HARRI STARK

Inflammatory Airway Responses Caused by *Aspergillus fumigatus* and PVC Challenges

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium 2, Kuopio University Hospital, on Friday 16th November 2007, at 1 p.m.

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National Public Health Institute
Kuopio
ABSTRACT

World-wide epidemiological studies have revealed that there is a strong linkage between inhaled indoor air pollutants and adverse health effects. Typical symptoms experienced by the exposed subjects in association with moisture damage are airway symptoms such as cough, phlegm, wheezing and nasal symptoms. Indoor air microbes including Aspergillus fumigatus can also cause ocular symptoms, fatigue, headache, joint pain and nausea in the exposed individuals, and even chronic diseases such as asthma have been reported. In addition, degradation of polyvinyl chloride (PVC) is associated with an increased prevalence of allergy, asthma and dermal symptoms. However, the causal health impacts of these indoor air contaminants are not well understood.

This study involved experimental challenges to A. fumigatus and degraded PVC flooring material or control challenges (allergen diluent, ceramic tile, respectively) and the active agent and control exposures were performed in a random order. There were 28 subjects in the A. fumigatus study and ten individuals were exposed to degraded PVC. The effects of the challenges on cytokine and nitric oxide (NO) concentrations in nasal lavage fluid (NAL), fractional exhaled NO (FE\textsubscript{NO}) and nasal NO (FN\textsubscript{NO}) and lung functions were studied. The subjects of the A. fumigatus challenge study formed three groups: one group had occupational mould exposure, one group consisted of atopic subjects and the third was a control group. Those 10 subjects participating in the PVC challenge had experienced occupational exposure to damaged PVC material. In addition, the short-term and seasonal reproducibility of FE\textsubscript{NO} and FN\textsubscript{NO} measurements techniques used in the challenge studies were assessed.

The A. fumigatus challenge evoked a rapid increase in FE\textsubscript{NO} levels and there was a significant difference when compared to control challenge (p<0.005) and the changes were not related to group. On the next morning after the A. fumigatus challenge, the subjects reported significantly more often respiratory tract symptoms compared to placebo (p=0.014). A. fumigatus challenge increased the levels of proinflammatory cytokines IL-1\(\beta\), TNF-\(\alpha\) and IL-6 in the subjects with the most distinct change being-observed in IL-1\(\beta\). In the subjects with occupational mould exposure, IL-4 concentrations also increased significantly.

On the next morning after the PVC challenge, the subjects reported significantly more frequently respiratory tract symptoms compared to control exposure (p=0.029). The PVC challenge did not affect the levels of inflammatory markers or lung functions.

The short-term and seasonal variations of the FE\textsubscript{NO} and FN\textsubscript{NO} measurements techniques were low. Diurnal variation was detected and the measurements performed in the mornings were more reproducible than the afternoon values.

In conclusion, due to rapid increase of FE\textsubscript{NO} levels after A. fumigatus challenge FE\textsubscript{NO} measurement can potentially be applied in the assessment of acute mould exposure. The increased cytokine levels provide a link between biochemical markers and acute mould exposure. Degraded PVC material evokes airway symptoms in the exposed individuals but it does not seem to cause an immediate asthma-like reaction. Serial FE\textsubscript{NO} and FN\textsubscript{NO} measurements can be used in the monitoring of respiratory tract inflammation. Due to the diurnal variation of FE\textsubscript{NO} and FN\textsubscript{NO} in long-term follow-up, the measurements should be performed at the same time of day, preferably in the morning.
To Minna, Elia, Niila, Miska, Akseli and Nuutti
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABPA</td>
<td>Allergic bronchopulmonary aspergillosis</td>
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<td>ATS</td>
<td>American Thoracic Society</td>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>CD4</td>
<td>Helper T lymphocyte</td>
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<tr>
<td>CD8</td>
<td>Cytolytic T lymphocyte</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cNOS</td>
<td>Constitutive nitric oxide synthase enzyme</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>CoV</td>
<td>Coefficient of variation</td>
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<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
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<tr>
<td>DEPH</td>
<td>Diethylhexyl phthalate</td>
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<tr>
<td>DINP</td>
<td>Diisononyl phthalate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DLCO</td>
<td>Pulmonary diffusion capacity</td>
</tr>
<tr>
<td>EBC</td>
<td>Exhaled breathe condensate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
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<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase enzyme</td>
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<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>EPX</td>
<td>Eosinophil protein X</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
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<tr>
<td>FE\textsubscript{\text{NO}}</td>
<td>Fractional exhaled nitric oxide</td>
</tr>
<tr>
<td>FEV\textsubscript{1}</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>FIOH</td>
<td>Finnish Institute of Occupational Health</td>
</tr>
<tr>
<td>FNAM</td>
<td>Finnish National Agency for Medicine</td>
</tr>
<tr>
<td>FN\textsubscript{\text{NO}}</td>
<td>Fractional nasal nitric oxide</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<tr>
<td>HES</td>
<td>Hypereosinophilic syndrome</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HNL</td>
<td>Human neutrophil lipokalin</td>
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<tr>
<td>IA</td>
<td>Invasive aspergillosis</td>
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<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
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<tr>
<td>IFN-\gamma</td>
<td>Interferon-gamma</td>
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<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>IPH</td>
<td>Idiopathic pulmonary hemosiderosis</td>
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<tr>
<td>MIP-1\alpha</td>
<td>Macrophage inflammatory protein 1 alpha</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>MVOC</td>
<td>Microbial volatile organic compound</td>
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<tr>
<td>NAL</td>
<td>Nasal lavage fluid</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase enzyme</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domains</td>
</tr>
<tr>
<td>PCD</td>
<td>Primary Ciliary Dyskinesia</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts per billion</td>
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<tr>
<td>ODTD</td>
<td>Organic dust toxic syndrome</td>
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<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SPT</td>
<td>Skin prick test</td>
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<tr>
<td>Th</td>
<td>T helper lymphocyte</td>
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<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TVOC</td>
<td>Total volatile organic compound</td>
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ORIGINAL PUBLICATIONS

This thesis is based on original publications which are referred to by Roman numerals.


TABLE OF CONTENTS

1 INTRODUCTION 15

2 REVIEW OF THE LITERATURE 17
   2.1 Health problems related to moisture-damaged buildings 17
      2.1.1 Microbes indicating moisture damages in building 19
      2.1.2 Aspergillus fumigatus 21
   2.2 Degraded PVC materials and adverse health effects 23
   2.3 Occupational lung diseases and exposure to indoor air bioaerosols 25
   2.4 Biochemical markers of respiratory tract inflammation 26
      2.4.1 Immune responses 26
      2.4.2 Nitric oxide 27
      2.4.3 Inflammatory cells 28
      2.4.4 Cytokines 29
   2.5 Non-invasive assessment of airway inflammation 31
      2.5.1 Fractional exhaled and nasal nitric oxide measurements 31
      2.5.2 Nasal lavage fluid 33
   2.6 Lung functions 34

3 AIMS OF THE STUDY 35

4 SUBJECTS AND METHODS 36
   4.1 Subjects 36
      4.1.1 Study I 36
      4.1.2 Studies II-III 36
      4.1.3 Study IV 37
   4.2. Methods 37
      4.2.1 Analysis of $F_{E_{NO}}$ and $F_{N_{NO}}$ 37
      4.2.2 Nasal lavage 38
      4.2.3 Analysis of cytokines and NO in NAL 39
      4.2.4 Analysis of protein and 2-ethylhexanol in NAL 44
      4.2.5 Lung function tests 44
      4.2.6 Skin Prick tests 44
      4.2.7 Symptoms related to challenges 45
      4.2.8 Cytospin 45
      4.2.9 IgE and ECP analysis 45
      4.2.10 Statistical methods 46
   4.3 Study protocols 47
      4.3.1 Study I 47
      4.3.2 Studies II and III 48
      4.2.3 Study IV 49
   4.4 Ethics 50
5 RESULTS

5.1 Short and long-term reproducibility of FE_{NO} and FN_{NO} 51
5.2 The induced effects of *Aspergillus fumigatus* challenge 54

5.2.1 Symptoms related to challenges 54
5.2.2 FE_{NO} and FN_{NO} levels 54
5.2.3 Lung functions 55
5.2.4 NO and cytokines in NAL 56
5.2.5 Cell differential count 60
5.2.6 IgE and ECP responses 60

5.3 The effects of PVC challenge 60

5.3.1 Symptoms related to challenge 60
5.3.2 TVOC and 2-ethylhexanol concentrations 62
5.3.3 Lung functions 64
5.3.4 FE_{NO} and FN_{NO} 64
5.3.5 Cytokines, NO and 2-ethylhexanol in NAL 64
5.3.6 Cell differential count 65

6 DISCUSSION 66

6.1 Short and long-term reproducibility of FE_{NO} and FN_{NO} 66
6.2 Inflammatory airway effects caused by *A. fumigatus* challenge 69
6.3 The effects of degraded PVC material challenge on airways 76
6.4 Clinical importance of the current results 78

7 CONCLUSIONS 80

8 REFERENCES 81

APPENDIX: ORIGINAL ARTICLES
1 INTRODUCTION

Indoor air pollutants in damp environments are a major public health concern since they are associated with harmful health effects, even chronic disease (Bornehag et al. 2001; Health Canada 2004; National Academy 2004). A wide variety of symptoms have been reported by the subjects exposed to indoor bioaerosols including upper and lower airway symptoms, conjunctival symptoms and clusters of asthma (Verhoeff and Burke 1997; Seuri et al. 2000; Zureik et al. 2002). Although the capability of indoor air microbes to produce secondary metabolites is well known (Jarvis and Miller 2004), the question of which agents in the indoor air contributes to the adverse health outcomes, remains unanswered. Although inflammatory markers such as proinflammatory cytokines and nitric oxide (NO) have been linked to indoor microbial exposure (e.g. Hirvonen et al. 1999; Purokivi et al. 2001, Roponen et al. 2001) the etiological factors linking indoor air bioaerosols and health problems are still unclear.

Moisture-damage in a building is thought to be an important source of the indoor air problems both in residential and occupational environments (Bornehag et al. 2001). In a Finnish study, 450 houses were investigated by civil engineers and in 55% of them were found to suffer from moisture-damage requiring renovation (Nevalainen et al. 1998). The prevalence of significant moisture damages in buildings was evaluated in Finland in the 1990s and estimated to be as high as 50% depending on how the damages was defined (Nevalainen et al. 1998; Koskinen et al. 1999). The proportion has been estimated to be approximately the same in other countries with similar climates e.g. the northern European countries and North America (Brunekreef et al. 1989). According to a recent Finnish questionnaire study, moisture damage was reported in 53% of the 1164 school buildings and serious mould damage with visible mould growth or mould smell in 26% of the schools (Kurnitski et al. 1996).
In addition to the adverse effects caused by the microbial exposure as such, the microbial growth in building materials may lead to degradation of polyvinyl chloride (PVC) flooring materials which release phthalates that are also hazardous to human health and may cause asthma in certain sensitive subjects (Bornehag et al. 2005b). Only few markers, except for IgE-mediated sensitization and to some extent IgG antibodies, can be used to assess exposure in the subjects residing in moisture problem buildings (Jarvis and Miller 2004).

As stated before, exposure to indoor air microbes is associated with inflammatory respiratory diseases and symptoms. Thus, an assessment of airway inflammation may serve as an indicator of the exposure. One parameter, fractional exhaled nitric oxide (FE\textsubscript{NO}) measurement, is used widely in clinical work for assessing lower airway inflammation. For example, among asthmatics, the FE\textsubscript{NO} levels are significantly increased when compared to those of healthy subjects (Kharitonov et al. 1994). Nasal nitric oxide (FN\textsubscript{NO}) levels can be used for examination of the upper airway inflammation. Normally the FN\textsubscript{NO} levels are clearly higher compared with FE\textsubscript{NO} levels (Jorissen et al. 2001). Nasal lavage fluid (NAL) sampling represents a simple and non-invasive way to investigate upper respiratory tract inflammation (Steerenberg et al. 1996).

In an attempt to investigate the health effects associated with exposure to microbes present in water-damaged buildings, we utilized two different approaches. In this study, it was assessed if experimental Aspergillus fumigatus and degraded PVC flooring material challenges could affect FE\textsubscript{NO} and FN\textsubscript{NO} levels, lung functions and nitric oxide (NO) and cytokines in NAL. Short-term and seasonal reproducibility of the FE\textsubscript{NO} and FN\textsubscript{NO} measurement techniques used in this study were also assessed.
2 REVIEW OF THE LITERATURE

2.1 Health problems related to moisture-damaged buildings

According to epidemiological data, exposure to the complex mixture of microbes and other chemical components of indoor air in moisture-damaged buildings is associated with a variety of symptoms (Bornehag et al. 2005; Portnoy et al. 2005). Most often the subjects have experienced upper and lower airway irritation symptoms such as cough, phlegm, blocked or itching of the nose, dyspnoea, wheezing, sore throat or hoarseness (Ruotsalainen et al. 1996; Seuri et al. 2000; Purokivi et al. 2001). The other symptoms include ocular distress, headache and joint pain (Verhoeff and Burke 1997; Roponen et al. 2001) and there is even a cluster of idiopathic pulmonary hemosiderosis (IPH) in infants exposed to fungal mycotoxins at their moisture-damaged homes (Dearborn et al. 1999). The irritation symptoms among the mould exposed subjects have been linked to endotoxins, β(1-3)-glucans and microbial volatile metabolites (MVOC) (Rylander 1998; Korpi 2001; Douwes 2005). In addition, fungal volatile substances have an unpleasant smell which can lead to psychological symptoms such as nausea and fatigue in the occupants of moisture-damaged buildings (Portnoy et al. 2005).

It has been claimed that exposure to indoor air fungi is associated with exacerbation of asthma in mould-sensitive asthmatics (Zureik et al. 2002; National Academy of Sciences 2004) and clusters of asthma have been found among occupants of moisture-damaged buildings (Smedje et al. 1996; Seuri et al. 2000; Bornehag et al. 2001). Moreover, increased risk of asthma has been shown to be related to the presence of visible mould or mould smell in occupational situations (Jaakkola et al. 2002). Allergic responses such as asthma, allergic rhinitis and organic dust toxic syndrome (ODTS) are the most common medical problems associated with inhalation exposure to fungi (Jarvis and Miller 2004). In a Finnish study, mould odour and visible mould in the workplace were connected to
the risk of asthma (Jaakkola et al. 2002). However, in a prospective cohort study, only mould odour was strongly associated with increased risk of developing asthma whereas other indicators of mould exposure such as history of water damage, moisture in the inferior surfaces and visible mould did not predict asthma. In addition, the joint effect of parental atopy and mould odour in asthma development was weaker than expected (Jaakkola et al. 2005).

