-Reactive Protein; C3: Complement C3; FRTN: Ferritin; ICAM-1: Intercellular Adhesion Molecule-1; IL-1alpha: Interleukin-1- alpha; IL-1ra: Interleukin-1-receptor antagonist; IL-8: Interleukin-8; IL-10: Interleukin-10; IL-15: Interleukin-15; IL-17: Interleukin-17; IL-18: Interleukin-18; MIP-1 α: Macrophage Inflammatory protein-1 alpha; MMP-3: Matrix-Metalloproteinase-3; MMP-9: Matrix-Metalloproteinase-9; MCP-1: Monocyte chemotactic protein-1; SCF: Stem Cell factor; TIMP-1: Tissue Inhibitor of Metalloproteinases-1; TNFR2: Tumor Necrosis-Factor receptor-2; VCAM-1: Vascular Cell Adhesion Molecule-1; VEGF: Vascular Endothelial Grow Factor; VDBP: Vitamin D-Binding Protein. HMW: high molecular weight; LMW: low molecular weight. %DS: percentage of nasal samples with proteins concentrations within the limits of detection. Data are expressed as medians (IQR). %DS: percentage of samples from control specific challenge day with levels ≥ LDD.

Table 4. Relationship between inflammatory markers after specific exposure to HMW agents

|  |
|--|
|  |
|--|

|             | Haptoglobin | Fibrinogen | VCAM-1 | VEGF   | CRP  | VDBP  |
|-------------|-------------|------------|--------|--------|------|-------|
| Haptoglobin |             | 0.94**     | 0.66*  | 0.82** | 0.60 | 0.52  |
| Fibrinogen  |             |            | 0.71*  | 0.73** | 0.48 | 0.62* |
| VCAM-1      |             |            |        | 0.55   | 0.55 | 0.66* |
| VEGF        |             |            |        |        | 0.60 | 0.53  |
| CRP         |             |            |        |        |      | 0.68* |
| VDBP        |             |            |        |        |      |       |

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Abbreviations: VCAM-1: Vascular Cell Adhesion Molecule-1; VEGF: Vascular Endothelial Grow Factor; CPR: C-Reactive Protein; VDBP: Vitamin D-Binding Protein  
Spearman’s rho;  
\* p <0.05  
\*\*p <0.01

**Supplemental Table 1.** Inflammatory proteins with values below the least detectable dose (LDD)

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Brain-Derived Neurotrophic Factor (BDNF)  
Eotaxin-1  
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)  
Interferon Gamma (IFN-gamma)  
Interleukin-1 beta (IL-1 beta)  
Interleukin-2 (IL-2)  
Interleukin-3 (IL-3)  
Interleukin-4 (IL-4)  
Interleukin-5 (IL-5)  
Interleukin-6 (IL-6)  
Interleukin-7 (IL-7)  
Interleukin-12 Subunit p40 (IL-12p40)  
Interleukin-12 Subunit p70 (IL-12p70)  
Interleukin-23 (IL-23)  
Macrophage Inflammatory Protein-1 beta (MIP-1 beta)  
Matrix Metalloproteinase-2 (MMP-2)  
T-Cell-Specific Protein RANTES (RANTES)  
Tumor Necrosis Factor alpha (TNF-alpha)  
Tumor Necrosis Factor beta (TNF-beta)  
von Willebrand Factor (vWF)

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