A search for missing pieces of the puzzle; the development of asthma and atopy

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Chapter 7

Smoke exposure interacts with *ADAM33* polymorphisms in the development of lung function and hyperresponsiveness

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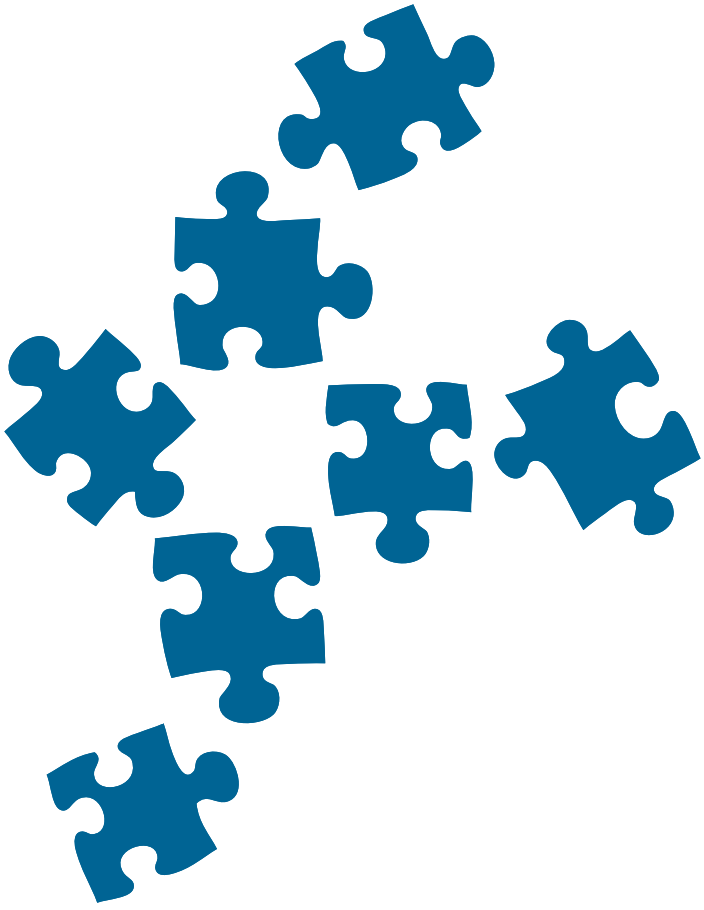

Abstract

Introduction: *ADAM33* is the first identified asthma gene by positional cloning, especially asthma combined with bronchial hyperresponsiveness (BHR). Moreover, *ADAM33* is associated with early life lung function and decline of FEV_1 in the general population. In utero and postnatal cigarette smoke exposure (CSE) is associated with reduced lung function, and development of BHR and asthma. We hypothesized that this may occur via interaction with *ADAM33*.

Aim: To replicate the role of *ADAM33* in childhood lung function and development of BHR and asthma. Furthermore, we investigated gene-environment interaction of *ADAM33* with in utero and postnatal CSE in the Dutch PIAMA cohort.

Methods: Six *ADAM33* SNPs were genotyped. Rint was measured at age 4 and 8 years, FEV_1 and BHR at age 8 years; asthma was based on questionnaire data at age 8.

Results: In the total cohort, the rs511898 A, rs528557 C and rs2280090 A alleles increased the risk to develop asthma (+BHR). There existed interaction between in utero, but not postnatal CSE and the rs528557 and rs3918396 SNPs with respect to development of BHR, the rs3918396 SNP with Rint at age 8 and the rs528557 SNP with FEV_1 % predicted.

Conclusions: We confirm associations between *ADAM33* and the development of asthma (+BHR). This is the first study suggesting that interaction of in utero CSE with *ADAM33* results in reduced lung function and the development of BHR, which needs further confirmation.

Introduction

Impaired lung function and bronchial hyperresponsiveness (BHR) in early childhood are well-known risk factors for the development of asthma later in life. [1-3] The origins of impaired lung function and BHR have not been elucidated so far, but it is plausible that biological processes that underlie in utero and early life lung development are important contributing factors. It is clear that asthma and BHR have strong genetic and environmental components. These environmental factors often start in pregnancy and early infancy, and include a.o. pre and postnatal cigarette smoke exposure (CSE). [4,5]

ADAM33 is the first asthma gene identified by positional cloning. [6] This gene was in particular found by combining a doctor's diagnosis of asthma with the presence of BHR. Interestingly, *ADAM33* SNPs have also been associated with impaired early life lung function at age 3-5 years and with FEV_1 decline in both adult asthma patients and the general population. [7-10]

Multiple ADAM33 protein isoforms exist in human embryonic lung [11], as is the case in the lungs of adult asthma patients as well. [11,12] Not only airway smooth muscle cells but also primitive mesenchymal cells of the fetal lung, forming a cuff at the end of the growing lung bud, stain with ADAM33. [11] Thus, ADAM33 may well play a role in orchestrating branching morphogenesis and polymorphic variations in the *ADAM33* gene may influence the subsequent susceptibility of the lung to asthma.