A total of 70 fungal allergens have been characterized and among atopic subjects the prevalence of fungal allergy ranges from 20-30 % compared to 6 % in general population (Kurup et al. 2000; 2002). It has been reported that e.g. *Alternaria, Cladosporium, Aspergillus* and *Penicillium* species are linked to the development of atopic disease (Husman 1996; Kurup 2000). On the other hand, fungal allergy has not been associated with microbial exposure in symptomatic schoolchildren (Taskinen et al. 1997; 1998; 2002). These data indicate that there are also pathways other than IgE-mediated allergy that mediate the adverse health effects of indoor air microbes and that non-allergic inflammatory responses seem to play an important role. Elevated serum fungal IgG antibody levels have been found in farmers with extensive occupational mould exposure (Erkinjuntti-Pekkanen et al. 1999). In a previous study, which examined teachers with indoor air mould exposure, the increased fungal-specific IgG concentrations were associated with a higher prevalence of sinusitis (Patovirta et al. 2003). However, the presence of fungal IgG antibodies in human sera indicates only that an exposure has occurred at some time in the past (Jarvis and Miller 2004).

In Finnish population-based incident case-control study an increased risk of developing asthma in adulthood was significantly related to IgG antibodies to *T. citrinoviride*, but not to the other moulds including *Aspergillus fumigatus, A. versicolor, Cladosporium cladosporioides, Fusarium oxysporum, Sporobolomyces salmonicolor, Stachybotrys chartarum, and Streptomyces albus* (Jaakkola et al. 2002).

In moisture-damaged buildings, the concentrations of viable fungi and bacteria (10^1-
10^4 cfu/m^3) are generally not much higher than those in reference buildings (Hyvärinen 2002). However, visible mould or dampness has been often linked to increased indoor air fungal levels (Johanning et al. 1999; Hyvärinen et al. 2001; Meklin et al. 2003). In addition, there are several studies indicating that remediation of the mouldy environment can decrease irritation symptoms among the occupants compared to situation before the renovation (Åhman et al. 2000; Jarvis and Morey 2001; Patovirta et al. 2004) linking the symptoms to exposure in these buildings. On the other hand, toxicological studies in vitro and in vivo have shown clear differences between these microbial strains in their ability to induce cytotoxicity and inflammatory responses (Ruotsalainen et al. 1998; Huttunen et al. 2000). Moreover, the growing conditions may significantly affect the evoked responses (Hirvonen et al. 2001; Murtoniemi et al. 2001).

2.1.1 Microbes indicating moisture damage in buildings

In moisture damaged buildings, certain microbial species normally not found indoors, start to grow on the wet material. The microbes that can be considered as indicators of a moisture problem are mostly moulds but also yeasts and bacteria are found (Andersson et al. 1996) These microbes include bacteria such as actinobacteria and non-enteric gram-negative bacteria, yeasts and several fungi like Aspergillus fumigatus, Aspergillus versicolor, Exophiala, Fusarium, Phialophora, Stachybotrys, Trichoderma and Ulocladium (Samson et al. 1994; Hyvärinen et al. 2002). They occur in clusters of species with the most important factor regulating the growth being the available water, defined as water activity (aw) (Jarvis and Miller 2004). Spores of these microbes can become airborne and then can be inhaled into the airways (Burge 2002) but no obvious threshold value can be defined below which no adverse health effects occur due to the fact that some microbes seem to be more harmful than others. Nonetheless, it has been generally considered that exposure to an average of more than 1000 colony forming units (cfu)/m3 in the indoor air represents a potential health risk (Flannigan et al. 1991).
The microbial elements that might be recognized by the host and which might evoke activation of the immune system are the spore, hyphae or part of the cell wall. They include a wide variety of molecules such as proteins, polysaccharides linked to proteins, lipids and carbohydrates. The immune system responds to microbial cell wall components through innate immune receptors such as Toll-like receptors (Netea et al. 2004). In addition, some proteins and glycoproteins may also be allergenic and trigger the activation of the adaptive immune system in susceptible individuals. Chitin, a common cell wall constituent of moulds, has also been shown to have immunomodulatory properties in airway inflammation (Strong et al. 2002).

Many of the filamentous fungal species found in damp buildings produce harmful secondary metabolites such as mycotoxins (Bennet and Klich 2003). Animal studies have revealed that mycotoxins evoke both cytotoxic and inflammatory changes. They also disrupt cellular structures and interfere with vital cellular processes such as RNA
and DNA synthesis (Jarvis and Miller 2004). In addition to mycotoxins, fungi can also produce other secondary metabolites that are potent immunostimulators such as enzymes with protease or proteolysing activities. This is of special interest since those proteases have been observed to trigger the production of pro-inflammatory cytokines in vitro (Kauffman et al. 2000).

Microbial volatile compounds (MVOC) are compounds accounting for mouldy odours and they are considered to evoke respiratory symptoms in the mould exposed individuals (Norbäck et al. 1999). It has been shown that most building structures release MVOCs into the indoor air and the production of these compounds is not only typical for microbes (Korpi 2001).

2.1.2 Aspergillus fumigatus

Aspergillus fumigatus was investigated in this study and, therefore, it will be examined in more detail.

A. fumigatus is a fungus that has been isolated frequently from moisture-damaged buildings in and its presence linked with a variety of upper and lower airway symptoms in the occupants (Hyvärinen et al. 1999). Within the Aspergillus spp, A. fumigatus, a saprophyte fungus, which grows on large variety of organic remains. These species causes a wide range of diseases including organic dust toxic syndrome (ODTS), allergic reactions, asthma, allergic alveolitis, allergic bronchopulmonary aspergillosis (ABPA) and systemic diseases such as invasive aspergillosis (IA) which has mortality ranging from 60 to as high as 90 % (Tekaia and Latge 2005). In immunodeficient individuals (e.g. patients who have undergone transplants, patients with leukaemia or HIV) the inhalation of A. fumigatus conidia may lead to fatal consequences (Rementeria et al. 2005). There are several reports linking aspergillosis to increased airborne levels of A. fumigatus spores (Andersson et al. 1996; Loo et al. 1996; Oren et al. 2001).
Since the conidia of A. fumigatus are small in size (from 2 to 3 µm) they can remain in the environment for a long time (Figure 1). They can reach the pulmonary alveoli in individuals constantly inhaling the air in which they are suspended (Latge 1999). A. fumigatus secretes highly toxic secondary metabolites such as gliotoxin, fumagillin and helvolic acids which are considered to cause the adverse health effects (Fischer et al. 2000).

Inhalation of A. fumigatus conidia by an immunocompetent individual activates the innate cellular system (alveolar macrophages, neutrophils) which is responsible for killing of the conidia. Conidial dihydroxynaphthalene-melanin has been recognized as a virulence factor of A. fumigatus and it is present on the conidial surface. On the other hand, A. fumigatus is a saprotrophic fungus that becomes a pathogen due to simple biological reasons: it is present in high concentrations in the atmosphere, it grows faster than any other airborne fungi at 40 °C and it can overcome the defence system of the host when the host has a very weak or impaired defence immunity (Tekaia and Latge 2005).

Indoor air exposure to A. fumigatus can evoke an IgE-mediated allergic reaction (Zureik et al. 2002) and a typical Th2-type response, including the elevated IL-4 levels, dominates the immune response (Schuh et al. 2003). In addition, production of the anti-inflammatory cytokine, IL-10, is also enhanced and it has been estimated to diminish both Th1- and Th2-type responses evoked by A. fumigatus (Schuh et al. 2003). On the other hand, it has been reported that fungal allergy (including A. fumigatus) is rare among symptomatic subjects exposed to indoor air microbes of a moisture-damaged building (Taskinen et al. 1997). Thus, there are also mechanisms other than allergy involved in evoking the symptoms attributed to A. fumigatus. In addition, part of the IgE binding to mould and yeast allergen extracts is explained by cross-reacting glycoproteins and, therefore, false-positive IgE and skin prick test results need be taken into account in any diagnosis of mould allergy (Leino et al. 2006). On the other hand, there is also
epidemiologic evidence that sensitization to *A. fumigatus* as well as to *Cladosporium herbarum* indicated by specific IgE is related to the risk of adult-onset asthma (Jaakkola et al. 2006).

2.2 Degraded PVC materials and adverse health effects

Polyvinyl chloride (PVC) is synthesized by polymerization of vinyl chloride monomers. PVC is a hard plastic material that is softened by plasticizers such as phthalates. PVC is an important product in the chemical industry since it has a wide range of applications including building materials, food packaging, credit cards, clothing, toys and medical devices.

Diethylhexyl phthalate (DEHP) and di-isononyl phthalate (DINP) are the best-known phthalates that release from degraded PVC products. Most of the environmental monitoring data is devoted to DEHP, almost 2 million tons of which is produced worldwide each year. The major source of human exposure to DEHP is contaminated food e.g. during production or packaging but the next important source is the indoor air. Degradation of PVC flooring material may result in the release of DEHP into the indoor air where the agent adheres to aerosols which are then inhaled by the exposed subjects (National Toxicology Program, 2000).

Damaged PVC materials have been related to asthma and allergy. Already in the 70s, some reports described occupational asthma among meat wrappers who are exposed to pyrolysis products of PVC when cutting the wrapper with hot wire rod (Sokol et al. 1973, Andrasch et al. 1976). In addition, epidemiologic studies have shown increased risk of ocular and respiratory symptoms and asthma among meat wrappers compared with unexposed staff (Polakoff et al. 1975, Falk and Portnoy 1976). Øie et al. (1997) suggested that DEHP can bind into the surface of building materials, particles etc. leading to large concentrations gaining access to the respiratory organs. They also suggested that
the plastic materials are potential sources of chemical emission into indoor air which may cause inflammation and elevate the risk of asthma.

Wieslander et al. (1999) and Norbäck et al. (2000) have studied nasal, ocular and asthmatic symptoms in relation to building dampness and the degradation of PVC flooring material among 87 workers of four geriatric hospitals. According to their studies, release of the DEHP from PVC flooring causes conjunctival and nasal irritation and increases asthma-like symptoms in the exposed subjects. They also detected the PVC degradation product 2-ethylhexanol in indoor air samples.

Jaakkola et al. (1999; 2000) have published studies on the problems associated with plastic materials at home and how this impacts on the respiratory health of young children. They concluded that the chemical emissions from PVC materials into indoor air could cause adverse respiratory effects. Bornehag et al. (2004) described a dose response relationship among children between asthma prevalence and the concentrations of DEHP in settled dust. In addition, it has been reported that DEHP concentrations were higher in buildings erected before 1960 (Bornehag et al. 2005a). That could reflect higher fractional concentrations in older products or higher emission rates as products degrade. It has been claimed that the combination of water leakage at home and PVC as flooring material in the rooms was associated with a higher prevalence of symptoms in the exposed subjects compared to only water leakage (Bornehag et al. 2005b).

Phthalate esters have been proposed to act as either allergens or adjuvants (Jaakkola et al. 1999; Øie et al. 1997) but even though there is a strong association between health problems and PVC materials, the etiological factors causing the symptoms are still unclear. However, it has been shown in animal models that phthalates can cause death and malformations of foetuses. They are also animal carcinogens and toxic to the reproductive system (Tyl et al. 1988; Li et al. 1998).
2.3 Occupational lung diseases and exposure to indoor air bioaerosols

Occupational lung disease is a work-related illness based on the frequency, severity and preventability of diseases. These illnesses have been attributed to due to extensive exposure to irritating or toxic substances that may cause acute or chronic respiratory ailments. World-wide, asthma is the most common occupational lung illness followed by asbestosis, mesothelioma, occupational lung cancer, byssinosis, coal workers’ pneumoconiosis and allergic alveolitis (National Institute for Occupational Safety and Health 2002). In Finland, 306 new occupational asthma cases were diagnosed during the year 2002, and the moulds of moisture-damaged buildings were the largest responsible factor causing this disease being responsible for 79 cases (Piipari and Keskinen 2005).

Increased prevalence of occupational disorders including respiratory tract irritation symptoms, allergic rhinitis, asthma and allergic alveolitis (e.g. “farmers lung disease”) has been reported in association with extensive bioaerosol exposure in farmers (Erkinjuntti-Pekkanen et al. 1999; Kimbell-Dunn et al. 2001), waste treatment and compost-handling workers (Wouters et al. 2002) and sawmill workers (Mandryk et al. 2000). There are also reports showing that exposure to indoor air fungi and phthalates may evoke asthma in some individuals (Zureik et al. 2002; Jaakkola et al. 1999; 2000). As stated before, Aspergillus fumigatus is a well-known indicator microbe of mould damages (Hyvärinen et al. 1999) which can cause a variety of respiratory disease including asthma (Rementeria et al. 2005; Jaakkola et al. 2006). Thus, occupational exposure in moisture and mould damaged work environment may lead to the development of occupational asthma induced by A. fumigatus.

In Finland, the diagnostic investigations of mould-induced asthma are performed in central hospitals or in the Finnish Institute of Occupational Health (FIOH). Skin prick tests (SPT) and specific IgE antibodies to the most common moulds are determined and the peak expiratory flow (PEF) values are followed at home and at the workplace.
Definite diagnoses are based on challenge tests to moulds which have been found in the material or indoor samples from the workplace. In practice, there are commercial challenge extracts only for *A. fumigatus, Cladosporium cladosporioides* and *Acremonium kiliense* (Piipari and Keskinen 2005).

### 2.4 Biochemical markers of respiratory tract inflammation

#### 2.4.1 Immune responses

The immune response is how the body recognizes and defends itself against harmful substances such as bacteria, viruses and fungi. An essential component of the immune system protection is the way that the cells recognize and respond to the antigens which are large molecules on the surface of cells, viruses, bacteria or fungi. After recognition, the immune system destroys micro-organisms containing the antigens.

Innate immunity means barriers such as skin, mucus and the cough reflex that prevent harmful antigens from gaining access to the body. It relies on receptors such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains proteins (NOD-like receptors, NLRs) which alert the immune system of the invading microbes (Albiger et al. 2007). In contrast, acquired immunity is the type of immunity that occurs when the body has been exposed to harmful antigens and, consequently, represents a specific defence against harmful antigens. Lymphocytes have an important role in acquired immunity and they will be described more detailed later.

The inflammatory response results in inflammation which represents tissue injury in the inflamed area. The damaged tissues release vasoactive chemicals such as histamine, bradykinin and serotonin which lead to fluid leakage from blood vessels. Subsequently, the tissues swell which prevents antigens from achieving further tissue contact. In addition to swelling, typical signs of inflammation are redness, pain and warmth in the inflamed area (Medical Encyclopedia 2006).

Nitric oxide, cytokines and inflammatory cells will be described in detail as a part of immune response in airways.
2.4.2 Nitric oxide

Nitric oxide (NO) is a potent biological mediator and it has an important role in a wide variety of cellular and tissue functions. Previously, NO was regarded as a noxious environmental pollutant but later several studies revealed that it is an essential molecule in the human body (Fabio et al. 2004). The existence of NO in exhaled air of healthy subjects was originally reported by Gustafsson et al. (1991). Later, it was shown that NO is clearly increased in exhaled air of asthmatic subjects (Kharitonov et al. 1994). NO is synthesized from the amino acid L-arginine and three isoforms of NO synthase (NOS) enzymes have been described (Moncada et al. 1993; Nathan et al. 1993). In the airways, NO participates in many different functions such as mediation in inflammation, bacteriostatic and virostatic activity, and dilatation of bronchial smooth muscle (Fabio et al. 2004).

