MATERNAL SMOKING DURING PREGNANCY INDUCES AIRWAY REMODELING IN MICE OFFSPRING

RUNNING TITLE: MATERNAL SMOKING REMODELS OFFSPRING AIRWAYS

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ABBREVIATIONS

HDM = House Dust Mite

$T_H$ = T helper

IgE = Immunoglobulin E

MCh = Methacholine

Ig = Immunoglobulin

IL = Interleukin

IFN  = Interferon-

TNF = Tumour Necrosis Factor

PC300 = Concentration of methacholine inducing 300% increase in Penh

Penh = Enhanced Pause

NSM = Non-Smoking Mother

SM = Smoking Mother
ABSTRACT

Introduction: Children from smoking mothers have an increased risk to develop asthma for reasons largely unknown. We investigated effects of maternal smoking during pregnancy on remodeling, allergic airway inflammation and hyperresponsiveness in offspring in an experimental asthma model.

Methods: Mice were exposed to fresh air or cigarette smoke from 3 weeks prior to conception until birth. Offspring were exposed to house dust mite (HDM) or PBS intranasally 4 times/week from week 5-10 after birth onwards.

Results: Maternal smoking increased airway smooth muscle layer, collagen III deposition and HDM-induced goblet cell numbers in offspring. It additionally increased methacholine responsiveness which correlated significantly with increased airway smooth muscle layer and collagen deposition. Maternal smoking increased HDM-induced numbers of neutrophils and mast cells in lung tissue. No further effects were observed.

Conclusions: Smoking during pregnancy induces airway remodeling in mice offspring, which may contribute to increased methacholine responsiveness. This takes place irrespective of allergen exposure but may worsen the outcome of the allergic stimulus, resulting in higher methacholine responsiveness in HDM exposed offspring from smoking mothers when compared to non-smoking mothers. Our results provide a possible mechanism behind the association between maternal smoking and asthma.
INTRODUCTION

Maternal smoking during pregnancy has been shown to increase physician-diagnosed asthma and wheezing during childhood and some studies reported this to be independent of postnatal smoke exposure [1,2]. In addition, two large studies in North America and several European countries have shown that maternal smoking during pregnancy is associated with reduced lung function [3,4], and that maternal smoking during pregnancy is a strong and significant independent predictor of wheezing after age 16 [5]. These studies mainly report effects in children and adolescents from 0 to 18 years of age. Whether maternal smoking during pregnancy also affects the development of asthma in adulthood is not certain, but it increases this risk in smoking adolescents [6].

The mechanisms underlying the increased risk for development of asthma in infants exposed to maternal tobacco smoking in utero are largely unknown. Several studies have shown that maternal smoking during pregnancy has effects on immune system development [7,8,9], lung development and lung function in offspring.

With respect to lung development, in utero smoke exposure in rats decreased the number and increased the size of alveoli in the offspring [10], whereas in rhesus monkeys in utero exposure to nicotine increased collagen deposition around large airways and vessels [11]. Lambs exposed to maternal nicotine in the last trimester of foetal life developed lung function in a way that is suggestive for smaller airways and/or smaller or stiffer lungs [12].

Furthermore, the inner airway wall thickness was higher in children from smoking versus non-smoking mothers in children who died from sudden infant death syndrome [13], showing evidence for lung remodeling due to in utero smoke exposure in humans too.

We set out to further unravel the consequences of in utero cigarette smoke exposure on susceptibility to develop allergic airway inflammation by studying house dust mite (HDM)-induced airway remodeling (smooth muscle layer thickness, collagen III deposition and goblet
cell hyperplasia), inflammation (T_{H2} cytokines and inflammatory cells) and methacholine responsiveness in adult offspring from smoking mothers.
METHODS

Animals
Female and male BALB/c mice, age 8-10 weeks, were obtained from Harlan (Horst, The Netherlands). Standard food and water were provided ad libitum, lights were set to a 12:12 hours light:dark cycle. All animal protocols were approved by the local Committee on Animal Experimentation and were performed under strict governmental and international guidelines on animal experimentation.

Cigarette smoke exposure
Mainstream cigarette smoke was generated by the smoke exposure system of the Tobacco and Health Research Institute of the University of Kentucky, which is similar to active smoking (described in [14]). Female mice were exposed to fresh air or two nose-only smoking sessions per day, 5 days per week. Smoking sessions started with two puffs from a 2R1 Reference cigarette (University of Kentucky), and increased daily until 24 puffs per session were reached after three weeks. Then females were introduced pair wise to one male to induce conception. Smoke exposure remained constant during the total pregnancy. Mothers and offspring were not exposed to cigarette smoke after offspring were born. 2R1 Reference cigarettes were chosen because of high tar and nicotine content, and because we were able to induce emphysema using these cigarettes [14].