Several epidemiologic studies have provided evidence that CSE in utero and early life is associated with reduced lung function and constitutes a risk factor for the development of asthma and BHR. [13,14] It is thus conceivable that in utero and early life CSE interact with genes important in asthma development, thereby affecting in utero and early life lung development, airway remodelling, and subsequent development of early life lung function changes and asthma.

We studied the role of *ADAM33* in early life lung function, BHR and asthma in a Dutch birth cohort (PIAMA) that allowed us to prospectively follow environmental exposure, symptoms and lung function, including BHR measurements up to 8 years of age. We analysed whether CSE in utero and in early life, in combination with *ADAM33* genotypes influences early life lung function, BHR and asthma.

Methods

Study design

Prevention and Incidence of Asthma and Mite Allergy (PIAMA) is a birth cohort study that started in 1996. At baseline, 4146 children were included, 1327 high-risk children (with a positive maternal history for atopy) and 2819 children of non-allergic mothers. The children were recruited during the first trimester of pregnancy. Parents completed annual questionnaires derived from the International Study of Asthma and Allergies in Childhood (ISAAC) [15] about allergic symptoms in the child, information about general health, indoor environment like CSE, socio-economic characteristics, lifestyle, breastfeeding and

demographic factors. All 1327 high risk children and a random sample of 663 low risk children were selected for medical examinations. Resistance interrupter technique (Rint; Micro Medical, Rochester, Kent, UK) measurements were obtained at age 4 yrs, and Rint as well as spirometry and BHR to methacholinebromide were measured at age 8 yrs. The Rint technique measures interrupter resistance, which is similar to airway resistance. It is a non invasive method that requires only tidal breathing. Therefore it can easily be performed in pre-school children. [16-18] A further description of the study protocol has been published previously. [19] All parents provided written informed consent.

DNA

Genomic DNA was extracted from buccal swabs or blood [20] at age 4 yrs (n=1037). Genetic studies were approved by local medical ethics committees of participating institutes. Six Single Nucleotide Polymorphisms (SNPs), i.e. rs511898, rs612709, rs3918396, rs528557, rs2280090 and rs2787094 were analysed. These SNPs were chosen since they were associated with asthma and lung function in the Dutch population. [8,9,21] Children with a non-Caucasian ethnicity were excluded (n=51).

For all SNPs, primers and probes were ordered from Applied Biosystems TaqMan® SNP Genotyping Assays (Nieuwekerk aan de IJssel, The Netherlands), using the Assay-by-Design service for which we provided sequences, since the rs528557 SNP previously failed [8] we used primers and probes with the Primer Express package and obtained primers from Biolegio (Malden, The Netherlands) and probes from Applied Biosystems. Sequences of all primers and probes are shown previously. [8] Reactions were performed in 5 µl volumes of 10 ng DNA, 1x Taqman Universal Mastermix (Applied Biosystems), 200 nM probe and 900 nM primer. Cycling conditions on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) were 10 min 95°C followed by 40 cycles of 15 seconds 95°C and 1 minute 60°C. End-point fluorescence was measured immediately after cycling. Alleles were assigned using SDS 2.1 software (Applied Biosystems). Genotyping call rates ranged from 89%-94.5%, comparable with the study by Schedel *et al.* [22]

Phenotypes

Rint measurements were performed as described previously. [23] Rint measurements at age 4 and 8 yrs and FEV_1 % predicted at age 8 yrs were used as lung function variables. BHR was defined as a fall of ≥ 20% in FEV_1 after inhalation of a maximum of 0.62 mg methacholinebromide. We used two definitions of asthma; 1) Asthma at age 8, based on at least 1 episode of wheeze and/or dyspnea in the last year and/or the use of inhaled steroids; and 2) Asthma in combination with BHR.