The NOS enzymes which form the system responsible for NO production were originally identified by Bult et al. (1990). Molecular cloning and protein purification methods have revealed three distinct isoforms of NOS: inducible NOS (iNOS), constitutive neuronal NOS (nNOS) and constitutive endothelial NOS (eNOS). Each of the three isoforms has a characteristic tissue-specific expression and all the isoforms are expressed in the airways (Lamas and Michel 1997). NO derived from the constitutive isoforms nNOS and eNOS (cNOS) has been found to moderate the bronchomotor tone. In contrast, NO derived from the iNOS isoform has been indicated to be a proinflammatory mediator (Fabio et al. 2004). iNOS isoform levels are induced by many triggers e.g. cytokines IL-1β, TNF-α and IFN-γ (Morris and Billiar 1994). After exposure the iNOS produces large amounts of proinflammatory NO and these high levels may be sustained for several hours (Fabio et al. 2004). In a recent case report, acute purulent sinusitis was triggered by topical nasal administration of an NO synthase inhibitor (Lundberg 2005).

In addition to several inflammatory diseases, elevated NO concentrations have been measured in NAL after exposure to indoor air microbes (Hirvonen et al. 1999; Roponen et al. 2001). On the other hand, it has been claimed that the presence of nitric oxide alone is an insufficient biomarker of exposure to microbes in a moisture-damaged building (Purokivi et al. 2002).
2.4.3 Inflammatory cells

Macrophages are large phagocytic cells secreting proinflammatory and antimicrobial mediators and they have a major role in innate and adaptive immunity (Gordon 1999). These cells exist in several tissues such as connective tissue (histiocytes), liver (Kupffer’s cells), lung (alveolar macrophages), lymph nodes (free and fixed macrophages), spleen (free and fixed macrophages), bone marrow (fixed macrophages), serous fluids (pleural and peritoneal macrophages) and skin (histiocytes, Langerhans’s cell). Macrophages have also been classified according to how they are activated. Classically activated macrophages exhibit a Th1-like phenotype promoting inflammation, extracellular matrix (ECM) destruction and apoptosis. Instead, alternatively activated macrophages display a Th2-like phenotype, promoting ECM construction, cell proliferation, and angiogenesis.

Neutrophilic granulocytes are peripheral blood cells which account for constituting normally 99 % of the circulating polymorphonuclear cells. The granules of neutrophils contain lysozyme, myeloperoxidase (MPO) and human neutrophil lipokalin (HNL). Neutrophils are involved in acute inflammatory responses, acting as a first line of defence against invading micro-organisms. In the inflammatory context, the neutrophils are known to produce proinflammatory cytokines (TNF-α and IL-1β), CC and CXC chemokines, macrophage inflammatory protein (MIP-1α) and angiogenic factors (Kasama et al. 2005).

Eosinophil granulocytes are produced in bone marrow and they are normally found in peripheral blood and gut lining. Eosinophils are essential mediators of allergic inflammation and increased eosinophil levels have been detected in atopic disease such as asthma and allergic rhinitis (serum, sputum) and also in parasitic infections (Keatings and Barnes 1997). Hypereosinophilic syndrome (HES) is a rare disorder that is characterized by persistent and marked eosinophilia combined with organ system dysfunction (Wilkins et al. 2005). As a response to inflammation eosinophils can produce eosinophil peroxidase (EPO), eosinophil protein X (EPX), oxygen radicals and cytokines (IL-5, IL-6, TNF-α).
Lymphocytes are divided into two broad categories called T- and B-lymphocytes. T-cells play an important role in cell-mediated immunity whereas B-cells are primarily responsible for humoral immunity. T-lymphocytes are grouped to CD4 and CD8 cells, and the CD4 cells are further divided according to which cytokine types they produce. CD4 cells are subgrouped to Th1 cells producing IFN-γ and Th2 cells inducing the production of IL-3, IL-4, IL-5 and IL-10. Th2 cells are activated in atopic disorders whereas IFN-γ inhibits IgE production in B-lymphocytes (Schuh et al. 2003).

2.4.4 Cytokines

Cytokines are small, soluble and multifunctional polypeptides produced by a variety of cells. Epithelial and inflammatory cells in the nasal mucosa can secrete several cytokines such as TNF-α, IL-1β, IL-4, IL-6 and interferon gamma (IFN-γ) (Barnes et al. 1998; Opal et al. 2000). The human immune response is regulated by a highly complex network of agents and cytokines form an essential part of this entity. Cytokines modulate leukocytes and structural cells during inflammatory and immune responses. They are able to eliminate pathogens by a number of approaches including free radical generation and phagocytic activation. The cytokines are capable of evoking (e.g. via TNF-α, IL-1β) or suppressing the inflammation (e.g. via IL-10) (Schuh et al. 2003). Cytokines IL-1β, IL-4, IL-6, IL-12, TNF-α and IFN-γ were assessed in this study and they are reviewed in more detail.

IL-1β is a proinflammatory cytokine mainly produced by airway macrophages as a part of the non-specific inflammatory response. It stimulates expression of endothelial adhesion molecules and chemokines and enhances the production of NO (Barnes et al. 1998; Opal et al. 2000). IL-1β stimulates the production of TNF-α which is also a proinflammatory cytokine (Yoshimura et al. 2003). In a similar manner to IL-1β, TNF-α stimulates the expression of endothelial adhesion molecules but also stimulates the recruitment of neutrophils and monocytes in inflammation (Barnes et al. 1998). Increased levels of IL-1β and TNF-α in nasal lavage fluid (NAL) have been detected after mould exposure in a moisture damaged building (Purokivi et al. 2001) and exposure
to swine dust (Wang et al. 1997). TNF-α is expressed by various cell types including lymphocytes, eosinophils, macrophages, monocytes and epithelial cells (Barnes et al. 1998).

IL-6 is also a proinflammatory cytokine but, on the other hand, it can inhibit the production of TNF-α and IL-1β and may protect the host cells from potentially destructive inflammatory responses (Opal et al. 2000). IL-6 is known to stimulate the synthesis of acute-phase proteins and the growth of B lymphocytes and it is expressed by lymphocytes, monocytes, macrophages, epithelial cells, fibroblasts and smooth muscle cells (Barnes et al. 1998). Several studies have reported increased IL-6 levels in NAL after exposure to indoor air microbes of moisture-damaged buildings (Hirvonen et al. 1999; Purokivi et al. 2001; Roponen et al. 2001). The spores of *Aspergillus versicolor*, isolated originally from the indoor air of a moisture-damaged building, have been shown to cause acute inflammation in mouse lungs assessed as a dose-dependent increase in the levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in bronchoalveolar lavage fluid (BALF). This was confirmed by the presence of histopathological changes in the lungs (Jussila et al. 2002).

* T cells are divided into subsets based on cytokine production. Those cells that produce IFN-γ are called Th1 cells and those producing IL-4 are designated Th2 cells. Mast cells can generate IL-4, while the natural killer cells are an important source of IFN-γ. IL-4 promotes the Th2-type response and it is connected to allergy and atopic disease. In addition, the Th2-type response including, the elevated IL-4 levels, dominates the immune response in *A. fumigatus* induced diseases such as pulmonary aspergillosis (Schuh et al. 2003). Elevated IL-4 levels in NAL have been found in association with occupational mould exposure (Roponen et al. 2001). Th1-type cytokine IFN-γ inhibits IL-4 production and cell proliferation and also enhances the cytotoxicity of TNF-α (Barnes et a. 1998). It has been demonstrated that the development of an IFN-γ producing capacity during the first 3 months of life is associated with farming, the existence of endotoxin in house dust and cat and dog exposure (Roponen et al. 2005).

IL-12 inhibits the production of Th2-type cytokines and enhances the Th1-induced differentiation and proliferation (Nutku et al. 2001). Increased IL-12 levels have been
detected in several respiratory disorders e.g. in rhinovirus infections (Ferreira et al. 2002).

2.5 Non-invasive methods for airway inflammation assessment

Several non-invasive methods have been developed for assessing respiratory tract inflammation. In this study, fractional exhaled ($F_{ENO}$) and nasal ($FN_{NO}$) nitric oxide (NO) measurements, lung function tests including spirometry and diffusion capacity (DLCO) and the assessment of cytokine concentrations in nasal lavage fluid (NAL) were used and, therefore, they will be described in detail. However, determining of inflammatory markers from induced sputum and exhaled breath condensates (EBC) are also widely used non-invasive techniques to assess airway inflammation (Effros et al. 2004; Brightling 2006).

2.5.1 Fractional exhaled and nasal nitric oxide measurements

Direct measurements of $F_{ENO}$ and $FN_{NO}$ are performed by means of chemiluminescence analysis. The instruments developed for the NO measurement are based on technology dating from the 1970’s which were originally used for environmental and atmospheric analyses (Fontjin et al. 1970). $F_{NO}$ measurement techniques are nowadays widely used in clinical work in the assessment of lower airway inflammation. Both ATS and ERS have provided recommendations on how the $F_{NO}$ and $FN_{NO}$ measurements should best be done (Kharitonov et al. 1997; ATS 1999; ATS/ERS 2005). The experimental data show that the normal $F_{NO}$ values of healthy subjects range from 10-20 ppb but there is not a full consensus about the reference values (Smith and Taylor 2005). Conventionally, the $F_{NO}$ and $FN_{NO}$ measurements have required the presence of several pieces of bulky, non-portable equipment. Novel hand-held devices for NO measurements have been developed and are claimed to be suitable for clinical studies (Alving et al. 2006). In addition to the previously reported techniques, new off-line methods for $FN_{NO}$ measurement have been developed (Oh et al. 2004).
The $\text{FE}_{\text{NO}}$ levels have been found to be inversely correlated to exhalation flow rate and therefore a fixed flow rate of 50 mL·s$^{-1}$ has been recommended (Kharitonov et al. 1997; ATS 1999). It has been found that higher exhalation flow rates (250 vs. 500 mL/minute) lead to larger intraday and inter-day variabilities in $\text{FN}_{\text{NO}}$ measurement, and the $\text{FN}_{\text{NO}}$ levels are inversely related to the flow rate (Silkoff et al. 1999).

The baseline levels of $\text{FN}_{\text{NO}}$ are high compared to $\text{FE}_{\text{NO}}$, with the highest levels being reported in paranasal sinuses (Lundberg et al. 1999; Jorissen et al. 2001). At present, there is no standardized technique for measuring $\text{FN}_{\text{NO}}$ and, thus the levels measured from different laboratories vary from 30 to 2000 ppb (Jorissen et al. 2001). This has been explained as being attributable to the different measurement techniques (Silkoff et al. 2001; Jorissen et al. 2001).

The short-term reproducibility of $\text{FE}_{\text{NO}}$ measurement techniques (intraday, day-to-day, week-to-week) is high according to several studies (Ekroos et al. 2000; Ekroos et al. 2002; Kharitonov et al. 2003). The reproducibility of $\text{FN}_{\text{NO}}$ has not been investigated as extensively as $\text{FE}_{\text{NO}}$ but, however, the short-term variation of $\text{FN}_{\text{NO}}$ has been indicated as being low (Bartley et al. 1999; Palm et al. 2000; Kharitonov et al. 2005). In spite of the intense interest in the assessment of NO, it is surprising that there are so few studies about the long-term reproducibility of $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ measurement techniques. This is an important issue because $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ measurements provide excellent tools for long-term follow up of an individual’s airway inflammatory status.

It has been stated that $\text{FE}_{\text{NO}}$ levels increase in asthma and decline from these high levels as a response to corticosteroid treatment (Kharitonov et al. 1994; Fabio et al. 2004). $\text{FE}_{\text{NO}}$ may also reflect disease severity and clinical control of asthma, particularly during exacerbations (Kharitonov et al. 1996; Sippel et al. 2000). $\text{FE}_{\text{NO}}$ levels correlate in asthmatic patients with airway hyperresponsiveness to metacholine, peak expiratory flow values and eosinophilic inflammation as determined in blood, bronchoalveolar lavage (BAL) and sputum (Fabio et al. 2004).

The role of $\text{FE}_{\text{NO}}$ in COPD is conflicting. Current smokers with severe COPD (particularly in combination with cor pulmonale) show lower $\text{FE}_{\text{NO}}$ levels than ex-smokers who have mild or moderate COPD (Clini et al. 2000). Smoking is known to
decrease FE\textsubscript{NO} and FN\textsubscript{NO} levels (Persson et al. 1994) but after smoking cessation, oral NO increases again to normal levels (Robbins et al. 1997).

Other disorders associated with increased FE\textsubscript{NO} levels are rhinitis, bronchiectasis, active pulmonary sarcoidosis, active fibrosing alveolitis and acute lung allograft rejection (Fabio et al. 2004). In contrast, low levels of FE\textsubscript{NO} have been linked to primary ciliary dyskinesia (PCD), cystic fibrosis (CF), PiZZ phenotype-related \(\alpha_1\)-antitrypsin deficiency and pulmonary hypertension (Fabio et al. 2004).

In the occupational environment, inhalation of organic dust from swine houses increased the levels of FE\textsubscript{NO} in the exposed occupants (Sundblad et al. 2002). Traffic-related air pollution has also been linked to peak FE\textsubscript{NO} levels among exposed subjects (Steerenberg et al. 2001). In addition, latex allergen exposure is known to increase FE\textsubscript{NO} concentrations (Baux and Barbinova 2005).

Low levels of FN\textsubscript{NO} have been detected in CF and both acute and chronic sinusitis (Lundberg and Weizberg 1999). There are controversial data about the FN\textsubscript{NO} concentrations in rhinitis. Several studies have reported increased FN\textsubscript{NO} levels in patients with allergic rhinitis (Lundberg and Weizberg 1999) but, in contrast, no alterations in FN\textsubscript{NO} concentrations were found in a group of children with perennial rhinitis (Lundberg et al. 1996). Increased FN\textsubscript{NO} levels have been described in upper airway infection and nasal polyposis (Selimoglu 2005). However, according to Jorissen et al. (2001), the FN\textsubscript{NO} levels in people suffering from an upper airway infection did not differ from the levels of healthy individuals. In children with Kartagener syndrome – a triad of sinusitis, bronchiectasis and situs inversus - FN\textsubscript{NO} levels are extremely low (Lundberg et al. 1994).

2.5.2 Nasal Lavage Fluid (NAL)

The nose is an easily accessible part of the human airways for repeated cytological and immunological assessments. Most of the inhaled air during normal breathing enters via the nose and, therefore, the nasal mucosa serves as the primary barrier against inhaled pollutants. NAL is a simple, non-invasive and well tolerated method to assess inflammatory changes in the upper airways (Steerenberg et al. 1996).
Increased levels of cytokines in NAL have been detected during viral infection (Linden et al. 1995), in allergic diseases (Hiltermann et al. 1997) and in occupational exposure (Hirvonen et al. 1999; Roponen et al. 2001; Purokivi et al. 2001). Total cell count (Blaski et al. 1996) and differential cell count of inflammatory cells (Prat et al. 1993) in NAL can be reliably used in the investigation of upper airway inflammation. In addition to cytokines, other inflammatory mediators such as EPO, MPO and NO can be measured from nasal lavage fluid (Noah et al. 1995; Hirvonen et al. 1999; Wålinder et al. 2001).

Variation in cell counts, NO and cytokine levels of NAL has been evaluated and there are differences between the genders. For example, baseline cytokine levels are significantly higher in males compared to females. However, the low intra-patient variability makes NAL samplings an appropriate method for assessing upper airway inflammation (Hauser et al. 1994; Roponen et al. 2003).