Antigen administration in offspring
Offspring were housed with the mother until the end of the weaning period (3 weeks). Then male and female pups were separated (n=8 pups per cage). Offspring (8 female and 8 male pups per group) were mildly anesthetized (inhaled isoflurane with oxygen) and exposed to
phosphate buffered saline (PBS) or purified whole body house dust mite (HDM) extract (Dermatophagoides pteronyssinus, obtained from Greer laboratories, Lenoir, NC) intranasally (25 µg in 10 µl PBS) to induce allergic airway inflammation, four times per week from week 5 until week 10 after birth.

**Assessment of methacholine responsiveness**

Twenty–four hours after the last allergen exposure, methacholine (MCh) responsiveness was assessed by measuring enhanced pause (Penh) in conscious, spontaneously breathing animals using a whole-body plethysmography system (Buxco Electronics, Petersfield, UK) as described earlier by our group [15]. An advantage of using this technique is that it allows detailed immunohistochemistry of the lung tissue after this procedure within the same mouse.

**Tissue and blood collection**

Mice were sacrificed under anaesthesia at 10 weeks of age. Blood was taken by heart puncture. The two smallest right lung lobes were snap frozen for multiplex ELISA, the two largest right lung lobes were snap frozen for immunohistochemistry, the left lung lobe was formalin fixed and embedded in paraffin for immunohistochemistry.

**Cytokines**

Concentrations of IL-2 (interleukin-2) IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFNγ (interferon-γ) and TNF (tumour necrosis factor) were measured in homogenized lung tissue with a multiplex ELISA system (Lincoplex Systems, St Charles, MO, USA) on a Luminex 100 system using Starstation software (Applied Cytometry Systems, Sheffield UK).

Eotaxin was determined using an ELISA kit (R&D, Minneapolis, USA), performed as described by the manufacturer.
Histology

Frozen lung sections were made in the caudal-cranial axis from the ventral end (the convex side) of the left azygous and diaphragmatic lobe (see figure 1). Sections were made where small/intermediate airways were present and large cartilaginous airways were totally or largely absent in the sectional plane. Paraffin embedded lung sections were made cross-sectionally halfway the left lobe, 45 degrees from the dorsal-ventral axis (to exclude the large cartilaginous bronchi close to the hilus). Sections were made when small/intermediate airways were present and large cartilaginous airways were totally or largely absent in this sectional plane as well.

Eosinophils and neutrophils were determined by staining 4 µm cryosections of lung tissue for cyanide resistant endogenous peroxidase activity with diaminobenzidine (Sigma Aldrich, Zwijndrecht, The Netherlands) or a monoclonal rat anti GR1 antibody (BD Biosciences, San Jose, CA, USA) respectively. The volume percentage of eosinophils was calculated using morphometric analysis using Leica Qwin image analysis software (Leica Microsystems, Rijswijk, The Netherlands).

Mast cells were determined by staining 3 µm paraffin embedded sections of lung tissue with Toluidin Blue. Mast cells and neutrophils were counted manually in whole lung sections. The tissue area was quantified by morphometric analysis and the numbers of cells were expressed per mm² lung tissue.

Goblet cells were stained with Periodic Acid Schiff’s (PAS) in 3 µm paraffin embedded sections and all PAS-positive cells in the section were counted manually. The length of all airways (measured at the basal end of the airway epithelium) in the section was determined, and the total number of PAS-positive cells was expressed per mm airway.
Collagen III and airway smooth muscle layer were stained on 4 µm frozen lung sections with polyclonal goat anti type III collagen antibody (SBA, Birmingham, AL, USA) and a monoclonal mouse anti α smooth muscle actin antibody (Progen Biotechnik, Heidelberg, Germany), respectively. Collagen III and α-SMA presence directly adjacent to the airway epithelium were quantified in the total lung section by morphometric analysis. The surface of positively stained tissue was expressed as mm2 per mm airway in the total lung section. Staining in the parenchyma and directly adjacent to blood vessels was excluded from the measurement. Cartilaginous airways, if present, were excluded from all analyses. Each analysis was performed blinded by the same observer.