In utero CSE was defined as a confirmative answer to the question "do you smoke cigarettes?" or "have you been smoking, and when did you quit smoking?" asked to the mother during the third trimester of pregnancy. CSE in the first year of life was defined as a confirmative answer to the question "does anyone smoke, at least once a week, in the home?" asked to the parents when the child was 1 year of age. Lung function, BHR, asthma and asthma with BHR were analyzed in the total group and in children with in utero CSE and CSE at age 1 year separately.

Statistical analysis

Lung function parameters were normally distributed and analyzed using analysis of variance (ANOVA) and linear regression. BHR, asthma and asthma with BHR were analysed using chi-squared tests and logistic regression. An interaction term of genotype and CSE (in utero and in the first year of life) was included into linear and logistic regression models. All analyses were corrected for gender. All genotypes were analysed in the best fitting, dominant model, i.e. by comparing children with one or two copies of the minor alleles with those carrying no copy. Analyses of *ADAM33* in combination with CSE analyses were corrected for gender, atopy or asthma of the mother, atopy or asthma of the father, siblings, breastfeeding, pet and in utero CSE or CSE at age 1 year. Calculations were performed using SPSS 14.0 statistical software.
Benjamini and Hochberg False Discovery Rate (FDR) was used to correct for multiple testing. [24]

Results

DNA and Rint values at age 4 yrs were available in 768 children, DNA and spirometry at age 8 yrs were available in 746 children. Since inhalation medication taken before Rint measurement could influence the outcome, we excluded children who took inhalation medication <12 hours prior to Rint measurements (n=25 at 4 yrs and n=7 at 8 years).
General characteristics of the study population are shown in Table 1.

Table 1. Characteristics

Characteristics	Genotyped Children
Number	1037
Boys (%)	532 (51.2)
Family history	
Atopy mother (%)	690 (66.5)
Atopy father (%)	327 (31.6)
Environmental exposures	
In utero smoke exposure (%)	154 (15.0)
Smoke exposure in first year (%)	230 (23.2)
Lung function	
number	779
FEV_1% predicted mean (SD)	107.0 (11.5)
Methacholine Provocation	
number	692
BHR (%)	313 (45.2)
Asthma (%)	153 (16.5)
Asthma with BHR# (%)	72 (10.2)

Within children with complete methacholine provocation measurements

Genotyping

All SNPs were in Hardy Weinberg equilibrium (P>0.05). There existed strong Linkage Disequilibrium (LD) between the SNPs rs511898, rs612709, rs3918396, rs528557 and rs2280090, (D'>0.8), (Figure 1). Allele frequencies are described in Table 2.

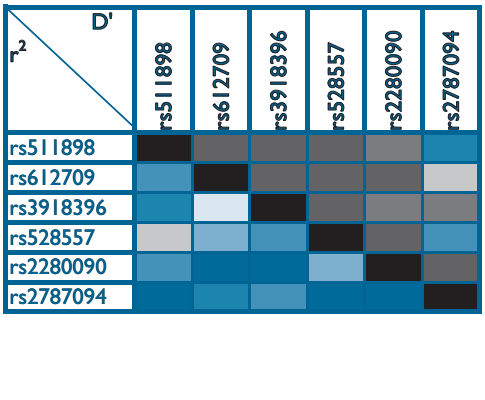

Figure 1. LD structure of *ADAM33* SNPs in the PIAMA cohort (r^2 below and D' above the diagonal)

Table 2. *ADAM33* SNPs and their minor allele frequency in PIAMA cohort

rs number	Alleles*	SNP**	Location	MAF***
rs511898	G/A	F+1	Catalytic domain	0.35
rs612709	C/T	Q-1	Cysteine rich domain	0.13
rs3918396	G/A	S_1	Trans membrane domain	0.09
rs528557	G/C	S_2	Trans membrane domain	0.28
rs2280090	G/A	T_2	Cytoplasmic domain	0.14
rs2787094	C/G	V_4	3'UTR	0.25

*First allele, wild type.
** SNP numbering as shown in previous publications[6;8;9;21]
**Minor Allele Frequency

Association analysis

Lung function

No association was found between SNPs in *ADAM33* and FEV_1 and Rint in the total cohort (data not shown).

BHR, asthma and asthma combined with BHR

No association was found between SNPs in *ADAM33* and BHR in the total cohort. Children carrying the rs511898 A allele or rs528557 C allele were more likely to develop asthma compared to children with the rs511898 GG or rs528557 GG genotypes. Children carrying the rs528557 C allele or rs2280090 A allele were more likely to develop asthma in combination with BHR compared to the rs528557 GG or rs2280090 GG genotypes (Table 3).

