

Received Date : 05-Mar-2013
Revised Date : 10-Jun-2013
Accepted Date : 14-Jul-2013
Article type : Original Article-Clinical Mechanisms in Allergic Disease

VEGFA variants are associated with pre-school lung function but not neonatal lung function

Eskil Kreiner-Møller, MD^{1,2}; Bo Chawes, MD, PhD^{1,2}; Nadja Hawwa Vissing, MD^{1,2}; Gerard H. Koppelman, MD, PhD³; Dirkje S. Postma, MD, PhD⁴; Jonna Skov Madsen, MD, PhD⁵; Dorte Aalund Olsen, MSc⁵; Florent Baty^{1,2}; Judith M. Vonk, PhD⁶; Marjan Kerkhof, MD, PhD⁶; Patrick Sleiman MSc, PhD⁷; Hakon Hakonarsson, MD, PhD⁷; Li Juel Mortensen^{1,2}; Porntiva Poorisrisak, MD, PhD^{1,2}; Hans Bisgaard, MD, DMSci^{1,2}; Klaus Bønnelykke, MD, PhD^{1,2}

1: COPSAC: Copenhagen Prospective Studies on Asthma in Childhood; Copenhagen University Hospital, Gentofte, Denmark.

2: The Danish Pediatric Asthma Center, Copenhagen University Hospital, Gentofte

3: Department of Pediatric Pulmonology and Pediatric Allergology, Beatrix Children's Hospital, University Medical Center Groningen, Griac research institute, University of Groningen, the Netherlands

4: Dept. of Pulmonary Medicine and Tuberculosis; University Medical Center Groningen, The Netherlands

5: Dept. of Clinical Biochemistry, Lillebaelt Hospital, Vejle, Denmark

6: Department of Epidemiology, University Medical Center Groningen, Griac research institute, University of Groningen, the Netherlands

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.12188

This article is protected by copyright. All rights reserved.

7: Center for Applied Genomics and Division of Human Genetics, The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Correspondence:

Eskil Kreiner-Møller

Copenhagen Prospective Studies on Asthma in Childhood

Danish Pediatric Asthma Center

Health Sciences, University of Copenhagen

Copenhagen University Hospital, Gentofte

Ledreborg Allé 34

2820 Gentofte

Denmark

Tel: +45 39 77 73 60

E-mail: eskil.kreiner@dbac.dk

Website: www.copsac.com

Authors Contributions

KB, EKM and HB designed the study. HB is responsible for design, initiation and conduct of the COPSAC cohort. EKM drafted the manuscript. EKM, KB, BC, NHV, GHK, DSP, JSM, JMV, MK, PS, LJM, PP, HH contributed to data acquisition, analysis and interpretation. All co-authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript.

The corresponding author had full access to the data and had final responsibility for the decision to submit for publication.

This article is protected by copyright. All rights reserved.

Funding

COPSAC is funded by private and public research funds listed on www.copsac.com. The Lundbeck Foundation; The Danish Strategic Research Council; the Pharmacy Foundation of 1991; Augustinus Foundation; the Danish Medical Research Council and The Danish Pediatric Asthma Centre provided the core support for COPSAC research center. No pharmaceutical company was involved in the study. The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript.

The PIAMA study is supported by the Dutch Asthma Foundation (grant 3.4.01.26, 3.2.06.022, 37 3.4.09.081 and 3.2.10.085CO), the ZonMw the ZON-MW Netherlands Organization for Health Research and Development (grant 912-03-031), the Stichting Astmabestrijding and the Ministry of the Environment.

Abstract

Background

Vascular endothelial growth factor (VEGF) is implicated in airway remodeling and asthma development. We studied *VEGFA* gene variants and plasma levels and the development of lung function, bronchial hyperresponsiveness and asthma in childhood.

Methods

We analyzed 13 SNPs in the *VEGFA* gene in 411 children from the COPSAC2000 high-risk birth cohort. Asthma was diagnosed prospectively and lung function measurements were obtained at birth and six years of age. Plasma VEGF levels were measured at 18 months of age. We used a Bonferroni adjusted significance level. Findings were replicated in the PIAMA birth cohort at age 8.