2.6 Lung functions

Spirometry is an important tool for a lung physician in clinical work since the spirometric values change in several lung diseases. For example, forced expiratory volume in one second (FEV1) is often decreased in patients with asthma or COPD. Lung diffusion capacity (DLCO) is decreased in parenchymal lung diseases such as allergic alveolitis (Erkinjuntti-Pekkanen et al. 1999).

In a previous study, cytokine and NO concentrations in NAL were increased in the mould exposed subjects even though the spirometric values remained unaffected (Roponen et al. 2001). In a Swedish study, no significant changes in FEV1 and forced vital capacity (FVC) were found in subjects exposed to indoor air microbes of a moisture-damaged building (Gunnbjörnsdottir et al. 2003). Conversely, reduced FEV1 and FVC levels have been reported in individuals exposed to mould in indoor air at home (Kilburn 2003). Furthermore, among waste collectors exposed to various bioaerosols, the FEV1 decreased significantly on the fourth day after a vacation (Heldal et al. 2003).
3 AIMS OF THE STUDY

The overall aim of this study was to assess the effects of experimental Aspergillus fumigatus and degraded PVC flooring material challenges on FE\textsubscript{NO}*, FN\textsubscript{NO}*, lung functions, cytokines and NO in NAL. The reproducibility of FE\textsubscript{NO} and FN\textsubscript{NO} measurement techniques was also studied.

The specific aims of the study were:

1. To study the short-term and seasonal reproducibility of FE\textsubscript{NO} and FN\textsubscript{NO} in healthy volunteers (I).

2. To assess the ability of experimental Aspergillus fumigatus challenge to cause inflammatory changes in airways as assessed by FE\textsubscript{NO}*, FN\textsubscript{NO}*, lung functions and NO in NAL and self-reported symptoms (II).

3. To assess the effects of experimental A. fumigatus inhalation challenge on cytokine levels (TNF-α, IL-1β, IL-4, IL-6 and IFN-γ) in NAL in subjects with or without occupational exposure in a moisture-damaged building (III).

4. To study the ability of experimental PVC flooring material challenge, simulating low concentrations of emissions found in indoor air conditions, to cause airway inflammation as assessed by FE\textsubscript{NO}*, FN\textsubscript{NO}*, lung function tests, cytokine levels in NAL as well as the symptoms reported by the subjects (IV).
4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Study I

To assess the short and long-term variations of $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$, twenty-one healthy, non-smoking, non-atopic subjects participated voluntarily in the study (Table 1). Twenty-one subjects participated in the study in the autumn, 18 in the winter and 17 during the summer. The exclusion criteria are described in Table 2. Fasting time was one hour prior to the exhalations and the subjects were asked to avoid nitrate-containing foods such as lettuce during the study periods.

4.1.2 Studies II-III

Twenty-eight subjects volunteered to participate in study II where the effects of $A. fumigatus$ challenge on $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ were assessed. All the subjects were hospital personnel and they were working in two different hospital buildings in Kuopio, eastern Finland. Moisture and mould problems in one of the buildings were confirmed by technical and microbiological investigations. The subjects formed 3 groups. Group 1 consisted of 13 subjects working in a moisture-damaged building (referred in text as a mould exposed group). Group 2 consisted of 5 atopic non-asthmatic subjects without mould exposure. Group 3 was a control group including 10 non-atopic non-asthmatic subjects with no known mould exposure. The subject characteristics of study II are given in Table 1.

In study III, in which the effects of $A. fumigatus$ challenge on cytokine levels were assessed, the subjects were the same as those in the study II but one atopic male subject was excluded as baseline cytokine levels in NAL are significantly higher in males
compared to females (Roponen et al. 2003). Thus, all the subjects in study III were females (Table 1).

Exclusion criteria in studies II-III are given in Table 2.

### 4.1.3 Study IV

In this study there were 10 volunteer subjects with occupational PVC exposure who were challenged with degraded PVC material under controlled conditions (Table 1). Five subjects had been diagnosed with asthma and three of these cases were diagnosed in the period during which they were exposed to degrading PVC flooring material at the workplace. The five non-asthmatic subjects had a variety of symptoms (upper and lower respiratory tract symptoms, conjunctival irritation and eczema). The exclusion criteria are shown in Table 2. The subjects were asked not to use inhaled steroids, long-acting $\beta_2$-adrenoreceptor agonists, leukotriene receptor antagonists and mast cell stabilizers for three days before the challenges and short-acting $\beta_2$-adrenoreceptor agonists for six hours before the challenges. All of the subjects worked in a building where the first indoor air evaluations were made in 1997 and later the cause of the symptoms in the occupants was related to PVC flooring materials as described earlier (Tuomainen et al. 2004).

### 4.2 Methods

#### 4.2.1 Analysis of $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ (I, II, IV)

$\text{FE}_{\text{NO}}$ was measured by chemiluminescence analyser (Sievers Model 280 NOA, Sievers Instruments INC., Boulder, CO, USA) according to ERS and ATS recommendations (Kharitonov et al. 1997; ATS 1999) using the same protocol described earlier (Purokivi...
et al. 2000; Figure 2). When measuring $\text{FE}_{\text{NO}}$, the subjects performed a slow vital capacity maneuver for 30 s against a fixed expiratory resistance. The pressure level during exhalation was optimized by following the computer screen on-line to reach a constant 50 mL·s$^{-1}$ flow rate. Exhaled air was led through a nonrebreathing valve into a Teflon tubing system connected to the analyser. The relative standard deviation between the three exhaled samples was permitted to be $< 10\%$ and the detection limit for NO was 1 part per billion (ppb). The measurements were performed in the same laboratory under constant conditions. The chemiluminescence analyzer was calibrated daily by using zero air and a certified concentration of NO.

$\text{FN}_{\text{NO}}$ levels were measured by an application of the fixed flow exhalation technique (Silkoff et al. 1999). Two soft, well-fitting nose pieces were placed at the entrance of both nostrils. The pieces were attached via a "Y" connector to a two-way valve and a resistor was placed in the exhalation limb, which required a pressure of 10 cm H$_2$O to produce a flow of 100 mL/minute. The subjects inhaled normal room temperature air to total lung capacity (TLC) via their mouths and exhaled nasally while targeting a flow signal displayed on a computer monitor. The expiration was continued until a steady NO plateau lasting at least 10 seconds was reached. The contribution of the oral NO was excluded. The measurement was repeated 3 times and the mean value was calculated.

4.2.2 Nasal Lavage (II, III, IV)

Nasal lavage samples were gathered according to the protocol described previously by Hirvonen et al. (1999) with some modifications. Prewarmed ($+37^\circ$C) Hanks’ balanced salt solution (4.5 ml) was instilled through a heat-softened catheter into the nostril (Figure 3). The subject held his or her chin down during the instillation, and held the catheter in place by pinching the nostrils closed. The cartilaginous bridge of the nose was vibrated by using a neonatal percussor (Neo-Cussor™, General Physiotherapy
Inc., St. Louis, MO) while the fluid was refluxed three times. The same protocol was repeated on the opposite nostril. The sample was centrifuged (425 x g, 10 min) and the cells were resuspended in 2 ml of the supernatant. The remaining cell suspension was incubated for 24 h at 37°C and then centrifuged (425 x g, 10 min). The supernatant and cells were frozen at −70°C.

In studies II and III, NAL samples for measurement of cytokines were collected immediately before both *A. fumigatus* and placebo challenges and exactly at 6 and 24 hours after *A. fumigatus* and placebo challenges (Table 2). In study IV, NAL samplings were performed immediately before and after the PVC and control challenges (Table 3).

**4.2.3 Analysis of Cytokines and NO in NAL (III, IV)**

Concentrations of IFN-γ (III), TNF-α (III, IV), IL-1β (III), IL-4 (III, IV), IL-6 (III, IV) and IL-12 (IV) in the NAL supernatant were analyzed by using ELISA kits (R&D Systems™, Minneapolis, MN, USA). Assays were analyzed according to the manufacturer's instructions with the ELISA microplate reader (iEMS Reader MFTM, Labsystems, Finland) at a wavelength of 450 nm by comparing the absorbances of the samples to the standard curve. Each standard and sample was run in duplicate. Extrapolated values below lowest standard points (IFN-γ, 31.3 pg/ml; TNF-α, 31.3 pg/ml; IL-1β, 15.6 pg/ml; IL-4, 8.2 pg/ml; IL-6, 15.6 pg/ml; IL-12, 8 pg/ml) were used as such instead of replacing them with a constant. NO in the NAL supernatant was assayed by the Griess reaction as the stable NO oxidation product nitrite (Green et al. 1982) as described in detailed elsewhere (Hirvonen et al. 1999). Cytokines and NO in NAL were analyzed from incubated supernatants.
Table 1. Subject characteristics in studies I-IV.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects:</td>
<td>21</td>
<td>28</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>Age years:</td>
<td>38</td>
<td>45</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>(range)</td>
<td>(22-57)</td>
<td>(27-60)</td>
<td>(27-60)</td>
<td>(28-53)</td>
</tr>
<tr>
<td>Gender:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>27</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Smoking:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Positive Skin Prick Tests:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic series or Storage mites</td>
<td>10</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moulds</td>
<td>1#</td>
<td>1#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doctor-diagnosed asthma:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Occupational mould exposure:</td>
<td>13</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occupational PVC exposure:</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

#Geotrichium candidum
Table 2. Exclusion criteria in studies I-IV

<table>
<thead>
<tr>
<th>Criterion</th>
<th>I</th>
<th>II-III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>All the subjects non-smokers</td>
<td>Prohibited during the study period</td>
<td>Prohibited during the study period</td>
</tr>
<tr>
<td>Vigorous Exercise</td>
<td>Prohibited during the study period</td>
<td>Prohibited during the study period</td>
<td>Prohibited during the study period</td>
</tr>
<tr>
<td>Occurrence of previous respiratory tract infection</td>
<td>at least 6 weeks prior to the study</td>
<td>at least 6 weeks prior to the study</td>
<td>at least 6 weeks prior to the study</td>
</tr>
<tr>
<td>Regular medication</td>
<td>Prohibited</td>
<td>Prohibited</td>
<td>Prohibited 3 days before the study</td>
</tr>
<tr>
<td>Short-acting $\beta_2$-agonists</td>
<td>Prohibited during the study period</td>
<td>Prohibited during the study period</td>
<td>Prohibited 6 hours before the study</td>
</tr>
</tbody>
</table>
Table 3. Protocols of studies II-III (A) and study IV (B).

### A.

<table>
<thead>
<tr>
<th>Time</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Lung functions (spirometry, diffusion capacity, PEF)</td>
</tr>
<tr>
<td></td>
<td>Cytokines and NO in NAL</td>
</tr>
<tr>
<td>During the exposure</td>
<td>Lung functions (FEV1, PEF)</td>
</tr>
<tr>
<td></td>
<td>Self-reported symptoms</td>
</tr>
<tr>
<td>Next morning after the exposure</td>
<td>Self-reported symptoms</td>
</tr>
<tr>
<td>3 hours after the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>PEF</td>
</tr>
<tr>
<td>6 hours after the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Cytokines and NO in NAL</td>
</tr>
<tr>
<td></td>
<td>PEF</td>
</tr>
<tr>
<td>24 hours after the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Lung functions (spirometry, diffusion capacity, PEF)</td>
</tr>
<tr>
<td></td>
<td>Cytokines and NO in NAL</td>
</tr>
<tr>
<td></td>
<td>Self-reported symptoms</td>
</tr>
</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th>Time</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Lung functions (spirometry and PEF)</td>
</tr>
<tr>
<td></td>
<td>Cytokines and NO in NAL</td>
</tr>
<tr>
<td>Exposure (4 h)</td>
<td>Self-reported symptoms</td>
</tr>
<tr>
<td></td>
<td>PEF at 1 h intervals</td>
</tr>
<tr>
<td></td>
<td>Exhaled breath sample (at 2 hours after PVC exposure)</td>
</tr>
<tr>
<td></td>
<td>VOC collection from the chamber air (sampling time 2 h)</td>
</tr>
<tr>
<td>Immediately after the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Lung functions (spirometry and PEF)</td>
</tr>
<tr>
<td></td>
<td>Cytokines and NO in NAL</td>
</tr>
<tr>
<td>2 h after the exposure</td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Lung functions (spirometry and PEF)</td>
</tr>
<tr>
<td>Next morning after the exposure</td>
<td>Self-reported symptoms</td>
</tr>
<tr>
<td></td>
<td>Exhaled and nasal NO</td>
</tr>
<tr>
<td></td>
<td>Lung functions (spirometry and PEF)</td>
</tr>
</tbody>
</table>
Figure 2. Performance of $FE_{20}$ measurement.

Figure 3. Collection of a NAL sample.
4.2.4 Analysis of protein and 2-ethylhexanol in NAL (IV)

Part of the NAL was centrifuged at 1800 rpm (10 min), after which 200 µl was separated into an Eppendorf tube for the protein measurement and it, as well as the rest of the supernatant were stored at -18 °C before the analysis of proteins and 2-ethylhexanol. The protein content of the samples was measured with the Bradford method in a microplate (Bradford 1976). Further, 2-ethylhexanol of the NAL was extracted with ethyl acetate and analyzed by gas chromatography and mass spectrometry.

4.2.5 Lung Function Tests (II, IV)

The standard spirometric values (Custo Med GmbH™, Munich, Germany) and pulmonary diffusion capacity (Type 2200 Sensor Medics BV™, Bilthoven, Netherlands) were measured according to American Thoracic Society guidelines (ATS 1995; ATS 1996). Reference values of Viljanen et al. (1982) for the Finnish population were used. Peak expiratory flow (PEF, Mini Wright Standard Peak Flow Meter, Clement Clarke International Ltd., Edinburgh, UK) values were followed during and after the challenges in studies II and IV.

4.2.6 Skin Prick Tests (II, III)

Atopic status was examined by ALK skin prick tests (ALK laboratories®, Copenhagen, Denmark). The prick tests included 14 common environmental allergens (two house dust mites (Dermatophagoides farinae and Dermatophagoides pteronyssinus), birch, alder, meadow fescue, timothy, meadow grass, mugwort, dandelion, horse, dog, cat, cow and natural rubber), three storage mites (Lepidoglyphus destructor, Acarus siro and Tyrophagus putrescentiae) and 13 moulds (Alternaria alternata, Cladosporium herbarum, Aspergillus fumigatus, Aspergillus versicolor, Aureobasidium pullulans,
Botrytis cinerea, Chaetomium globusum, Geotrichium candidum, Mucor racemosus, Penicillium brevicompactum, Penicillium expansum, Phoma herbarum and Trichoderma viride). ALK allergen diluents and histamine hydrochloride (10 mg/mL) were used as negative and positive controls, respectively. If there was at least one positive reaction with a diameter 3 mm or more then the atopy was defined.

4.2.7 Symptoms Related To Challenges (II, IV)

During the challenges (Aspergillus fumigatus vs. placebo in study II, PVC vs. ceramic tile in study IV) the subjects were asked to report if they experienced any symptoms. In addition, they reported any possible symptoms which occurred on the next morning after the challenge. The subjects wrote down the symptoms in the PEF follow-up form.