Table 3. Association of *ADAM33* SNPs with asthma and asthma combined with BHR in the PIAMA cohort

SNP	Allele	N*	BHR	Asthma	Asthma with BHR
rs511898	GG	397	1	**1**	1
	GA	397	1.1 (0.8-1.5)	**1.4 (1.0-2.1)**	1.5 (0.8-2.8)
	AA	122	1.2 (0.8-2.0)	**1.7 (1.0-2.9)**	2.0 (0.9-4.3)
rs612709	CC	684	1	1	1
	CT	216	0.8 (0.5-1.2)	1.2 (0.8-1.9)	1.1 (0.6-2.1)
	TT	11	0.2 (0.0-1.9)	1.9 (0.5-7.4)	1.2 (0.1-12.2)
rs3918396	GG	745	1	1	1
	GA	153	1.0 (0.6-1.5)	1.4 (0.9-2.3)	1.2 (0.6-2.4)
	AA	9	0.4 (0.0-3.9)	0.7 (0.8-5.3)	1.3 (0.1-13.1)
rs528557	GG	458	1	**1**	**1**
	GC	330	1.1 (0.8-1.6)	**1.4 (0.9-2.0)**	**1.8 (1.0-3.2)**
	CC	73	0.8 (0.4-1.5)	**1.9 (1.0-3.6)**	**1.8 (0.1-4.6)**
rs2280090	GG	659	1	1	**1**
	GA	219	1.2 (0.8-1.7)	1.5 (1.0-2.3)	**1.7 (0.9-3.2)**
	AA	17	2.6 (0.8-8.9)	1.0 (0.2-4.4)	**3.8 (0.6-23.9)**
rs2787094	CC	520	1	1	1
	CG	334	0.9 (0.7-1.3)	(0.7-1.5)	1.0 (0.6-1.8)
	GG	59	1.1 (0.8-1.5)	0.4 (0.1-1.0)	0.9 (0.3-2.8)

*** numbers genotyped**
Bold: P ≤ 0.05, in a dominant model **Odds ratios and (95% confidence intervals),**
wild type is reference group.
BHR: Bronchial hyperresponsiveness

Interaction of ADAM33 genotypes and in utero smoke exposure

FEV$_1$: In children whose mother smoked during pregnancy, the rs528557 C allele was associated with lower FEV$_1$ % predicted compared to children with the GG genotype. In children whose mother did not smoke during pregnancy, the rs528557 C allele was associated with higher FEV$_1$ % predicted levels compared to children with the GG genotype (interaction term p=0.006, figure 2A). A similar trend was present for rs3918396 (smoke exposed children mean FEV$_1$% predicted 104.0% (A allele) vs. 105.9% (GG genotype) and children not exposed to smoke in utero mean FEV$_1$% 108.1% (A allele) vs. 106.9% (GG genotype))

Rint: A similar type of interaction as seen with FEV$_1$ was present for Rint at age 8 years and rs3918396 SNP, showing increased Rint values in children with the rs3918396 A allele in children whose mother smoked during pregnancy (interaction term p=0.02). A similar trend was present for rs528557. No interaction was present for Rint at age 4 yrs and in utero CSE.

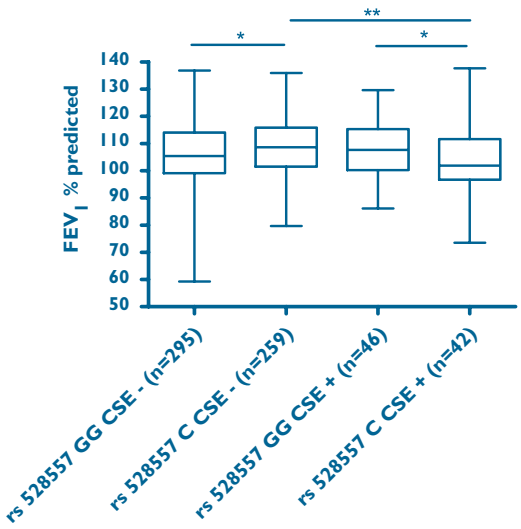

Figure 2A
Interaction of *ADAM33* SNPs and in utero CSE in level of FEV_1 at 8 years of age