Results

At age six, three SNPs from the same linkage block, were associated with FEV1 (rs699947, $P=1.31E-05$), independent of asthma, and there were suggestive associations between FEV1/FVC ratio and rs833052 and MMEF and rs6900017. Replication in the PIAMA cohort showed borderline association between FEV1 and rs699947 and significant meta-analysis result

SNPs upstream and nearby rs699947 were nominally associated with VEGF plasma levels. VEGF levels were not associated with asthmatic symptoms or lung function measures.

Conclusion

VEGF gene variants are associated with lung function at school-age but not at birth suggesting a role of *VEGF* in postnatal lung function development.

Keywords, MeSH:

Vascular Endothelial Growth Factor A; Child; Infant; Respiratory Function Tests; Asthma

Abbreviations

COPSAC - the Copenhagen Prospective Studies on Asthma in Childhood

FEF50 - forced expiratory flow at 50% of the functional vital capacity

FEV0.5 - forced expiratory volume in 0.5 seconds

FEV1 - forced expiratory volume in 1 seconds

MMEF - maximal mid-expiratory flow

PD15 - provocative dose causing a 15% drop in lung function

PD20 - provocative dose causing a 20% drop in lung function

PIAMA - the Prevention and Incidence of Asthma and Mite Allergy birth cohort

VEGF - Vascular endothelial growth factor

INTRODUCTION

Asthma is a heterogeneous complex genetic inflammatory disease of the airways[1] and is the most common chronic disease in children. The disease is associated with substantial structural alterations in the airways[2] that occur early in life[3] independent of atopy[4]. Changes in the vascularization of the airways, such as increased vessel number, vessel size, vascular surface area and vascular leakage play a pivotal role in the airway remodeling[5] and important correlation between these alterations and disease severity is evident[6]. Vascular endothelial growth factor (VEGF) is suspected to be an important factor in this process inducing these vascular changes[5, 7] but is also involved in the Th2-mediated inflammatory response[6] and is hence of special interest when investigating the pathogenesis of childhood asthma.

Few studies have investigated the role of *VEGFA* gene variants and *VEGF* levels in the inception of childhood asthma: Common variants in the *VEGFA* gene have been suggested to be of importance for the development of asthma and increased airway hyperresponsiveness[8, 9]; VEGF levels in sputum and plasma have been associated with the burden of asthma symptoms[10-13] and VEGF serum levels in children are associated with severity of bronchial hyperresponsiveness[13], but, not conclusive or consistent.[3, 8, 9, 14] A recent study by Simpson and colleagues suggest an association between lung function measured prospectively in children and a genetic variant in the *VEGFA* gene, rs3025028, between exon 7 and 8 thought to influence splicing of active and inhibitory isoforms of VEGFA.[15]

We hypothesized that polymorphisms in the *VEGFA* gene and *VEGF* levels are associated with the development of asthma and related objective measurements such as lung function and airway hyperresponsiveness in children. Therefore, we investigated the role of *VEGF*

polymorphisms and *VEGF* plasma levels (at 18 months of age) on asthma and lung function prospectively from birth to age 6 years in children from the clinical birth cohort study the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC). [16] Findings were replicated in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort up to age 8. [17]

METHOD

Study population, COPSAC

The COPSAC study is a single-centre, prospective clinical birth-cohort of 411 Caucasian children born to asthmatic mothers, with the primary objective of investigating gene-environment-phenotype interactions in the development of atopic diseases. Children attended the research unit at age 1 month and subsequently every 6 months for scheduled clinical investigations. Additional visits were arranged immediately upon onset of any respiratory symptoms.[16] Key exclusion criteria were gestational age <36 weeks, severe congenital abnormality, neonatal mechanical ventilation, and symptoms of lower airway infection prior to inclusion. The study was approved by the Copenhagen Ethics Committee (KF 01-289/96) and the Danish Data Protection Agency (2008-41-1754). Informed consent was obtained from the parents at enrolment.[16]