**Measurement of HDM specific serum IgE**

HDM-specific serum IgE was measured using an enzyme-linked immunosorbent assay (ELISA). A flat bottomed 96-well plate was coated overnight with rat-anti-mouse IgE (BD Biosciences, San Jose, CA USA). Serum samples were added (dilution 1:10) and incubated for 2h. Biotinylated HDM was added for 1 h and horseradish peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) was added for 30 min. Plates were developed using tetra methyl benzidine (TMB) substrate (Sigma Aldrich, Zwijndrecht, Netherlands), stopped and optical densities were read at 450 nm using a Varioscan ELISA reader (Thermo Scientific, Breda, The Netherlands). HDM specific IgE levels were expressed as arbitrary ELISA units by correcting OD’s for the dilution factor of the serum sample.

**Statistical analysis**

When residuals were not normal distributed, appropriate log10 or 1/x transformation of the data was performed. The interaction of the effect of smoking during pregnancy and the effect
of HDM exposure was investigated with a multiple linear regression model (SPSS 14.0 software, SPSS Inc. Chicago, Illinois, USA). The multiple linear regression analysis can distinguish between a positive interaction and a negative interaction. A significant positive interaction (the only interaction type found in our analyses) means that the effect of HDM exposure is greater in offspring from smoking mothers.

When no interaction was found, the effect of smoking during pregnancy and the effect of HDM were assessed separately with linear regression analysis. To assess differences between subgroups, we tested normal distribution of the data in the subgroups with a Kolmogorov-Smirnov and a Shapiro-Wilk test for normality of data. When data were normally distributed according to these both tests, we tested differences between subgroups with a two-sided independent samples T-test. When data were not normally distributed we used two-sided Mann-Whitney-U tests.

Correlations between parameters were established using Spearman’s rho (SPSS software). To further assess predictors of MCh responsiveness, parameters that were independently associated with PC300 were identified and subsequently a stepwise linear regression model was used to explain the variability in PC300. A value of P<0.05 was considered significant.
RESULTS

Maternal smoking during pregnancy induces airway remodeling in offspring

Maternal smoking during pregnancy and HDM exposure independently increased the thickness of the airway smooth muscle layer (Figure 2A and Figure 3A-B) and deposition of collagen III around the airways from offspring (Figure 2B).

The number of goblet cells was analysed as an additional feature of airway remodeling. HDM exposure increased the number of goblet cells in the airways (Figure 2C), an effect that was stronger in offspring from smoking mothers than from non-smoking mothers (indicated by a significant interaction between the effects of smoking during pregnancy and HDM exposure).

Maternal smoking during pregnancy increases methacholine responsiveness in offspring

Exposure to HDM induced a significant increase in Penh in offspring from smoking and non-smoking mothers, as calculated by the area under the Penh (a unit-less index of airway hyperresponsiveness) curve (Figure 4A).

Maternal smoking during pregnancy increased methacholine (MCh) responsiveness in offspring, as shown by a decreased concentration of MCh inducing a 300% increase in Penh from baseline (PC300, figure 4B). This increased MCh responsiveness was independent from HDM exposure. Offspring exposed to HDM had a lower PC300 than offspring exposed to PBS, which yields the lowest PC300 in HDM-exposed offspring from smoking mothers. PC300 correlated inversely with airway smooth muscle layer thickness (Figure 4C) and collagen III deposition (rho -0.62, p<0.001 and rho = -0.41, p=0.001 respectively).

Effect of maternal smoking during pregnancy on HDM-induced inflammatory cells and cytokines in offspring
HDM exposure increased numbers of neutrophils and mast cells in lung tissue in offspring from smoking mothers, but not from non-smoking mothers. HDM exposure increased eosinophils in lung tissue irrespective of maternal smoking (Table 1).

### Table 1: inflammatory cells in lung tissue from offspring

<table>
<thead>
<tr>
<th></th>
<th>Non-smoking mother</th>
<th>Smoking mother</th>
<th>Linear regression analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>HDM</td>
<td>PBS</td>
</tr>
<tr>
<td><strong>HDM effect:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SM effect</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Eosinophils</strong></td>
<td>0.05 (0.03-0.07)</td>
<td>0.36 (0.05-0.43) **</td>
<td>0.05 (0.04-0.07)</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td>181 (134-245)</td>
<td>220 (177-293)</td>
<td>163 (116-207)</td>
</tr>
<tr>
<td><strong>Mast cells</strong></td>
<td>0.00 (0.00-0.00)</td>
<td>0.08 (0.00-0.31)</td>
<td>0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