CSE + = Smoke exposure during pregnancy
CSE - = No smoke exposure during pregnancy
* P ≤ 0.05, **P ≤ 0.01

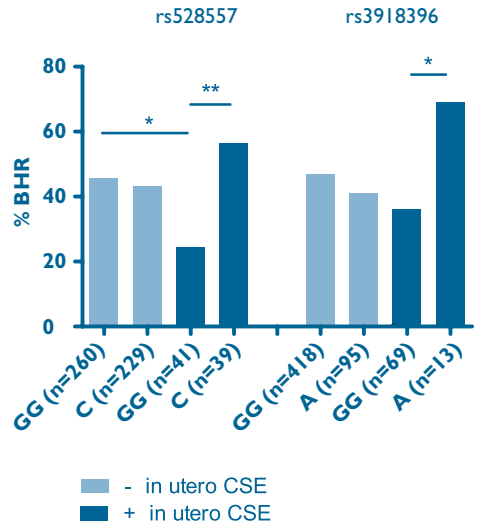

Figure 2B
Interaction of *ADAM33* SNPs and in utero CSE in the development of BHR at 8 years of age

* P ≤ 0.05, **P ≤ 0.01

BHR: We found a significant interaction between in utero CSE and the *ADAM*33 SNPs rs528557 and rs3918396 with respect to the development of BHR. Within children whose mother smoked during pregnancy, the rs528557 C or rs3918396 A allele increased the risk for BHR development compared to rs528557 GG or rs3918396 GG (rs528557 Odds Ratio (OR) 4.0; 95% Confidence Interval (CI) 1.5-10.4 p-value interaction term = 0.004; rs3918396 OR 4.0; 95% CI 1.1-14.2, p-value interaction term = 0.02). This genotype effect was not found in children whose mothers did not smoke during pregnancy. Conversely, in children with the rs528557 GG or rs3918396 GG genotype in utero CSE decreased the risk of BHR, compared to all the children

who were not exposed to cigarette smoke (figure 2B). The same trend was present for the rs511898 SNP (data not shown).
The groups became too small to analyse an interaction between in utero CSE and *ADAM33* genotypes with respect to asthma development.

Interaction of ADAM33 genotypes and smoke exposure in the first year of life

No significant interaction was found between CSE in the first year of life and the *ADAM33* genotypes with respect to the development of BHR and level of lung function at the age of 4 and 8 years (data not shown).

Correction for multiple testing

None of the above described analyses reached significance after correction for multiple testing.

Discussion

Our results confirm the previously reported association of *ADAM33* SNPs with asthma as well as asthma combined with BHR. [6] We extend previous findings in that we provide data suggesting that gene-environment interaction of *ADAM33* genotypes and in utero CSE exist with respect to the level of lung function and the development of BHR at age 8 years, an effect not observed with CSE in the first year of life.
ADAM33 is expressed in embryonic lung tissue on mesenchymal cells [11] which are responsible for branching, a lung developmental phase that continues until $\pm$ 17 weeks of gestation. [12] The respiratory airways and future respiratory units develop during the third trimester. This is followed by an alveolar phase of lung development, which proceeds into the first 2-3 years of postnatal life, during which the number of alveoli increases. [25] ADAM33 may thus play a role especially in prenatal lung development, for instance by orchestrating branching morphogenesis. In this way it can affect the development of lung function, BHR, and asthma. Furthermore soluble ADAM33 in bronchial alveolar lavage fluid is increased in asthma patients and correlates with disease severity and reduced lung function. [26] A recent *in vitro* and *in vivo* study showed that soluble ADAM33 causes endothelial cell differentiation and affects angiogenesis in embryonic lung tissue. [27] Angiogenesis is considered an important aspect of tissue inflammation and remodelling. How would in utero CSE influence ADAM33 shedding? This may well run by increased levels of TGF-β by smoke exposure [28], since Puxeddu *et al* showed that TGF-β_2 enhances ADAM33 shedding from cells expressing ADAM33. [27]
A recent report on another prospective birth cohort study, the MAAS cohort, showed at the age of 5 yrs that four SNPs in *ADAM33* were associated with reduced FEV_1 (rs511898, rs3918395, rs2280091, and rs2280090; $p < 0.04$). [7] Our study extends the latter observations. It appears that smoking by mothers during pregnancy is an important contributor to the effects of *ADAM33* on lung function, since the rs528557 SNP was associated with reduced FEV_1 in children with in utero CSE, with an opposite

effect on FEV_1 in children not exposed. Interestingly, the percentage of children with in utero CSE in the MAAS cohort is much higher than in the PIAMA cohort (respectively 22.3% and 15%). [29] It is therefore plausible that the association of *ADAM33* with reduced lung function in the MAAS cohort is predominantly present in the children whose mother smoked during pregnancy. In addition to the interaction of in utero CSE with *ADAM33* SNPs on lung function we found interaction on the development of BHR as well. In utero CSE is known to influence lung function development and to increase the risk to develop asthma and BHR. [13,30-32] Our data suggests that this association may at least in part be due to an interaction with *ADAM33*.