Genotyping of the *VEGFA* gene (chromosome 6, 43830931-43877201) was performed in both the child and parents, by high throughput genome-wide SNP genotyping, using the Illumina Infinium II HumanHap550 BeadChip technology (Illumina, San Diego) at the Center for Applied Genomics at CHOP, as described previously.[18] 13 SNPs were genotyped in this region corresponding to 30% of the polymorphic unambiguous SNPs genotyped in the Utah residents with ancestry from northern and western Europe population

(CEU) from the Hapmap study tagging pairwise 17 other SNPs (with $r^2 > 0.5$) within this region leaving 14 SNPs untaggable.[19]

Lung function measurements in COPSAC were obtained at 1 month of age by the rapid raised volume thoracoabdominal compression technique (RVRTC) assessing forced expiratory volume in 0.5 seconds (FEV0.5) and forced expiratory flow at 50% of the functional vital capacity (FEF50) both at baseline and during methacholine challenge with quadrupling dose steps. The provocative dose causing a 15% drop in lung function and transcutaneous oxygen saturation (PtcO₂) (PD15) was estimated from the dose response curves fitted with a logistic function. Our previous sensitivity analyses showed PtcO₂ to be more sensitive than any of the forced flow indices of infant spirometry; therefore PtcO₂ PD15 was used in the analyses. Spirometric measurements of FEV1, FVC and maximal mid-expiratory flow (MMEF) was assessed at six years of age before and during methacholine challenge measuring the provocative dose resulting in a 20 percent drop in FEV1 (PD20) as the test outcome. The lung function measurements are described in the online supplement and elsewhere in detail.[16, 20-23]

Asthmatic symptoms were evaluated, treated and classified strictly based on predefined standard operating procedures and treatment algorithms. Symptoms were solely evaluated by the COPSAC doctors according to the GINA guidelines as previously detailed based on symptom diaries and the need of intermittent rescue use of inhaled β_2 -agonist: Troublesome lung symptoms (TLS) were defined as breathlessness, wheeze or a whistling sound recurrent cough severely affecting, sleep, activity and/or the well being of the child recorded in the diaries and reviewed by the research unit doctors. Recurrent troublesome lung symptoms (TLS) in three consecutive days five times within 6 months or troublesome lung symptoms

for four consecutive weeks or one episode of asthmatic symptoms requiring hospitalization) were treated with a 3-month course of inhaled corticosteroids. Response to treatment and presence of a relapse (two episodes of troublesome lung symptoms in three consecutive days within three months or two consecutive weeks) within the first year after the three months course was the diagnostic criteria for asthma. Definitions, standard operating procedures and treatment algorithms are described in further details elsewhere.[24-26] Recurrent troublesome lung symptoms, as defined above, within the first 18 months of age and current asthma at age 6-7 were used as outcomes.

Total VEGF levels were determined in plasma aliquots from blood samples drawn at 18 months of age. [16] A commercially available enzyme-linked immunosorbent assay (cat. no. DVE00, R&D Systems, Minneapolis, MN, USA) was used and performed according to the manufacturer's protocol. The ELISA kit measures both the pro-angiogenic isoform VEGF165 and the anti-angiogenic isoform VEGF165b. The detection range and the detection limit of the assay were 31.2-2000pg/ml and 9.0pg/ml, respectively. Interassay coefficients of variation on three levels were between 7% and 14%.

Smoking exposure was determined by measurements of Nicotine in a tuft of hair at one year of age as described in detail earlier.[27]

Replication population, PIAMA

The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study is a birth cohort study that started in 1996. At baseline, 4146 children were included, 1327 high-risk children (born to allergic mothers) and 2819 children of non-allergic mothers. The children were

recruited during the first trimester of pregnancy. All 1327 high-risk children and a random sample of 663 low-risk children were selected for medical examinations.

Genotypes on *VEGF* were obtained from 956 Caucasian children. At age 8 years, spirometry and bronchial hyperresponsiveness were measured in 724 and 645 of these children, respectively. Bronchial hyperresponsiveness was defined as a fall of $\geq 20\%$ in FEV1 after inhalation of a maximum of 0.62 mg methacholine bromide.