4.2.8 Cytospin (III, IV)

Cytocentrifuge preparations were made by using 100 µl of resuspended cell suspension, in which the mucus was broken by 0.5 % dithiothreitol/0.1 % bovine serum albumin. The solution was centrifuged and the slides were stained with May-Grunwald-Giemsa staining (Prat et al. 1993) for the cell differential counts. The standard was to count 100 cells per a sample. The cell differential counts were collected before and at 6 and 24 hours after the challenges (A. fumigatus vs. placebo, PVC vs. control).

4.2.9 IgE and ECP analysis (II)

From serum samples, total IgE (IU/L) was determined by EIA (Immulite total IgE®, Diagnostic Products Corporation, Los Angeles, CA, USA), ECP (IU/L) by radioimmunoassay (RIA®, Pharmacia et Upjohn, Uppsala, Sweden), and Aspergillus fumigatus IgE (IU/L) by UniCAP-FEIA (Model Unicap 100®, Pharmacia AB, Uppsala, Sweden).
4.2.10 Statistical Methods

**Study I:** Means and standard deviations (SD) were used to describe the NO data. Day-to-day and week-to-week reproducibility were visualised by using Bland-Altman plots (Bland and Altman 1986). Intraday, day-to-day, week-to-week and seasonal variations of $FE_{NO}$ and $FN_{NO}$ as well as the correlation between $FE_{NO}$ and $FN_{NO}$ were examined by intra-class correlation analysis (ICC). ICC-values > 0.6 were considered clinically significant (Faul et al. 1999). The coefficients of variation were also calculated. In addition, intraday changes of $FE_{NO}$ and $FN_{NO}$ were studied by variance analysis of repeated measures.

**Study II:** Differences in changes from baseline on the course of time (e.g. $FE_{NO}$, $FN_{NO}$, NO in NAL and lung functions) between the *A. fumigatus* and placebo exposures were compared by variance analysis for repeated measures. In addition to variance analysis, the change in NO levels was calculated as NO concentration [6 or 24 hours after the challenge] - NO concentration [baseline before the challenge] for both *A. fumigatus* and placebo challenges and Wilcoxon signed rank test was used to compare the changes between active agent and placebo inhalations. Logarithmic transformations were used for variables not normally distributed. Means between the groups were compared by one-way ANOVA and mean ranks by Kruskall-Wallis test. The percentages were compared by using chi-square test.

**Study III:** The change in cytokine production was calculated as cytokine concentration [6 or 24 hours after the challenge] - cytokine concentration [baseline before the challenge] for both *A. fumigatus* and placebo challenges. Despite standard transformations, the normal distribution of the cytokine data was not achieved. Therefore, the non-parametric Wilcoxon signed rank test was used to compare the changes of cytokine production between the challenges (*A. fumigatus* vs. placebo) at different time points (before the
challenges, 6 and 24 hours after the challenge). Mean ranks between groups were compared by Kruskall-Wallis test.

**Study IV:** The changes in different parameters during and after the exposures were studied by variance analysis of repeated measures (e.g. \( FE_{NO} \), \( FN_{NO} \), NO and cytokines in NAL and lung functions). Logarithmic transformations were performed for data not normally distributed. In addition, induced cytokine responses were calculated as cytokine concentration [immediately after the challenge] - cytokine concentration [baseline before the challenge] for both PVC and control tests and Wilcoxon signed rank test was used to compare the changes between the tests. Between groups differences were analyzed by the Fisher’s exact test. The Pearson’s test was used to determine correlations. The percentages were compared by using the Chi-square test.

In all of the studies the data was analysed by SPSS for Windows 11.5 (SPSS Inc.™, Chicago, USA) and p-values < 0.05 were considered to be statistically significant.

**4.3 Study Protocols**

**4.3.1 Study I**

\( FE_{NO} \) and \( FN_{NO} \) measurements were performed in three periods: the first in the autumn, the second in winter and the third in summer. During the first study day of the autumn period, the \( FE_{NO} \) and \( FN_{NO} \) values were measured in the morning (at 7 to 9 o’clock) and again six hours later in the afternoon (at 13 to 15 o’clock). In the autumn period, both the morning and afternoon measurements of the \( FE_{NO} \) and \( FN_{NO} \) were repeated 24 hours and seven days after the first measurement day. In the winter and summer, the \( FE_{NO} \) and \( FN_{NO} \) measurements were performed on one day in the morning (at 7 to 9 o’clock).
4.3.2 Studies II and III (Table 3A)

Each subject underwent randomly two challenges where they inhaled *Aspergillus fumigatus* or placebo solution via a nebulizer (Spira elektro 2 flowmeter™, Respiratory Care Center, Hameenlinna, Finland) using an oronasal mask. The inhalation aerosol was generated from a 1-mL sample reservoir with the nebulizing pressure adjusted to 2 bar (flow rate 7.5 mL/min). The nebulization time was 0.4 s, equivalent to a mean volume uptake of 6.5+/−0.3 µL (SD) of the solution per breath. The subjects inhaled each solution 60 times using controlled tidal volume respiration during a period of approximately 5 min and this was followed by a rest period of 5 min and subsequent spirometric measurements.

FEV1 was measured in triplicate before the challenges, and the greatest value was used as the baseline. Four series of 10-fold dilutions of commercial *A. fumigatus* allergen (ALK-ABELLO®, Madrid, Spain) were prepared immediately before the challenges and the inhalation was started with the lowest dilution. *A. fumigatus* solution was a purified allergen product approved to be marketed in Finland by National Agency for Medicine. The subjects inhaled the allergen dilution from a concentration of 0.01 mg/mL until the maximal concentration of 10 mg/mL was administered. FEV1 was measured 5 min after each inhaled concentration. The difference between the baseline and lowest FEV1 was observed. The allergen diluent (physiological salt solution) was used as placebo. The placebo challenge protocol was identical to the *A. fumigatus* challenge but the concentration of the solution did not change during the challenge. The challenges were performed one week apart at the same time of the day.

In study II, FE$_{NO}$ and FN$_{NO}$ were measured before the challenges and at 3, 6 and 24 hours after the challenges. In addition to FEV1 follow-up during the challenges, the lung functions (spirometry, diffusion capacity) were assessed before the challenges and at 24 hours after them in study II.
In study III, the NAL samplings for cytokine (IFN-γ, TNF-α, IL-1β, IL-4 and IL-6) and cell differential count assessments were performed before the challenges and subsequently at 6 and 24 hours later.

4.3.3 Study IV

The exposure chamber (volume 1.2 x 1.2 x 2.2 m) was constructed of stainless steel. It was equipped with a small window and had separate adjustable ventilation (Figure 4). The ventilation was adjusted so that the carbon dioxide concentration during the exposure session in the chamber stayed below 1000 ppm. The chamber was tested to ensure it was tight and clean (total volatile organic compounds (TVOC) below 10 µg/m³). The exposure material used in the exposure tests in the chamber was a PVC flooring material recently removed from an office where the occupants had suffered respiratory and dermal irritation symptoms related to degrading PVC. The area of this

Figure 4. The exposure chamber used in degraded PVC material challenge study.
PVC material placed in the chamber was 1 m², with a new piece of PVC being replaced every week. During the control tests, unused ceramic tiles (area 1 m²) were placed in the chamber floor. TVOC and 2-ethylhexanol concentrations were measured during every PVC exposure test in the chamber air (Tuomainen et al. 2004).

Volatile organic compounds from the chamber air and from the exhaled breath were collected and analyzed two hours after the beginning of the exposure in laminate bags according to the method described by Tuomainen et al. (2001). For example, similar laminate bags have been used in collecting air samples such as sulphur oxides and the bags do not release any emissions themselves. In the chamber, the sample was always collected near to the breathing zone of the test subject. Levels of TVOC and 2-ethylhexanol were measured from the samples.

The protocol of the chamber challenge tests for the subjects is shown in Table 3B. The challenges lasted 4 hours and the interval between the two exposures ranged from three to seven weeks. PVC and control exposures were performed in a random order and the subjects were blinded to the type of exposure.

4.4 Ethics

The studies I, II and III were approved by the Ethics Committee of Kuopio University Hospital and the study IV by the Ethics Committee of Helsinki University Hospital. All of the studies were performed according to principles of the Helsinki Declaration (World Medical Association 2004). All the subjects were asked a written approval for the participation in the studies.
5. RESULTS

5.1 Short and long-term reproducibility of $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ levels (I)

In all the measurements, the levels of $\text{FN}_{\text{NO}}$ were lower in the morning than in the afternoon. In the autumn, the afternoon values of $\text{FN}_{\text{NO}}$ were significantly higher compared to those taken in the morning (p=0.003) and the correlation between morning and afternoon levels was not clinically significant ($r=0.565$, Table 4A). The day-to-day correlation of $\text{FN}_{\text{NO}}$ levels was significant in the mornings but not in the afternoons ($r=0.868$, $r=0.543$, respectively, Table 4B, Figure 5A).

In line with $\text{FN}_{\text{NO}}$, the mean levels of $\text{FE}_{\text{NO}}$ were regularly lower in the mornings compared to the afternoon values of the same day. There was a significant difference between the morning and afternoon values in the autumn (p<0.001) and there was also a clinically significant correlation between the values ($r=0.830$, Table 4A). Day-to-day correlation of $\text{FE}_{\text{NO}}$ was clinically significant when measured 24 hours apart both in the mornings and the afternoons ($r=0.784$, $r=0.779$, respectively, Table 4B, Figure 5B).

In addition, the week-to-week correlation of $\text{FN}_{\text{NO}}$ was clinically significant for both morning and afternoon values ($r=0.637$, $r=0.781$, respectively, Table 4C, Figure 6A). With the seven days interval, the correlation between $\text{FE}_{\text{NO}}$ measurements was also high in the mornings ($r=0.738$, Table 4C, Figure 6B). When comparing the afternoon week-to-week values of the $\text{FE}_{\text{NO}}$ measurements, no clinical significance was reached ($r=0.560$, Table 4C).

Seasonal variations of both $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ were low, and the correlations were clinically significant ($r=0.709$, $r=0.624$, respectively, Table 4D). $\text{FE}_{\text{NO}}$ levels were clearly lower than the $\text{FN}_{\text{NO}}$ levels and they did not correlate with each other at any measurement point (data not shown).
Table 4. Short-term and Seasonal Variations of FN\textsubscript{NO} and FE\textsubscript{NO} studied by intra-class correlation analysis (ICC) and coefficients of variation (CoV) (I).

A. Intraday variation of FN\textsubscript{NO} and FE\textsubscript{NO} (ppb) during three days in autumn (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>Mornings</th>
<th>Afternoons</th>
<th>n</th>
<th>r</th>
<th>p</th>
<th>CoV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN\textsubscript{NO}</td>
<td>127.1±28.9</td>
<td>149.2±40.2</td>
<td>54</td>
<td>0.565</td>
<td>0.005</td>
<td>11.0</td>
</tr>
<tr>
<td>FE\textsubscript{NO}</td>
<td>14.8±5.8</td>
<td>17.4±6.0</td>
<td>54</td>
<td>0.830</td>
<td>0.000</td>
<td>13.7</td>
</tr>
</tbody>
</table>

B. Day-to-day variation of FN\textsubscript{NO} and FE\textsubscript{NO} (ppb) during the first week in autumn (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>1\textsuperscript{st} day</th>
<th>2\textsuperscript{nd} day</th>
<th>n</th>
<th>r</th>
<th>p</th>
<th>CoV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN\textsubscript{NO}</td>
<td>121.4±37.5</td>
<td>119.8±29.6</td>
<td>21</td>
<td>0.868</td>
<td>0.000</td>
<td>8.1</td>
</tr>
<tr>
<td>FE\textsubscript{NO}</td>
<td>14.6±6.7</td>
<td>14.2±5.1</td>
<td>21</td>
<td>0.784</td>
<td>0.000</td>
<td>16.1</td>
</tr>
</tbody>
</table>

C. Week-to-week variation of FN\textsubscript{NO} and FE\textsubscript{NO} (ppb) during the autumn period (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>1\textsuperscript{st} week</th>
<th>2\textsuperscript{nd} week</th>
<th>n</th>
<th>r</th>
<th>p</th>
<th>CoV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN\textsubscript{NO}</td>
<td>121.4±37.5</td>
<td>128.0±32.1</td>
<td>18</td>
<td>0.637</td>
<td>0.001</td>
<td>12.3</td>
</tr>
<tr>
<td>FE\textsubscript{NO}</td>
<td>14.6±6.7</td>
<td>15.4±7.3</td>
<td>18</td>
<td>0.738</td>
<td>0.000</td>
<td>19.4</td>
</tr>
</tbody>
</table>

D. Seasonal variation of FN\textsubscript{NO} and FE\textsubscript{NO} (ppb) in autumn, winter and summer mornings (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>autumn</th>
<th>winter</th>
<th>summer</th>
<th>n</th>
<th>r</th>
<th>p</th>
<th>CoV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN\textsubscript{NO}</td>
<td>121.4±37.5</td>
<td>133.5±29.7</td>
<td>138.1±47.6</td>
<td>17</td>
<td>0.624</td>
<td>0.000</td>
<td>21.0</td>
</tr>
<tr>
<td>FE\textsubscript{NO}</td>
<td>14.6±7.2</td>
<td>16.6±6.4</td>
<td>17.4±8.0</td>
<td>17</td>
<td>0.709</td>
<td>0.000</td>
<td>15.7</td>
</tr>
</tbody>
</table>
Figure 5. Repeatability of $\text{FN}_{\text{NO}}$ (A) and $\text{FE}_{\text{NO}}$ (B) measurements performed in consecutive mornings 24h apart visualized by Bland-Altman plots. It is anticipated that 95% of the differences between measures will be $< 2\ SD$.

Figure 6. Repeatability of $\text{FN}_{\text{NO}}$ (A) and $\text{FN}_{\text{NO}}$ (B) measurements collected one week apart in the mornings expressed by Bland-Altman plots. It is anticipated that 95% of the differences between measures will be $< 2\ SD$. 
5.2 The induced effects of Aspergillus fumigatus challenge (II, III)

5.2.1 Symptoms related to the challenge

On the next morning after the challenges, the subjects reported significantly more frequently airway symptoms after the A. fumigatus challenge compared to placebo inhalation (57 % vs. 25 %, respectively, p=0.014, n=27). Symptoms of both upper (blocked nose, rhinorrhea, sneezing) and lower (cough, phlegm) airways were more common during the A. fumigatus challenge compared to placebo inhalation but the differences were not statistically significant (data not shown).

5.2.2 FE\textsubscript{NO} and FN\textsubscript{NO} levels

The baseline levels of FE\textsubscript{NO} prior to the A. fumigatus and placebo inhalations (20.4 ppb and 21.7 ppb, respectively) did not differ significantly. A significant increase in FE\textsubscript{NO} levels of all the subjects was observed after the A. fumigatus inhalation (p=0.002) and the highest FE\textsubscript{NO} values were achieved three hours after the A. fumigatus inhalation. No such responses were detected after the placebo inhalation and there was a significant difference between the mould and placebo challenges (p<0.05) (Figure 7A). The difference was not dependent on the group to which the subjects belonged. In statistical analysis, the atopy was not related to changes in FE\textsubscript{NO} levels. When one subject who experienced an asthmatic reaction during the A. fumigatus challenge (25 % decrease in FEV1) was excluded, there was still a tendency but not a significant difference in FE\textsubscript{NO} levels between the challenges (p=0.082).