Values are medians (25% percentile – 75% percentile)
Subgroup analysis: HDM vs PBS group (* p<0.05, ** p<0.01, *** p< 0.001)

Smoking during pregnancy had no effects on lung cytokines as investigated by linear regression (Table 2). HDM exposure increased levels of IL-2, IL-4, IL-5, IL-6, IL-13, IL-17 and TNF in lung homogenate, whereas it decreased levels of IFNγ and IL-10. Particularly IL-17 was strongly upregulated after HDM exposure, which correlated with neutrophil (rho=0.51, p=0.003) and eosinophil numbers (rho=0.75, p<0.001) and eotaxin level (rho=0.61, p<0.001) in lung tissue.

### Table 2: cytokines in lung tissue from offspring

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Non-smoking mother</th>
<th>Smoking mother</th>
<th>Linear regression analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>HDM</td>
<td>PBS</td>
</tr>
<tr>
<td><strong>HDM effect:</strong></td>
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<tr>
<td><strong>SM effect</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>2.1 (1.7-2.7)</td>
<td>9.1 (2.9-26.9) ***</td>
<td>1.9 (1.7-1.9)</td>
</tr>
<tr>
<td>IL-5</td>
<td>12.5 (9.9-17.8)</td>
<td>17.8 (10.6-26.7)</td>
<td>8.6 (8.6-14.2)</td>
</tr>
</tbody>
</table>

Values are medians (25% percentile – 75% percentile)
Subgroup analysis: HDM vs PBS group (* p<0.05, ** p<0.01, *** p< 0.001)
<table>
<thead>
<tr>
<th>Parameter</th>
<th>HDM vs PBS Group</th>
<th>Subgroup Analysis</th>
<th>HDM Specific IgE Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>112 (96-124)</td>
<td>149 (109-286) *</td>
<td>103 (83-118)</td>
</tr>
<tr>
<td>IL-10</td>
<td>1399 (1109-1513)</td>
<td>868 (715-1245) **</td>
<td>1276 (1028-1443)</td>
</tr>
<tr>
<td>IL-13</td>
<td>542 (500-613)</td>
<td>542 (472-724)</td>
<td>500 (444-549)</td>
</tr>
<tr>
<td>IL-17</td>
<td>12.4 (10.9-14.5)</td>
<td>1018 (83.9-3144) ***</td>
<td>11.7 (10.9-12.4)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>95.3 (69.8-124.6)</td>
<td>62.5 (28.3-96.6)</td>
<td>72.7 (50.0-102.1)</td>
</tr>
<tr>
<td>TNF</td>
<td>14.2 (11.8-16.2)</td>
<td>15.4 (11.8-19.3)</td>
<td>12.2 (10.7-13.7)</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>1588 (1376-1803)</td>
<td>2222 (1678-2475)**</td>
<td>1450 (1313-1637)</td>
</tr>
</tbody>
</table>

Values are medians (25% percentile – 75 % percentile)
Subgroup analysis: HDM vs PBS group (* p<0.05, ** p<0.01, *** p< 0.001)

HDM-specific IgE levels in serum were increased after HDM exposure, without a significant effect of maternal smoking status (data not shown).

**Parameters explaining variability in MCh responsiveness in offspring**

To further assess whether the observed increased remodeling could underlie the increased MCh responsiveness, we first identified whether smooth muscle layer thickness, collagen III deposition and goblet cell hyperplasia were independently associated with PC300. They were all independently associated (p<0.05). These 3 remodeling parameters explained 40% (R²) of the variability in PC300 in a linear regression model. A model with only smooth muscle layer thickness accounted for 39% of the variability in PC300. Interestingly, eosinophils in lung tissue were also significantly correlated with PC300 and adding eosinophils to the first model increased the explained variability in PC300 to 46%, with a further increase to 59% when adding smoking during pregnancy to this model.
DISCUSSION

This study has three important observations. Firstly, maternal smoking during pregnancy increased airway smooth muscle layer thickness and collagen III deposition around the airways in offspring and increased HDM-induced goblet cell hyperplasia. Secondly, maternal smoking during pregnancy increased HDM-induced numbers of neutrophils and mast cells, and thirdly, maternal smoking increased methacholine responsiveness in 10 weeks old offspring.