Genotyping in the PIAMA study was performed by Competitive Allele-Specific PCR using KASParTM genotyping chemistry (K-Biosciences, Herts, UK) as described previously.[28] Information on respiratory health was obtained from annual questionnaires completed by the parents at the time of the child's birthday until the age of 8 years. "Doctor-diagnosed asthma" was defined as asthma ever diagnosed by a doctor and asthma present in the last 12 months. A detailed description of the study design has been published previously.[17]

Statistical analyses

Standard genetic description and quality control was carried out using PLINK[29] as described in the online supplementary. The Linkage Disequilibrium and haplotype block plot was made in Haploview using the Gabriel method for haplotype block definition.[30]

Statistical analyses and plots were performed using the Stats, Graphics, Rmeta and Survival packages in the R statistical software.[31] For single marker analysis we used an additive genetic model in a linear or logistic regression analysis: Neonatal outcomes tested in linear regression models were FEV0.5, FEF50 and PD15. Outcomes at age 6 in COPSAC and at age 8 in PIAMA tested in linear regression models were FEV1, FEV1/FVC, MMEF (only COPSAC) and PD20. In COPSAC asthma time to onset was modeled in Kaplan Meier graphs. A logistic regression and a generalized estimation equation were used to analyze asthma in COPSAC and PIAMA respectively. The models were adjusted for height and sex,

when analyzing FEV1, MMEF and FVC. Neonatal PD15, MMEF at age 6 and PD20 at age 6 and 8 (PIAMA) were log10 transformed to normalize residuals. To assess the effect of the SNPs on the development of lung function we adjusted the analyses at age 6 for the measured inborn lung function: the analyses of FEV1 and MMEF were adjusted for neonatal FEV0.5 and FEF50 respectively.

Analyses of the main lung function outcomes (FEV1, FEV1/FVC, FVC and MMEF) and statistically associated SNPs were studied more in detail and were stratified on asthma status, tested for interaction with asthma (Table E2), adjusted for smoking exposure and tested for smoking interaction (Table E3).

Linear regressions were used to assess association between VEGF levels and SNPs, lung function measurements and asthma. VEGF levels were log10 transformed. The VEGF level analyses were analyzed with and without adjustment for steroid use at time of measurement (from 3 weeks before until 3 weeks after).

We used an alpha level of $P < 0.05$ and corrected for multiple testing using the Bonferroni correction as level of significance ($P < 3.80e-03$). SNPs that were significantly associated with any outcomes were replicated in the PIAMA cohort. The discovery and replicated signals were meta-analyzed using fixed effects and the reciprocal of the estimated variances as weights.

Functional analyses of the SNPs

We analyzed the 13 SNPs of interest using the FastSNP online tool to analyze each SNPs potential functional effect on gene product. The tool combines several online databases to predict the functional effect of the input SNP. This prediction is used to categorize the SNP using a decision tree to give an estimate of the possible change in functional effect and gives

a score between 0 and 5 to how likely the SNP will affect the gene product.[32] The tool was accessed 24th of august 2011.

RESULTS

Baseline

Of the original 411 children included 336 children were investigated at age 6 and 14 per cent (47/336) had current asthma. Lung function at 1 month of age was obtained in 404 children. Between age 5 and 6.5 years, 318 children had completed a lung function measurement. VEGF levels were determined in 280 children from blood samples (plasma aliquots) drawn at 18 months of age. (Figure 1) After quality control of the genotype data 365 children had data on the 13 SNPs in the *VEGFA* gene. Descriptives of all genotyped children in the discovery and replication cohorts are presented in table 1 and minor allele frequencies, genotype distributions and call-rates in table E1 in the online supplement. All genotyped trios were used to calculate linkage disequilibrium and haplotype block structure. We identified three distinct haplotype blocks in this region. (Figure 2) These blocks are comparable to haplotype blocks in the Hapmap data (CEU population) (Figure E1).[19]

Discovery, Lung function and bronchial responsiveness

The SNPs in the middle LD block were highly associated with FEV1 at six years of age with the top-SNP rs699947 (Beta=-0.048 per extra A allele, SE=0.011, P= 1.31e-05) (Table 2, Figure 2). Adjusting for neonatal FEV0.5 did not alter this association.