Of interest and requiring further functional study is the observation that the rs528557 GG and rs3918396 GG genotype have a protective effect on BHR when in utero smoke exposure occurred. It is yet unclear, and has to be determined whether this implies an effect on smooth muscle proliferation, epithelial integrity and/or airway branching.

The interaction of *ADAM33* with in utero CSE and CSE in childhood was also analysed by Schedel *et al* [22] in children from the ISAAC II and MAS cohorts. They did not find evidence for interaction of *ADAM33* SNPs with asthma, BHR, or lung function. The difference in outcome between their and our study may be caused by, up to now unidentified environmental factors, or by methodological differences between the studies (e.g. methacholine provocation versus histamine and cold air provocation).

We replicate the initial association of *ADAM33* genotypes with asthma and asthma with BHR as reported by van Eerdewegh [6], thereby confirming the importance of ADAM33 in asthma. Not all studies have confirmed association of *ADAM33* SNPs and asthma [33,34], and the studies that do replicate the results often find association with different SNPs or different alleles. There are several explanations for this discrepancy. First, there is variability in the definition of asthma used in the different studies. We defined asthma at age 8 based on at least 1 episode of wheeze or dyspnea in the last year and/or the use of inhaled steroids. Furthermore we analysed asthma in combination with BHR, since linkage became stronger combining asthma and BHR in the initial study by van Eerdewegh. [6] Some studies with negative results analysed asthma solely based on a doctor's diagnosis. [34,35] Secondly, a meta-analysis showed that *ADAM33* has only a moderate effect on the development of asthma (odds ratio of 1.4 for SNPs known to date). [36] Therefore, as previously suggested [22,36], it is plausible that even with large datasets the chance of finding associations with such a moderate risk is limited. Thirdly, it may be possible that discrepant results between the studies are due to differences in environmental factors and population heterogeneity. We show in our study that the effects of *ADAM33* polymorphisms on lung function and BHR in the total population were only present in combination with CSE, suggesting that *ADAM33* is a gene that can be influenced by environmental factors. Finally, it is also possible that the associated SNPs are not the causative ones, but in linkage disequilibrium with the causative SNPs within *ADAM33* or in genes located near *ADAM33*. This might also explain why different SNPs and alleles are associated in the studies. This phenomenon is also known as loose genotypic replication. [37]

This study has some pitfalls, which should be acknowledged. First our study was

based on the selection of SNPs that were previously associated with lung function and asthma. We found strong LD between several genotyped SNPs, so it is plausible that the associations we observed with different SNPs and similar phenotypes is caused by one single SNP that is in strong LD with the other SNPs. Therefore a full description of *ADAM*33 in terms of polymorphic variations and LD structure is needed. Secondly, our results did not remain significant after correction for multiple testing. However, since this is a hypothesis testing study and (part of) the results are a replication of previously published data we feel that correction for multiple testing will underestimate the results. Notwithstanding this we do recognise that our results might be due to chance and that other studies have to replicate especially our new findings. Furthermore since mothers who are smoking during pregnancy are likely to smoke after pregnancy it is difficult to study the independent effect of CSE. However several studies have shown a strong effect of especially in utero smoke exposure on lung function and asthma development. [30-32] In our study, the majority of the children who were exposed in the first year of life (59%) were not exposed during pregnancy. CSE in the first year of life only did not show an interaction with *ADAM*33.

We conclude that *ADAM*33 genotypes increase the risk to develop asthma and asthma in combination with BHR. Furthermore, we have provided data that need confirmation, but suggest that gene-environment interaction of *ADAM*33 genotypes and in utero cigarette smoke exposure with the level of early life lung function and development of BHR. In combination with data in the literature, our study highlights that it is important to investigate cigarette smoking during pregnancy as a risk factor for early life lung function and BHR development, in interaction with genetic factors like *ADAM*33.

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