The baseline levels of FN\textsubscript{NO} did not differ significantly between the A. fumigatus and placebo challenges (129.5 ppb and 127.0 ppb, respectively). During the 24 hour follow-up after the challenges, the levels FN\textsubscript{NO} of all the subjects increased significantly after the placebo inhalation (p=0.003) and a similar tendency was seen after the A. fumigatus challenge (p=0.051). The peak levels of FN\textsubscript{NO} after both challenges were measured six hours after the inhalations. The FN\textsubscript{NO} profiles after the challenges were almost identical (p=0.81) (Figure 7B).
5.2.3 Lung functions

The baseline FVC (3.37 l, 4.16 and 3.44, respectively, p=0.046) and FEV1 (2.78 l, 3.56 l and 3.44 l, respectively, p=0.004) were significantly lower among the subjects with occupational mould exposure, compared to atopic and control groups. The baseline pulmonary diffusion capacities were significantly higher among the atopic individuals compared to subjects in the mould exposed and control groups (11.9 mmol/min/kPA, 8.7, 9.3, respectively, p=0.033).

FEV1 decreased significantly during the A. fumigatus (3.04-2.94 l, p=0.013) and placebo (3.03-2.98 l, p=0.029) challenges. There was also a significant difference in FEV1 changes between the challenges (p=0.049) but the changes were not dependent on the group. However, there was one subject who exhibited a decrease of 25% in FEV1 after A. fumigatus challenge and a 12% decrease after placebo inhalation. When this subject was excluded, the difference in changes of FEV1 between the inhalations did not reach a statistically significant level (p=0.320). There were no significant differences in spirometric values or in diffusion capacity between A. fumigatus and placebo inhalations at 24 hours after the challenges. In addition, PEF-levels were similar in both groups and they did not change significantly after A. fumigatus or placebo challenges.

![Figure 7A](image1.png)  ![Figure 7B](image2.png)

Figure 7. The changes in mean levels of a) $FE_{NO}$ (all of the subjects) and b) $FN_{NO}$ after A. fumigatus (—) and placebo (...) challenges (measured before and at 3, 6 and 24 hours later). There was a significant difference between the challenges in $FE_{NO}$ ($p<0.05$), but not in $FN_{OCR}$.
5.2.4 NO and Cytokines in NAL

There was an increase in NO levels, assessed as the nitrite concentration in NAL, at six hours after the *A. fumigatus* challenge but differences were not statistically significant when compared to placebo (data not shown).

The mean values of TNF-α, IL-1β, IL-4, IL-6 and IFN-γ in NAL before the first challenge are given in Table 5. Before the challenges, the lowest mean levels of TNF-α, IL-1β and IL-4 were detected among mould exposed subjects and IL-6 as well as IFN-γ among the atopic subjects. The highest basal concentrations of all the cytokines were measured in the samples of the control subjects. The only statistically significant difference between the groups was detected in TNF-α levels (*p*=0.029).

Mean levels of IL-1β in NAL increased time-dependently after the *A. fumigatus* challenge with the peak concentrations being achieved 24 hours after the challenge. In contrast, IL-1β levels decreased after the placebo challenge compared to the baseline. When all the subjects were examined as a single group, the changes of the IL-1β levels from the baseline were significantly higher both at 6 (*p*=0.013) and 24 hours (*p*=0.005) after the *A. fumigatus* challenge compared to the placebo (Table 6, Figure 8).

At the group level, significant differences in changes between *A. fumigatus* and placebo challenges were also seen in the control group at 6 (from baseline 24.1 to 47.9 pg/ml vs. from baseline 69.6 to 26.2 pg/ml, respectively, *p*=0.01) and 24 (from baseline 24.1 to 71.7 pg/ml, from baseline 69.2 to 22.2 pg/ml, respectively, *p*=0.008) hours after the challenges but not in the other study groups. In addition, there was a significant difference in baseline levels of IL-1β between the *A. fumigatus* and placebo challenges (Table 6, *p*=0.004).

There was an increase of the IL-4 concentrations in NAL after the *A. fumigatus* challenge whereas the placebo challenge decreased the levels (Table 6). The increase in IL-4 levels tended to be higher both at 6 (*p*=0.057) and 24 (*p*=0.099) hours after the *A. fumigatus* inhalation as compared to placebo (Table 6). In the group of subjects with occupational exposure in the moisture-damaged building, there was a significant difference between *A. fumigatus* and placebo challenges in the induced changes of IL-4
levels both at 6 hours (from baseline 0.3 to 0.7 pg/ml vs. from baseline 0.6 to 0.3 pg/ml, respectively, p=0.046) and 24 hours (from baseline 0.3 to 0.7 pg/ml vs. from baseline 0.6 to 0.2 pg/ml, respectively, p=0.008) after the inhalations when compared to the baseline values but no such changes were found in the other study groups.

The IL-6 levels in NAL increased after the *A. fumigatus* challenge and the peak levels were achieved 6 hours after the inhalation (Table 6). In contrast, IL-6 concentrations decreased after the placebo challenge compared to baseline levels. If all the subjects were examined as a single group, then the change in IL-6 levels tended to be higher at 6 hours after the *A. fumigatus* challenge compared to the placebo inhalation (p=0.058). No significant changes in IL-6 concentrations between the mould and placebo challenges were found at the subgroup level.

In all the subjects, the TNF-α concentration of NAL increased after *A. fumigatus* challenge and decreased slightly after the placebo inhalation but the differences did not reach statistical significance (Table 6). However, in the control group, the change from the baseline in TNF-α levels was significantly higher at 6 hours after the *A. fumigatus* challenge compared to the placebo challenge (from baseline 8.8 to 18.6 pg/ml vs. from baseline 31.9 to 21.7 pg/ml, respectively, p=0.028). In the other subgroups no significant changes in TNF-α concentrations were found between mould and placebo challenges.

Only minor changes in the IFN-γ levels were detected after *A. fumigatus* or placebo challenges and no significant differences were noted at any time point between the challenges (Table 6).

When the atopic subjects were treated as a single group (i.e. all of the atopic subjects with or without previous mould exposure) there was a tendency but not a statistically significant difference between *A. fumigatus* and placebo challenges in the extent of the changes of IL-1β levels at 24 hours after the challenges (from baseline 19.9 to 34.8 pg/ml vs. from baseline 27.7 to 23.2, respectively, p=0.066, n=9). No significant differences or tendencies in changes of any of the other cytokine between the mould and placebo challenges were found at any time point when comparing the atopic subjects to the other subjects.
Table 5. Baseline cytokine levels in different groups before the first challenge* (III).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mould exposed (n=13)</th>
<th>Atopic (n=4)</th>
<th>Controls (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α**</td>
<td>0.1 (0-0.6)</td>
<td>0.3 (0-1.0)</td>
<td>31.4 (0-230)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>19.7 (0-89)</td>
<td>47.4 (1-150)</td>
<td>51.5 (5.2-200)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.4 (0-1.9)</td>
<td>0.5 (0-1.9)</td>
<td>14.7 (0-120)</td>
</tr>
<tr>
<td>IL-6</td>
<td>7.2 (0-29.9)</td>
<td>6.7 (0-18)</td>
<td>69.3 (1.6-370)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.3 (0-14.9)</td>
<td>7.1 (0-16.9)</td>
<td>8.1 (0-14.9)</td>
</tr>
</tbody>
</table>

Data presented as mean values (range) pg/ml

*Aspergillus fumigatus and placebo challenges were performed in a random order

**significant difference between the groups (p=0.029)

Figure 8. The changes of IL-1β levels from the baseline after the A. fumigatus and placebo challenges. There was a significant difference between the challenges measured at 6 (*, p=0.013) and 24 (**, p=0.005) hours later.
Table 6. Changes in cytokine levels 6 and 24 hours after *A. fumigatus* and placebo challenges among all the studied subjects (n=27) (III).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Challenge</th>
<th>Baseline</th>
<th>aChange 6 hours</th>
<th>bChange 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td><em>A. fumigatus</em></td>
<td>3.3 (0-52.7)</td>
<td>3.6 (-7.7-42.2)</td>
<td>8.2 (-17.5-150)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>11.9 (0-230)</td>
<td>-3.6 (-87.4-8.1)</td>
<td>-3.9 (-190-46.9)</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.064</td>
<td>0.121</td>
<td>0.379</td>
</tr>
<tr>
<td>IL-1β</td>
<td><em>A. fumigatus</em></td>
<td>18.1 (0-150)</td>
<td>6.7 (-65.7-180)</td>
<td>23.7 (-37.6-520)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>38.3 (0.3-200)</td>
<td>-15.3 (-125.1-190)</td>
<td>-19.0 (-170-99.1)</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.004</td>
<td>0.013</td>
<td>0.005</td>
</tr>
<tr>
<td>IL-4</td>
<td><em>A. fumigatus</em></td>
<td>1.5 (0-10)</td>
<td>1.0 (-1.3-13)</td>
<td>1.1 (-3.7-21)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>5.8 (0-120)</td>
<td>-2.9 (-73.5-3.9)</td>
<td>-4.1 (-110-5.6)</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.256</td>
<td>0.057</td>
<td>0.099</td>
</tr>
<tr>
<td>IL-6</td>
<td><em>A. fumigatus</em></td>
<td>8.1 (0-35.3)</td>
<td>14.0 (-32-200)</td>
<td>8.3 (-20.5-220)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>31.1 (0-370)</td>
<td>-18.4 (-220-40.8)</td>
<td>-13.2 (-300-77.8)</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.143</td>
<td>0.058</td>
<td>0.848</td>
</tr>
<tr>
<td>IFN-γ</td>
<td><em>A. fumigatus</em></td>
<td>7.4 (0-16.8)</td>
<td>-0.2 (-4.5-6.7)</td>
<td>-0.1 (-4-13.7)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>7.5 (0-17.2)</td>
<td>-0.2 (-4.2-2.2)</td>
<td>-0.3 (-2.9-7.2)</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.411</td>
<td>1.000</td>
<td>0.168</td>
</tr>
</tbody>
</table>

Data presented as mean values (range) pg/ml

p-values are for the differences between *A. fumigatus* and placebo challenges at the same time point

aCytokine concentration [6 hours after challenge] - cytokine concentration [baseline]
bCytokine concentration [24 hours after challenge] – cytokine concentration [baseline]
5.2.5 Cell differential count

Neutrophilic cells dominated the NAL cell profile both before and after the *A. fumigatus* and placebo challenges. No changes in the proportions of lymphocytes, neutrophils or eosinophils in the NAL were seen after the challenges. Moreover, no differences in the changes of the cell counts were detected between the *A. fumigatus* and placebo challenges (data not shown).

5.2.6 IgE and ECP responses

Serum total IgE levels were significantly higher among the atopic subjects compared to subjects in the mould exposed and control groups (247.7 IU/L, 57.8 and 71.4, respectively, p<0.05). There was no significant difference in serum ECP concentrations between the groups and none of the subjects exhibited elevated *A. fumigatus* IgE levels (data not shown).

5.3 The effects of PVC challenge (IV)

5.3.1 Symptoms related to the challenge

The number of subjects reporting respiratory tract symptoms (such as nasal symptoms, cough, phlegm) was significantly higher on the following morning after they had been exposed to the degrading PVC material compared to the situation after the control exposure (50 % vs. 0 %, respectively, p=0.029, n=10). During the PVC exposure the subjects experienced more often respiratory tract and conjunctival symptoms compared to the control challenge but the differences did not reach statistical significance (Table 7).
Table 7. Symptoms reported by the subjects during exposures to the PVC flooring material and placebo (ceramic tile) and on the following morning after the exposures (IV).

<table>
<thead>
<tr>
<th>Symptoms during the exposures:</th>
<th>PVC flooring</th>
<th>Placebo</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Airway</td>
<td>2 (20)</td>
<td>0 (0)</td>
<td>0.263</td>
</tr>
<tr>
<td>Upper Airway</td>
<td>7 (70)</td>
<td>5 (50)</td>
<td>0.430</td>
</tr>
<tr>
<td>Lower and Upper Airway</td>
<td>8 (80)</td>
<td>5 (50)</td>
<td>0.259</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1 (10)</td>
<td>1 (10)</td>
<td>0.737</td>
</tr>
<tr>
<td>Skin</td>
<td>2 (20)</td>
<td>2 (20)</td>
<td>0.712</td>
</tr>
<tr>
<td>Eye</td>
<td>3 (30)</td>
<td>1 (10)</td>
<td>0.333</td>
</tr>
</tbody>
</table>

Table 8. Cytokines (TNF-α, IL-4, IL-6 and IL-12) (mean and range, pg/ml) and nitrite (NO) (mean and range, µM) in the nasal lavage samples of the study before and immediately after the challenges to PVC and control material (ceramic tiles). The results also show the values of a sample assessed after at least one week’s holiday away from the workplace.

<table>
<thead>
<tr>
<th></th>
<th>After holiday</th>
<th>Before PVC</th>
<th>After holiday</th>
<th>Before control</th>
<th>After control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>179.7 (0-1208.8)</td>
<td>152.2 (0-749.0)</td>
<td>267.2 (0-1146.8)</td>
<td>103.9 (0-517.6)</td>
<td>177.5 (0-803.0)</td>
</tr>
<tr>
<td>IL-4</td>
<td>51.6 (0-379.6)</td>
<td>56.4 (0-276.4)</td>
<td>102.9 (0-529.3)</td>
<td>39.6 (0-234.7)</td>
<td>80.1 (0-533.8)</td>
</tr>
<tr>
<td>IL-6</td>
<td>76.9 (0.2-572.4)</td>
<td>71.2 (0.3-383.9)</td>
<td>108.6 (0.1-536.1)</td>
<td>49.3 (0.5-278.4)</td>
<td>106.4 (0-525.9)</td>
</tr>
<tr>
<td>IL-12</td>
<td>308.4 (0-1863.0)</td>
<td>413.1 (0-2218.1)</td>
<td>453.2 (0-2647.8)</td>
<td>171.8 (0-1027.6)</td>
<td>435.5 (0-2650.9)</td>
</tr>
<tr>
<td>NO</td>
<td>6.8 (1.7-46.6)</td>
<td>4.8 (1.6-10.1)</td>
<td>6.6 (1.3-18.9)</td>
<td>6.1 (1.0-18.0)</td>
<td>4.9 (1.6-23.4)</td>
</tr>
</tbody>
</table>
5.3.2 TVOC and 2-ethylhexanol concentrations

Concentrations of TVOC and 2-ethylhexanol in the chamber air during the PVC challenges and TVOC during the control (ceramic tiles) tests are shown in the figure 9. During the control exposure, 2-ethylhexanol concentrations were very low in the chamber air (≤ 2.4 µg/m³). The correlation between TVOC levels in exposure chamber and in exhaled breath samples during the PVC exposure is shown in figure 10. Figure 11 shows the correlation between 2-ethylhexanol concentrations in the chamber and their concentrations in exhaled breath samples during the exposure to damaged PVC flooring material. During the PVC exposure tests, all of the exhaled breath samples of the subjects studied contained 2-ethylhexanol with the concentration ranging from 1.2 to 9.2 µg/m³ (mean 5.2 µg/m³).

Figure 9. Concentrations of TVOCs and 2-ethylhexanol during the PVC exposure and TVOCs during the control (ceramic tile) test in exposure chamber air.
Figure 10

Correlation between TVOCs in exposure chamber air and in exhaled breath samples during exposure to PVC. $R^2=0.84$.