We found a nominal significant association between FEV1/FVC at age six and rs833052 (Beta= -0.023 per extra A allele, SE= 0.008, P= 4.09e-03) (Table 2, Figure 2) and between FVC and rs699947 (Beta= -0.0334 per extra A allele, SE= 0.0131, P=1.15e-02) (Table 2, Figure E3) and MMEF and rs6900017 from the third linkage disequilibrium block (Beta= -

0.044 per extra T allele, SE= 0.017, P= 9.17e-03) (Table 2, Figure 2). These associations were however not below the multiple testing significance threshold. The association between MMEF and rs6900017 was not altered when furthermore adjusting for neonatal FEF50. There was no evidence of interaction between the top SNP rs699947 and asthma or smoking exposure in the stratified, adjusted or interaction analyses (Table E2 and E3). These analyses though revealed some evidence of a possible interaction for the two SNPs rs833052 and rs6900017 and asthma and smoking exposure. (Table E2 and E3)

Neonatal lung function measures and hyperresponsiveness at birth and at age six years were not associated with any of the SNPs in the *VEGFA* gene (Figure E4-6).

Replication, Lung function and bronchial responsiveness

We selected rs699947 and the two SNPs rs833052 and rs6900017 for replication in the PIAMA cohort. The findings of rs699947 showed a trend to FEV1 adjusted for height and sex (Beta=-0.018 per extra A allele, SE=0.01, P= 0.067) and significant association for PD20 at 8 years of age (Beta=-0.073 per extra A allele, SE= 0.33, P= 0.025). There was furthermore a trend between FEV1/FVC and rs6900017 (Beta = -0.01, SE = 0.01, P = 0.07). No association was found between FVC, FEV1/FVC and PD20 and rs833052 and rs699947 and FEV1/FVC and for the SNP rs6900017 and FEV1 and PD20 (Table 2).

Meta-analysis of discovery and replication results

The results of the meta-analysis revealed a significant association between rs699947 and FEV1 (Beta=-0.032 per extra A allele, SE= 0.007, P= 1.42e-5) and a nominal significant association between rs699947 and PD20 and rs6900017 and FEV₁/FVC (Table 2). There was however a significant heterogeneity between the two studies when comparing FEV1 and rs699947 (P= 0.043). Interaction with maternal asthma was analyzed in the PIAMA cohort

showing no significant interaction ($p=0.06$) and with a tendency towards lower effect of the rs699947 risk allele on lung function if the mother had asthma (data not shown).

Furthermore, there were no association between the three SNPs and asthma in the PIAMA cohort (rs833052: CC- reference; CA $P=0.23$; AA $P=0.53$; rs699947: CC – reference, CA $P=0.21$, AA $P=0.13$; and rs6900017: CC - reference; TC $P=0.85$, TT - none in this group).

Asthma

We found no association between asthma at age 6-7 or recurrent troublesome lung symptoms at 18 months of age and any of the SNPs (Figure E7 and E8). Kaplan-Meier graphs did not reveal any difference in time to onset of asthma between the different genotype groups of rs699947, rs833052 and rs6900017 respectively (Figure E9-11).

VEGF levels in plasma

There was no association between VEGF plasma levels at 18 months of age, asthma at age six or recurrent wheeze at 18 months of age or any of the lung function measurements. Three SNPs upstream of the *VEGFA* gene were associated with VEGF plasma levels with p-values very close to the significance level (however not below) (rs833052 $P=0.01$; rs866236 $P=0.0071$ and rs833057 $P=0.0046$) (Figure 2 and E2). We found, however, no association with the rs699947 linkage disequilibrium block. Adjustment for steroid use at time of VEGF level measurement did not alter the associations.

Functional analyses of the 13 SNPs

We were able to analyze 8 of the 13 SNPs. For five SNPs there were no available data.

Rs699947 and rs833069 were the two SNPs with the highest estimated functional risk with

the score 1-3 (score range from 0-5) with a possible functional effect on the promoter and/or regulatory regions.

DISCUSSION

Principal Findings

Common polymorphic variants in the *VEGFA* gene were associated with lung function development after birth independent of asthma. Our overall findings suggest a biological role of *VEGF* gene variants in lung function development independent of asthma during pre-school age, but no association with other markers of asthma, like hyperresponsiveness.