This is the first study to show that smoking during pregnancy, without subsequent postnatal exposure to tobacco smoke, increases and sustains remodeling in 10 weeks old offspring. An additional new finding is that increased smooth muscle layer thickness is the best predictor of increased airway responsiveness in offspring from smoking mothers. Since contraction of airway smooth muscle upon antigen stimulation causes airway narrowing, the increase in smooth muscle found in offspring from smoking mothers may account for the increased MCh responsiveness we observed. Our finding that MCh responsiveness is linked to airway smooth muscle thickening in the airways is supported by Southam et al, who found that (without introducing maternal smoking during pregnancy) persistent airway hyperresponsiveness is associated with increased smooth muscle area in mice exposed to HDM [16]. In a study from Elliot et al, however, the increase in acetylcholine chloride responsiveness in offspring from smoking guinea pig mothers was not coupled to increased smooth muscle area, possibly because changes in smooth muscle layer thickness are not apparent yet at 21 days after birth [17].

In mouse offspring exposed to maternal smoking in utero, MCh responsiveness was reported to associate with a decreased presence of cyclic AMP in lung tissue [18]. This is of interest since cyclic AMP is involved in relaxation of smooth muscle in the airway. Therefore,
together with an increased smooth muscle area, other smooth muscle characteristics in the offspring exposed to maternal smoking during pregnancy may be a major contributor to airway hyperresponsiveness.

The effects of the *in utero* smoke-induced increase in airway smooth muscle contraction upon Mch challenge could even be enhanced by a decreased adaptive capacity of the airway wall caused by the increased collagen III deposition as we observed in our study. Collagens are responsible for the compliance and structural integrity of the lung and increased collagen deposition as seen in remodeled airways is thought to render a stiffer airway. Interestingly, Sekhon *et al* found previously that maternal nicotine exposure increased collagen III mRNA levels and protein expression in foetal monkey lungs, which was indeed coupled to increased pulmonary resistance and decreased expiratory flows [19] probably due to stiffness of the airways. However, the latter study did not reveal whether these changes would remain throughout adult life or be reversible due to further lung development and/or maturation after birth. Our study indicates that effects of maternal smoking on collagen III deposition remain present even after a prolonged period of absence of smoke exposure. Thus both increased smooth muscle area and collagen III deposition may contribute to the observed increased MCh responsiveness in adult offspring exposed to smoke *in utero*.

We found a larger increase in HDM-induced goblet cells in offspring from smoking than from non-smoking mothers. Goblet cell hyperplasia has been shown before to occur in response to active smoke exposure in animal models and COPD patients. Our study is the first report demonstrating increased goblet cell hyperplasia in response to maternal smoke exposure. So far, increased goblet cell numbers have been reported in asthma [20] and the main consequence of goblet cell hyperplasia is hypersecretion of mucin glycoproteins, causing
mucous plugs that block the airways, contributing to increased airway hyperresponsiveness in these patients.

In addition to the effect of smoking during pregnancy on remodeling of the airways, numbers of HDM induced neutrophils and mast cells were increased in offspring from smoking mothers but not in offspring from non-smoking mothers. Several studies have shown that neutrophils are able to contribute to airway smooth muscle cell growth via production of elastase [21]. The mast cells present can also play an important role in proliferation of airway smooth muscle cells, collagen synthesis and hyperresponsiveness [22], mediated via release of e.g. tryptase, histamine and activin A. Although the increase in mast cell numbers in our study was limited, these cells were mainly located in the airway wall. Thus, the combined increase in HDM induced mast cells and neutrophils, could underly changes in airway remodeling in HDM exposed offspring from smoking mothers. The mechanism behind the increase in neutrophils and mast cells, but not in eosinophils, IgE and Th2 cytokines in association with maternal smoking during pregnancy remains unknown.

To further assess whether remodeling could predict MCh responsiveness, we used a linear regression model showing that smooth muscle layer thickness explained a considerable part of MCh responsiveness independently from smoking during pregnancy. Interestingly, addition of numbers of lung tissue eosinophils to the model increased the explained variability of the model substantially. Thus, both increased smooth muscle area and allergic eosinophilic inflammation contributed independently to the severity of MCh responsiveness in adult mice. This may suggest that interventions to reduce airway hyperresponsiveness need to assess both features of asthma. Finally, the addition of smoking during pregnancy to the model also increased the explained variability substantially, indicating an independent effect of smoking during pregnancy.
during pregnancy on the severity of airway hyperresponsiveness. This shows that other factors affected by smoking during pregnancy play a role, like airway geometry or airway size due to changes in lung development in utero, or changes in innate immune responses as proposed by Noakes et al [9]. Nasal obstruction caused by intranasal HDM exposure could also be a factor inducing changes in Penh, thereby affecting the variability in PC300 explained by the different factors in our linear regression model. However, this effect is not fully explanatory since nasal obstruction can not have affected effects of maternal smoking during pregnancy on Penh in PBS exposed offspring.