Figure 11

Correlation between 2-ethylhexanol concentrations in exposure chamber air and in exhaled breath samples during exposure to PVC. $R^2=0.4792$. 
5.3.3 Lung Functions

Before the PVC flooring challenge, the baseline values of forced expiratory volume in one second (FEV1) were 109% and forced vital capacity (FVC) 120% of the predicted values. The spirometric values did not change significantly during or after the PVC challenge, neither were there any significant differences in lung functions between the PVC and control exposures. The baseline levels of lung functions did not differ significantly in asthmatic subjects or smokers (including current and ex-smokers) compared to the others (data not shown). Moreover, PEF values did not change significantly during or after the challenges, and there was no significant difference in PEF values between the PVC and control exposures (data not shown).

5.3.4 $F_{NO}$ and $FN_{NO}$

The $F_{NO}$ levels did not change significantly during or after the PVC challenge and no significant difference in the $F_{NO}$ concentrations between the PVC and control exposures (data not shown). Furthermore, there were no significant changes in $FN_{NO}$ levels during or after either the PVC exposure, nor any statistically significant difference between the PVC and control challenges (data not shown).

5.3.5 Cytokines, NO and 2-ethylhexanol in NAL

The levels of all of the cytokines in NAL (TNF-α, IL-4, IL-6 and IL-12) increased immediately after exposure to either PVC flooring or the control material and there were no statistically significant differences in the cytokine concentrations between the challenges. No changes were detected in NO concentrations of NAL after the PVC exposure nor was there any statistically significant difference when compared to the control situation (Table 8).

The cytokine and NO levels in NAL of samples taken after at least one week vacation away from work did not differ from the baseline levels before the exposures. The
presence of 2-ethylhexanol in the NAL samples collected after the PVC exposure was not detected.

5.3.6 Cell Differential Count

Neutrophilic cells dominated the NAL cell profile both before and after both exposures (i.e. PVC flooring and control). No changes in the proportions of lymphocytes, neutrophils or eosinophils in the NAL were seen after the challenges. Moreover, no differences were detected in the changes in the cell counts between the PVC and control challenges (data not shown).
6. DISCUSSION

In this thesis, the reproducibility of the studied $F_{NO}$ and $FN_{NO}$ measurement techniques was shown to be high. Thus the techniques are suitable for long-term follow-up of an individual’s respiratory tract inflammatory status as demonstrated when they were utilized in the studies on *A. fumigatus* and degraded PVC material challenges. It was shown that inhalation challenges to *A. fumigatus* provoked inflammation in the upper and lower respiratory tract as indicated by increases in $F_{NO}$ and NAL cytokine levels as well as the airway symptoms reported by the subjects in this study. Thus, $F_{NO}$ measurement may serve as an indicator of mould exposure and the changes in cytokine levels may provide a link between mould exposure and biochemical inflammatory markers. In the other experimental study, even minuscule exposure to degraded PVC material caused inflammation in airways, as indicated by the self-reported symptoms of the subjects. In contrast, minor exposure to PVC degradation products did not evoke inflammation detectable by $F_{NO}$, $FN_{NO}$ or cytokine measurements.

6.1 Short and long-term reproducibility of $F_{NO}$ and $FN_{NO}$ (I)

The short-term and seasonal reproducibilities were good for both $F_{NO}$ and $FN_{NO}$ measurements in healthy subjects. Due to the strict exclusion criteria used the present $F_{NO}$ and $FN_{NO}$ data are not affected by inflammatory interference such as that associated with allergy and respiratory tract infections. The present observations of both day-to-day and week-to-week reproducibilities of $F_{NO}$ are in line with earlier findings showing high reproducibility of a single determination of $F_{NO}$ (Gabbay et al. 1998; Ekroos et al. 2002) as well as of the week-to-week reproducibility of the $F_{NO}$ measurement (Ekroos et al. 2000). However, in the present study, the week-to-week correlation of $F_{NO}$ measured in the afternoon did not reach a clinically significant level. It has been suggested that a change of 30-35 % or more in the $F_{NO}$ levels within an interval of 1-3 weeks could be considered abnormal (Ekroos et al. 2000).
The morning levels of both FENO and FNNO were consistently lower than those measured in the afternoon of the same day. The difference was more distinct in FNNO. In previous reports, the rise in the NO levels during the day has been attributed to dietary as well as metabolic factors (Zetterquist et al. 1999; Palm et al. 2000). On the other hand, in this study the FENO levels did not rise to abnormal levels in the afternoons in comparison to reference values (Kharitonov et al. 1997; ATS 1999; ATS/ERS 2005). The instrument used in this study was calibrated each morning and from time to time in the afternoon and no drift in the values was found. Thus, the intraday changes in the nitric oxide levels are interpreted as normal physiological variation. In contrast with our results, Kharitonov et al. (2003) did not find any diurnal variation in FENO levels. However, these present results indicate that the diurnal variation must be taken into consideration when planning clinical trials.

The present data suggests that seasonal changes in air temperature do not have any impact on the physiological NO production in the lower airways. This is of special interest, since cold air has been reported to be associated with inflammatory cell leakage into bronchial mucosa after exercise, but changes in NO levels have not supported the presence of active inflammation (Bonsignore et al. 2003). It must be noted that in Finland temperature and humidity varies extensively between the seasons. According to the Finnish Meteorological Institute, the mean outdoor temperatures (years 1900-2000) in the area that the study was performed were +9.0 C° in autumn (September), -8.8 C° in winter (January) and +16.5 C° in summer (June). In Finland, the pollen season lasts from the beginning of May until the end of August. Thus, significant changes in NO levels during the different seasons are probably related to airway inflammation such as allergy, asthma and respiratory infections.

In line with a previous study, the day-to-day and week-to-week variations of the FNNO levels were low (Bartley et al. 1999). However, the day-to-day reproducibility of FNNO measured in the afternoon was slightly below the limit of clinical significance.
Therefore, as with the FE\textsubscript{NO} measurements, it is advisable to perform the serial FN\textsubscript{NO} measurements in the morning to avoid these confounding effects.

The seasonal reproducibility of FN\textsubscript{NO} measurement was high in this healthy population. Since elevated FN\textsubscript{NO} concentrations have been detected in subjects with allergic rhinitis it could be anticipated that the FN\textsubscript{NO} levels vary seasonally in such patients. However, Henriksen et al. (1999) did not find any elevation in FN\textsubscript{NO} levels during the pollen season in subjects with allergic rhinitis though among the same population the FE\textsubscript{NO} levels had increased as expected. In this study, the FN\textsubscript{NO} levels in the healthy subjects were not affected by climatic factors such as cold outdoor temperature or the presence of pollen in the air.

FN\textsubscript{NO} levels were high compared to FE\textsubscript{NO} levels and this is in accordance with previous findings (Lundberg et al. 1999; Djupesland et al. 2001; Jorissen et al. 2001). This study revealed that the FN\textsubscript{NO} and FE\textsubscript{NO} levels did not correlate with each other. The FN\textsubscript{NO} and FE\textsubscript{NO} concentrations reflect the physiological status of the upper and lower airways respectively and NO is known to have different roles in the upper and lower airways.

The technique for FE\textsubscript{NO} measurement used in this study is well-documented and the mean FE\textsubscript{NO} values are in agreement with previous studies (Kharitonov et al. 1997; ATS 1999; ATS/ERS 2005). FN\textsubscript{NO} levels vary between laboratories with values from 30 to 2000 ppb being reported. This has been explained as being due to different measurement techniques (Jorissen et al. 2001; Kharitonov et al. 2005) but recently detailed instructions for FN\textsubscript{NO} measurement have been provided (ATS/ERS 2005). According to an earlier report, higher exhalation flow rates in FN\textsubscript{NO} measurement (250 vs. 500 mL/minute) lead to higher intraday and inter-day variability, and the FN\textsubscript{NO} levels are inversely related to the flow rate (Zetterquist et al. 1999). In this study, for the FN\textsubscript{NO} measurements a low, steady flow rate of 100 mL/minute was used in order to minimize the variation in the FN\textsubscript{NO} levels and this partly explains the high reproducibility of the present results.

The methods used in this study for FE\textsubscript{NO} and FN\textsubscript{NO} measurements require bulky,
non-portable equipment even though the methods were highly reproducible. Novel portable hand-held devices for NO measurements have been developed and, in future, they will represent more flexible tools for monitoring an individual’s respiratory tract inflammation.

6.2 Inflammatory airway effects caused by *A. fumigatus* challenge (II and III)

In study II, *A. fumigatus* challenge evoked a rapid increase in $\text{FE}_{\text{NO}}$ levels and there was a significant difference compared to the placebo test. The increased $\text{FE}_{\text{NO}}$ values indicate that inflammatory changes in lower airways had been induced by mould challenge. *A. fumigatus* has been reported to stimulate NO production in rat alveolar macrophages (Green et al. 1999). The results of this thesis are also in line with a study reporting increased NO concentrations in NAL in subjects with occupational mould exposure (Hirvonen et al. 1999). This is of interest because increased $\text{FE}_{\text{NO}}$ levels are associated with inflammatory lung diseases such as asthma and respiratory tract infections (Kharitonov et al. 1994; Ricciardolo 2003). In addition, a previous study reported that allergen challenge evoked a late, though not an immediate, increase in $\text{FE}_{\text{NO}}$ values in asthmatic subjects (Paredi et al. 1999).

Baseline $\text{FE}_{\text{NO}}$ levels were slightly higher in comparison to ERS and ATS recommendations (ATS 1999; Kharitonov et al. 1999; ATS/ERS 2005). On the other hand, the levels did not differ from previous measurements in the same laboratory and the differences can be explained by ambient NO levels (Purokivi et al. 2000). There was one current smoker and four ex-smokers among the subjects of studies I and II. Smoking decreases $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ levels (Persson et al. 1994) but after smoking cessation the values rise again to normal levels (Robbins et al. 1997). Smoking was prohibited during the study periods and thus did not affect the results.
FN\textsubscript{NO} levels increased in a similar way after mould and placebo challenges and no significant differences were found between these two exposures. Consistently, NO in NAL did not change significantly after the \textit{A. fumigatus} inhalation. The changes in FN\textsubscript{NO} and the minor increase of FE\textsubscript{NO} after the placebo challenge have been attributed to normal diurnal variation (Palm et al. 2000), a conclusion confirmed by the results of study I of this thesis. In summary, the inflammatory changes evoked by \textit{A. fumigatus} cannot be detected by FN\textsubscript{NO} measurement or by assessing NO in NAL.

There was a significant decrease in FEV1 after the mould challenge compared to placebo inhalation but the decrease was minor when observing the changes in absolute values. On the other hand, exposure to various bioaerosols among waste collectors was found to be connected with a significant decrease of FEV1 levels (Heldal et al. 2003). In addition, in study II, the baseline FVC and FEV1 values were significantly lower among the mould exposed subjects compared to their controls. However, because of the small group sizes the baseline FVC and FEV1 levels are poorly comparable with those in study II.

One of the atopic subjects experienced an asthmatic reaction during the \textit{A. fumigatus} challenge (25\% decrease in FEV1). After exclusion of this subject, there was no longer a significant difference in FE\textsubscript{NO} levels between the challenges but a distinct tendency could still be seen. The same subject had also a relatively large decrease in FEV1 after placebo challenge (12\%). This is an indication that bronchial hyperresponsiveness is not only related to \textit{A. fumigatus}, but nonetheless the decrease in FEV1 doubled after the \textit{A. fumigatus} challenge. The said subject belonged to the group of atopic subjects. It is well established that some non-asthmatic atopic subjects may have subclinical asthma not previously detected. After the exclusion of the subject experiencing an asthmatic response to \textit{A. fumigatus} inhalation, no significant difference or tendency was detected in FEV1 values between the challenges. Thus, it cannot be claimed that \textit{A. fumigatus} challenge affects lung functions.
The data of study III show that IL-1β concentrations in the NAL increased in a time dependent manner after the *A. fumigatus* challenge among all the subjects. IL-1β is a proinflammatory cytokine mainly produced by airway macrophages as a part of the nonspecific inflammatory response (Opal & DePalo 2000). *A. fumigatus* is also known to secrete proteases that promote the release of proinflammatory cytokines (Schuh et al. 2003). In a previous study, increased IL-1β concentrations in NAL were found in association with occupational mould exposure (Purokivi et al. 2001). IL-1β is also known to stimulate production of NO (Barnes et al. 1998). In accordance with the findings of this thesis it has been noted that an experimental *A. fumigatus* challenge can cause a rapid increase in $F_{ENO}$ levels. Thus, it can be proposed that *A. fumigatus* exposure activates IL-1β production which is involved in the pathway leading to inflammation in the upper airways.

TNF-α levels increased significantly at 6 hours after the *A. fumigatus* challenge compared to placebo inhalation also in the control subjects but no significant responses were seen in all the subjects or in the other subgroups. The elevated TNF-α levels were found to parallel to the increased IL-1β concentrations since both cytokines are proinflammatory and are activated as a part of the immune response against stimuli such as microbial infections (Sedgwick et al. 2002). In addition, IL-1β is known to promote the production of TNF-α (Yoshimura et al. 2003). In accordance with this study, elevated TNF-α concentrations in NAL have been observed in association with microbial exposure in a moisture-damaged building (Purokivi et al. 2001) and after exposure to swine dust (Wang et al. 1997).

The IL-6 levels in NAL of all the subjects tended to be higher after the *A. fumigatus* challenge in comparison to placebo but no significant differences were found in the changes of IL-6 production between the challenges at the group level. IL-6 is also considered to be a proinflammatory cytokine and the increased levels are in line with the changes of IL-1β and TNF-α. On the other hand, IL-6 inhibits TNF-α and IL-1β and may
protect host cells from potentially destructive inflammatory responses (Opal & DePalo 2000). Previous reports have also shown a significant elevation of IL-6 in NAL among subjects exposed to the microbes present in a moisture-damaged building (Hirvonen et al. 1999; Purokivi et al. 2001; Roponen et al. 2001). Moreover, consistent with results of study III, increased IL-6 concentrations have been measured from airway epithelial cell lines after acute *A. fumigatus* challenge (Borger et al. 1999). In summary, the present data supports the hypothesis that proinflammatory cytokines play a significant role in the inflammation occurring in the upper airways after *A. fumigatus* challenge.

The increased IL-4 levels in NAL observed after *A. fumigatus* challenge are similar to the findings of a previous study showing a link between elevated IL-4 concentrations in NAL and occupational exposure to microbes in a moisture-damaged building (Roponen et al. 2001). This is of interest since IL-4 is a Th2-type cytokine that is known to be activated in allergy and atopic diseases (Olivenstein et al. 1999). In addition, the Th2-type response, including the elevated IL-4 levels dominates the immune response in *A. fumigatus* induced diseases such as pulmonary aspergillosis (Schuh et al. 2003).

The changes in IL-4 levels after the *A. fumigatus* challenge were most distinct in the occupants of the moisture-damaged building. Therefore, inflammation caused by acute mould challenge may be linked to a Th2-type response in individuals with previous chronic mould exposure.