Strength and Limitations of the Study

The single-centre clinical, prospective and objective monitoring of the discovery cohort is an important strength of this study minimizing risk for information bias. Measurements of lung function in a large group of children at birth allowed analysis of effect of *VEGFA* on early life lung function, which has not previously been performed. Misclassification of asthma diagnosis was minimized as all children were followed, diagnosed and treated solely by the COPSAC clinical research doctors according to predefined standard operating procedures and treatment algorithms. A limitation of the current study may be the low statistical power in the dichotomized analysis of asthma as compared to the quantitative analyses and further the selected nature of the two cohorts. Other limitations to this study lies in the measurements of the VEGF-levels: this was measured at 18 months of age in the COPSAC cohort and measuring levels at same time points as lung function measures would strengthen the interpretation. Moreover measurement of specific inhibitory and active isoforms of VEGF might have increased the interpretation[15].

The high-risk nature of the COPSAC cohort with all mothers having asthma could be the reason for a stronger effect of risk variants on lung function. However, this seems not to be the case since stratified and interaction analyses by maternal asthma in PIAMA showed no significant interaction and a lower effect estimate if mothers had asthma.

The confirmation of the findings on the association of *VEGF* SNPs and early childhood lung function in two well defined cohorts is the major strength of this study and reduces the risk of chance findings.

Meaning of the Study

Here we studied the role of *VEGFA* gene variants and plasma levels in lung function development from birth to school age. Our findings suggest a role of *VEGFA* gene variants in postnatal lung function development, with a similar effect in healthy children and children with asthma. One study has assessed the role of SNPs in the *VEGFA* gene and lung function in children: Sharma et al investigated ten SNPs in the *VEGFA* gene in two independent cohorts using family data and found a borderline significant association between rs2146323 and FEV1.[8] This SNP is not genotyped in the COPSAC cohort but lies in the same linkage disequilibrium block as the top SNPs associated with FEV1. This confirms the importance of our findings. Sharma et al also reported an association between rs4711750 and FEV1/FVC, but this was not significant after correction for multiple testing. Rs4711750 is genotyped in the 1000genomes populations and in high LD with rs1358980 ($r^2=0.8$). We found no association between this SNP and FEV1/FVC.

Sharma et al also reported an association between rs833058 and asthma and nominal significant between rs833058 and hyperresponsiveness. This SNP is in close proximity to and has moderate linkage disequilibrium with the majority of the SNPs in the rs699947 linkage

Accepted Article

disequilibrium block (in HapMap data) (rs833058-rs699947 $R^2=0.54$). However, we did not find any association with bronchial hyperresponsiveness or asthma for any of the SNPs. One other study, Lachheb et al, investigated three polymorphisms in the *VEGFA* gene in relation to asthma in 434 Tunisian children and found a borderline significant association with rs2010963, although they did not correct for multiple testing and did not replicate their findings.[9] This SNP is in high linkage disequilibrium with our top SNP rs699947,[33] but also here we did not find an association with asthma for this SNP. The discrepant findings for asthma and hyperresponsiveness could be due to differences in environmental exposures, statistical methods and genetic models used or statistical power or lack of a true connection. Further studies are needed to confirm this.

The most consistent paper published so far is by Simpson and colleagues investigating 5 tagging SNPs in the gene in 4 different populations.[15] Rs3025028 was consistently associated (not corrected for multiple testing) to different lung function measures over time from 2 months of age, and at 3, 5, 6, 8, 16 and 22 years of age. Our closest SNP (rs3025033) is not tagging this SNP ($r^2=0.127$, 1000genomes data) and hence cannot use our data to replicate their findings.

We did not find an association between VEGF plasma levels at 18 months of age and asthma at age 6 possibly because of the difference in time at assessment. It might also be explained by the fact that we measured the total level of VEGF and not specific isoforms and their ratio. It would have strengthened the study if in addition to total VEGF measurements also the anti-angiogenic isoform VEGF165b had been measured. Furthermore information on mechanisms regulating differential splicing would be of great interest. These aspects ought to be examined in future studies.