Besides the serious effects of smoking during pregnancy on lung function and structure in offspring, we provide new insights in effects of HDM exposure with respect to cytokines and cell types involved in the inflammatory response. We show that particularly IL-17 levels strongly increased, which correlated with neutrophil and eosinophil influx and eotaxin production. This is in accordance with literature showing that IL-17 induces neutrophil recruitment [23] and is involved in eosinophil development [24] and eotaxin production [25]. Furthermore, we found support for some of the known effects of HDM exposure, such as increased eosinophils and goblet cells in lung tissue, increased IL-4, IL-5, IL-13 (previously investigated in BALF and at mRNA level) and eotaxin in lung tissue, as well as increased (HDM-specific) serum IgE levels and remodeling of the lung [26,27,28]. Whether changes in cytokine levels are caused by changes in the levels in a specific compartment of the lung (e.g., airways, parenchyma) can not be answered with our current approach using whole lung tissue.
In conclusion, these data indicate that smoking during pregnancy in mice induces several aspects of airway remodeling in adult mice offspring, which likely contribute to increased MCh responsiveness, even after a prolonged period without smoke exposure. Smoking during pregnancy induces remodeling irrespective of allergen exposure but worsens the outcome of the allergic stimulus, resulting in the highest MCh responsiveness in HDM exposed offspring from smoking mothers. Our study provided the opportunity to dissect the relative contribution of maternal smoking during pregnancy from the contribution of postnatal passive smoke exposure, showing long-lasting effects on lung structure and function in mice offspring. Our results support data from epidemiological studies on the association between maternal smoking and childhood or adolescent asthma. Moreover, our data provide suggestive evidence that in particular eosinophilic airway inflammation and increased airway smooth muscle area independently contribute to severity of airway hyperresponsiveness in asthma.
ACKNOWLEDGEMENTS

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Competing interests: none
Reference List


Figure 1: Lung lobe sampling

**Figure 1**

1: trachea  
2: hylus  
3: apical lobe  
4: cardiac lobe  
5: azygous lobe  
6: diaphragmatic lobe  
7: left lobe  
--- : sectional plane

Figure 2: Remodeling in lung tissue from offspring exposed to PBS (circles) or house dust mite (HDM, triangles) from non-smoking mothers (NSM, open symbols) and smoking mothers (SM, closed symbols), expressed as: A) smooth muscle layer thickness around airways, B) collagen III deposition around airways and C) goblet cell hyperplasia in airways. P values shown in the graph are from subgroup analyses. *Italic p values* are from linear regression analysis: “HDM effect” indicates a difference between both PBS exposed groups vs both HDM exposed groups. “SM effect” indicates a difference between both NSM groups
vs both SM groups. “Interaction” indicates that the effect of HDM is greater in offspring from SM.

Figure 2

Figure 3: A) Immunohistochemical staining of airway smooth muscle (red) around airways in lung tissue from offspring from a non-smoking mother and B) a smoking mother. Original magnification 40x.
Figure 4: Methacholine responsiveness from offspring exposed to PBS (circles) or house dust mite (HDM, triangles) from non-smoking mothers (NSM, open symbols) or smoking mothers (SM, closed symbols), expressed as: A) Penh and B) PC300. C) Correlation of PC300 with airway smooth muscle layer. P values shown in the graph are from subgroup analyses. *Italic p values* are from linear regression analysis: “HDM effect” indicates a difference between both PBS exposed groups vs both HDM exposed groups. “SM effect” indicates a difference between both NSM groups vs both SM groups.
Figure 4

A. Graph showing the effect of MCh (mg/ml) on Peth for different groups (NSM PBS, NSM HDM, SM PBS, SM HDM). The HDM effect (NSM and SM groups): p<0.05.

B. Graph showing the effect of PBS and HDM on PC300 (mg/ml). The SM effect: p<0.001, p<0.0001, p<0.018, p=0.009, p<0.0001.

C. Graph showing the relationship between PC300 (mg/ml) and smooth muscle layer (mm²/mm airway). The rho=-0.62, p<0.001.