IFN-γ is known to inhibit cell proliferation and IL-4 production and also to enhance the cytotoxicity of TNF-α (Yoshimura et al. 2003). In study III, no changes of IFN-γ levels in NAL after the *A. fumigatus* challenge were detected and the responses were similar to those noted after the placebo inhalation. This is in agreement with the results seen in mouse models where increased IL-4, IL-5, IL-10 and IL-13 levels have been detected after acute *A. fumigatus* exposure but IFN-γ levels remained unaffected (Kurup et al. 1994; Grunig et al. 1997).
In study III, there were differences between the groups in the measured baseline cytokine levels which were highest among the control subjects. This may be due to several factors. The quality of NAL samples received from atopic and occupationally mould exposed subjects was lower than the samples of the control subjects which may be reflected in the cytokine concentrations obtained. It has been shown in a previous study that there may be considerable differences in cytokine baseline levels in NAL even between healthy individuals (Roponen et al. 2003). It can also be assumed that the anatomy of each subject's nasal cavity may contribute to the between-subject variability, since the same volume of the instilled fluid may lavage a different total area of the nasal cavity in different individuals. However, because of the small group sizes, the differences in cytokine baseline levels before the first challenge are not comparable.

In study III, the cytokine concentrations in NAL decreased at 6 hours after the placebo challenge and returned at 24 hours towards the level which had been present before the challenge. In addition, there was only a slight increase in the detected cytokine concentrations after the A. fumigatus inhalation at 6 hours possibly due to an attenuation of the responses caused by reperforming the NAL protocol 6 hours after the first sampling. Thus, 6 hours is probably too short a time to allow for recovery after the first nasal lavage. The biological basis of this phenomenon remains unknown.

Within-subject variation of cytokine levels in serial NAL measurements is low but there may be a major variation between individuals and genders. Baseline cytokine concentrations in NAL have been reported to be significantly higher among males compared to females (Roponen et al. 2003). Thus, in study III, one male subject with high cytokine baseline values was excluded and thus all the subjects in that study were females. In addition, there are differences between NAL sampling methods as to how the samples are obtained, handled and analyzed (Roponen et al. 2003). Thus, there are no universal reference values for levels of cytokines in NAL and the laboratory specific validation of the method remains crucial.
The group to which the subjects belonged was not associated with significantly increased FE\textsubscript{NO} levels. For example, it could have been anticipated that atopic subjects would exhibit a different response to the mould challenge. It is well known that atopic status as such is connected with increased FE\textsubscript{NO} values (Riccardolo 2003). In addition, Piipari et al. (2002) reported that those individuals with higher baseline FE\textsubscript{NO} values displayed only a minor increase in FE\textsubscript{NO} compared to those with lower baseline levels after mould challenge including \textit{A. fumigatus}. Consistently, in study III, a tendency was seen in IL-1β levels but no significant changes in cytokine concentrations among the atopic subjects including those with and without mould exposure were detected after the mould challenge. On the other hand, due to the exclusion criteria the group of atopic subjects became smaller than planned and, therefore, it was not feasible to perform a comprehensive statistical analysis.

\textit{A. fumigatus}-induced airway inflammation is known to be associated with e.g. eosinophilia (Schuh et al. 2003). Therefore, one might have expected to see changes in nasal cell proportions after mould challenge since clear changes in cytokines in NAL were detected in this study. However, no significant differences in the changes of cell differential counts in NAL between the \textit{A. fumigatus} and placebo challenges were found. On the other hand, consistent with the current results, there are several previous studies where mould exposure has been found to significantly increase cytokine concentrations in NAL but the proportions of NAL cells have remained unaltered (Hirvonen et al. 1999; Purokivi et al. 2001; Roponen et al. 2001). This suggests that the proportions of nasal lavage cells do not necessarily represent the proportion of all the cells in the nasal cavity contributing to the cytokine concentrations detected in NAL.

In line with the increased FE\textsubscript{NO} and cytokine concentrations, respiratory tract symptoms on the morning after the challenge were significantly more common after the \textit{A. fumigatus} inhalation compared with the placebo test in all of the studied groups. This finding is also supported by previous studies establishing that subjects exposed to indoor air microbes report airway symptoms more frequently than their control counterparts
and, consistent with this thesis, the symptoms were associated with increased cytokine levels (Hirvonen et al. 1999; Purokivi et al. 2001; Roponen et al. 2001).

It has been suggested that exposure to the indoor air fungi of moisture-damaged buildings can cause an IgE mediated allergic reaction (Zureik et al. 2002). In our study, none of the subjects had increased *A. fumigatus* specific IgE levels, and only one subject in the group of mould exposed individuals exhibited a positive reaction to the fungus *Geotrichum candidum* in skin prick tests. There is also epidemiologic evidence that sensitization to *A. fumigatus* as well as to *Cladosporium herbarum* indicated by specific IgE is related to the risk of adult-onset asthma (Jaakkola et al. 2006). On the other hand, it has been reported that fungal allergy is rare in individuals exposed to mould in a moisture-damaged school (Immonen et al. 2000). This suggests that there are mechanisms other than immediate IgE-type reaction evoking the respiratory symptoms after exposure to indoor air fungi of moisture-damaged buildings. Nonetheless, it has been reported that sinusitis among teachers with occupational mould exposure is associated with increased fungal specific IgG antibodies (Patovirta et al. 2003). In a Finnish population-based incident case-control study an increased risk of developing asthma in adulthood was significantly related to IgG antibodies to *T. citrinoviride*, but not to the other moulds including *A. fumigatus, A. versicolor, Cladosporium cladosporioides, Fusarium oxysporum, Sporobolomyces salmonicolor, Stachybotrys chartarum*, and *Streptomyces alb us* (Jaakkola et al. 2002). However, the presence of fungal IgG antibodies in human sera indicates only that exposure has occurred at some time in the past (Jarvis and Miller 2004). In addition, only a few fungal allergens have been accurately characterized with respect to their various morphology and antigenicity as well as prevalent antigenic crossreactivity (Malling 1992). Furthermore, part of the IgE binding to mould and yeast allergen extracts is due to cross-reaction with glycoproteins and the possibility of false-positive IgE and skin prick test results needs be taken into account in any diagnosis of mould allergy (Leino et al. 2006).
Microbial extracts from moisture-damaged buildings produce secondary metabolites which are known to be harmful to human health (Hendry and Cole 1993; Fischer et al. 2000). Since they may provoke unexpected health effects it is unethical to challenge human beings with these extracts, and a purified *A. fumigatus* product i.e. a product not containing e.g. mycotoxins was used in this study. Due to commercial considerations, the exact contents of the extract were not revealed by the manufacturer but the approval of the Finnish National Agency for Medicine (FNAM) for the product guarantees the safety of the challenging agent.

Mycotoxins secreted by *A. fumigatus* could have potentially caused inflammatory responses that were not capable of being detected in this study. In addition, several batches of the *A. fumigatus* were used and there may be differences between batches. Unfortunately, the possible variation between the batches is also unknown for the same commercial reasons. However, this thesis demonstrates that inhalation exposure to *A. fumigatus* extract without secondary metabolites is sufficient to evoke respiratory tract inflammation even if there may be possible differences between batches.

### 6.3 The effects of degraded PVC material challenge on airways (IV)

Ten subjects exposed to degraded PVC flooring material at work were challenged to PVC under controlled conditions. This is the first study to expose subjects to very low concentrations of chemical emissions similar to those found in indoor air conditions.

The morning following the PVC challenge, the subjects suffered significantly more often respiratory tract symptoms compared to the situation after control exposure. This result is in agreement with studies where the subjects have experienced similar irritation symptoms during the exposure to damaged PVC products (Wieslander et al. 1999; Wålinder et al. 1999; Bornehag et al. 2005). According to the results of this study, even minuscule emissions of degraded PVC flooring materials which may be found in indoor air are sufficient to evoke respiratory tract symptoms.
The PVC and control test were performed blindly and in a random order but it is possible that the subjects recognized the degraded PVC material due to its smell. However, no significant differences were found between PVC and control tests in self-reported irritation symptoms during the exposures. Instead, the significant differences in symptoms were found on the morning after the challenges. Therefore, it seems likely that the results have not been biased by the fact that the subjects were possibly aware of material to which they were challenged.

The PVC flooring material challenge did not cause any significant changes in lung functions, $FE_{NO}$ or $FN_{NO}$ levels when compared to the control test. Moreover, there were no individual asthmatic reactions such as any decrease in FEV1 after these short exposure times, although half of the subjects had asthma and their asthma medications had been withdrawn prior to the study. In occupational exposures, the development of asthma requires long and repeated exposure and the situation may be similar when the occupants are exposed to the damaged PVC material (Tuomainen et al. 2004). There is clear evidence that damaged PVC products are associated with asthma and atopic disorders (Øie et al. 1997; Bornehag et al. 2004).

The baseline $FE_{NO}$ levels were slightly higher than the normal levels defined by the ATS and ERS recommendations (Kharitonov et al. 1997; ATS 1999; ATS/ERS 2005). This is probably due to the presence of five subjects with asthma in the studied group. The baseline $FN_{NO}$ levels were also higher than those reported in study II of this thesis. There were two subjects with very high $FN_{NO}$ values which increased the mean levels of this small study group.

Cytokine concentrations in NAL increased after both PVC and control exposures but no significant differences in cytokine levels attributable to the active challenge were detected. Consistent with unaltered $FN_{NO}$ levels, the degraded PVC material challenge did not change the NO concentrations in NAL. Moreover, the cytokine or NO levels in NAL after a vacation did not differ from the baseline levels taken before the challenges. The timing of the NAL samplings was not entirely optimal because on the morning following the PVC exposure the subjects reported a great many of respiratory tract symptoms but at that time no NAL samples were collected.
Ceramic tile was selected as the control material because it was considered to represent a “clean” material with very low emissions. However, it is possible that exposure to ceramic tiles also evoked inflammatory changes which were seen as increased cytokine levels in NAL. Thus another material may be more appropriate for use as control. On the other hand, it is possible that the minuscule PVC exposure does not cause inflammation in upper airways which could be detected as increased cytokine or NO concentrations in NAL.

No 2-ethylhexanol was found in the NAL samples collected after the PVC challenge. The compound could have already evaporated from the nose before the sampling which was performed after the lung function tests and \( FE_{\text{NO}} \) and \( FN_{\text{NO}} \) measurements. It is also possible that at least part of the compound had been absorbed into the blood circulation.

All of the exhaled breath samples collected during the PVC exposure contained traces of 2-ethylhexanol. The presence of 2-ethylhexanol in exhaled breath is evidence that this compound had been breathed into the lungs, transferred into blood and then transported to other parts of the body. It is known to be a specific, biologic marker for exposure to damaged PVC flooring material and its presence describes recent exposure (Wiglus et al. 1998). In normal situations (i.e. when there is no exposure), there is no 2-ethylhexanol present in exhaled breath (Phillips et al. 1999). Although there were only ten subjects in this study, the exhaled breath 2-ethylhexanol concentrations correlated well with the chamber air 2-ethylhexanol levels. In spite of the ventilation of the exposure chamber after PVC exposures, there were still minimal concentrations of 2-ethylhexanol in the chamber air at the time the control tests were performed. These low levels are probably not due to emissions from ceramic tiles but are more likely connected to the air exchange in the exposure chamber.

6.4. CLINICAL IMPORTANCE OF THE CURRENT RESULTS

\( FE_{\text{NO}} \) levels are known to increase in inflammatory airway diseases such as asthma and, thus, they are widely measured in clinical work (ATS/ERS 2005). Inhaled corticosteroids decline \( FE_{\text{NO}} \) concentrations in asthmatic individuals and low levels
during the treatment demonstrate a good steroid response (Kharitonov et al. 1994; Fabio et al. 2004). Repeated $F{E_{NO}}$ measurements are well appropriate to treatment follow-up of asthmatic patients due to the high long-term reproducibility indicated in this study. In the study I, it was also shown that $F{N_{NO}}$ measurements were highly repeatable. However, there are controversial results about how $F{N_{NO}}$ changes in inflammatory upper-airway diseases and partly because of that the $F{N_{NO}}$ measurements are not routinely used in clinical work (Djupesland et al. 1999; Jorissen et al. 2001; ATS/ERS 2005).

*A. fumigatus* is a common fungus found in mouldy building materials (Hyvärinen et al. 1999). As stated in this thesis, increase in $F{E_{NO}}$ levels may indicate an acute exposure to that fungus. Nowadays, there are portable nitric oxide measurement devices which provide a possibility for measurements inside the moisture-damaged buildings (Alving et al. 2006). On ground of the study II, the first $F{E_{NO}}$ measurements could be performed when the occupants enter the building and the second time at 3 hours after the exposure. If a rapid increase in $F{E_{NO}}$ levels is observed it may indicate a mould exposure. However, the occupants of a moisture-damaged building are exposed to several different indoor air microbes and their secondary metabolites. Thus, further studies are needed to indicate if a real life mould exposure can be demonstrated by assessing changes in $F{E_{NO}}$ levels.

The changes in cytokine levels of NAL, especially IL-$\beta$, may also be indicators of exposure to mould. However, NAL is technically difficult to perform and, thus, it is not easily suited for clinical examination of mould exposed individuals. According to this thesis, changes of $F{N_{NO}}$ levels do not indicate an exposure to *A. fumigatus*.

In the study IV, degraded PVC materials provoked respiratory tract symptoms in the exposed individuals. Thus, it would be useful to replace surfacings containing PVC to less harmful materials in older buildings and PVC materials should be avoided when setting up new buildings. Interestingly, the study IV of this thesis was referred to when Prague University Hospital Motol informed that it substituted PVC floors by harmless rubber (Arnika Press Releases 2007). The biological inflammatory changes caused by degraded PVC materials are still unknown and, thus, need further research.
7. CONCLUSIONS

1. Short-term and seasonal reproducibilities of $\text{FE}_{\text{NO}}$ and $\text{FN}_{\text{NO}}$ levels are very high and serial NO measurements are a reliable tool in monitoring airway inflammation in both upper and lower airways. The diurnal variation needs to be taken into account such that in long-term follow-up, the measurements should be performed at the same time of the day, in the preferably mornings rather than the afternoons.

2. Inhalation of *A. fumigatus* solution evokes inflammatory changes in lower airways seen as a rapid increase in $\text{FE}_{\text{NO}}$ levels and respiratory tract irritation symptoms reported by the challenged subjects. $\text{FE}_{\text{NO}}$ measurement may serve as an indicator of acute mould exposure.

3. *A. fumigatus* challenge causes inflammation in the upper airways which can be detected as increased cytokine levels in NAL with the most distinct response being seen in IL-1$\beta$ levels. These results provide a link between acute mould exposure and biochemical markers of inflammation.

4. Minuscule emissions of degraded PVC flooring materials that can be detected in the indoor air are sufficient to evoke respiratory tract irritation symptoms in the exposed subjects. Exposure to small amounts of degrading PVC material do not cause airway inflammation as assessed by induced changes in cytokines, $\text{FE}_{\text{NO}}$, $\text{FN}_{\text{NO}}$ and lung functions.
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APPENDIX: ORIGINAL ARTICLES
Kuopio University Publications D. Medical Sciences


D 401. Andrušionyte, Laura. Transcription factors as candidate genes for type 2 diabetes: studies on peroxisome proliferator-activated receptors, hepatic nuclear factor 4α and PPARγ coactivator 1α. 2007. 112 p. Acad. Diss.