Furthermore we were not able to find an association between recurrent troublesome lung symptoms and VEGF levels at 18 months of age. No other studies have assessed VEGF levels and asthmatic symptoms in this age group. The two studies with the closest age range to our study did and did not find an association: Lee et al, age range 2-6 years and Kato et al, mean age 4.3 years of age.[12, 14] The Lee study was rather small with 54 asthmatic children and 16 matched controls with a very heterozygous asthma definition and great variability in VEGF levels in the severe asthma group that seemed to drive the association. However, many of the children in the COPSAC cohort with the troublesome lung symptoms would receive steroids at the time blood samples were taken and this may have masked the relationship between symptoms and VEGF levels. We furthermore did not see any association between levels at 18 months of age and later lung function measures at age 6, and the level measured at 18 months of age was not a good predictive factor.

We found a borderline association between three SNPs upstream of the gene and *VEGF* plasma levels that could explain the functional effect of these SNPs (or tagged variants) perhaps affecting an enhancer region of the *VEGFA* gene. We did not find an association between plasma VEGF levels and the rs699947 linkage block, which is in line with some studies[34, 35] but others do find an effect of these SNPs on levels: in serum levels in healthy adults; in the supernatant from peripheral blood mononuclear cell cultures from healthy adults and in in vitro studies of cloned glioma cells[36-38]. However it is clear that these polymorphisms and corresponding haplotype block lies in (rs699947 lies upstream and rs833069 downstream) an important regulatory promoter region regulating *VEGF* expression[39] which is also indicated by the functional analyses of these SNPs. Our lack of association could also be that the rs699947 tags a variant within the gene which affects the function of the gene product and not the expression level or only affects expression levels in certain tissues.

Many studies have underlined the importance of VEGF in lung maturation and development due to influence as angiogenesis and endothelial cell differentiation [7] and it has been shown in mice studies that the inhibition of VEGF levels or increase was associated with respiratory distress syndrome and impaired lung maturation or a protection against this respectively [40], underling the importance of VEGF and possibly the *VEGFA* gene as a susceptibility locus for lung function development. Interestingly this was not associated to asthma suggesting only a role for VEGF in normal lung function development in this present study. The association of VEGF levels to asthmatic symptoms could be argued as a general up-regulation of inflammatory and other mediators such as VEGF.

Our data demonstrates an effect of *VEGFA* gene variants on lung function at age 6, but not an inborn effect on neonatal lung indices. Thus, this effect becomes prominent during pre-school life suggesting a possible interaction between the *VEGFA* gene variants and environmental exposures.

Conclusion

VEGFA gene variants were associated with postnatal lung function development in both asthmatic and healthy children. This suggests a role of *VEGF* and *VEGFA* gene variants in normal lung function development. Possible interaction between *VEGFA* gene variants and post-natal environmental risk factors should be targeted in future studies.

ACKNOWLEDGEMENTS

We gratefully express our gratitude to the children and families of the COPSAC and PIAMA cohorts for their participation, all their support and commitment. We acknowledge and appreciate the unique efforts of the COPSAC and PIAMA research teams.

REFERENCES

1. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, von Mutius E, Farrall M, Lathrop M, Cookson WO. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med* 2010; 363: 1211-1221.

2. Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. *J Allergy Clin Immunol* 2011.
3. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, Zanin ME, Zuin R, Maestrelli P, Fabbri LM, Saetta M. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006; 174: 975-981.
4. Turato G, Barbato A, Baraldo S, Zanin ME, Bazzan E, Lokar-Oliani K, Calabrese F, Panizzolo C, Snijders D, Maestrelli P, Zuin R, Fabbri LM, Saetta M. Nonatopic children with multitrigger wheezing have airway pathology comparable to atopic asthma. *Am J Respir Crit Care Med* 2008; 178: 476-482.
5. Detoraki A, Granata F, Staibano S, Rossi FW, Marone G, Genovese A. Angiogenesis and lymphangiogenesis in bronchial asthma. *Allergy* 2010; 65: 946-958.
6. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, Cohn L, Kim YK, McDonald DM, Elias JA. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med* 2004; 10: 1095-1103.
7. Tudor RM, Yun JH. Vascular endothelial growth factor of the lung: friend or foe. *Curr Opin Pharmacol* 2008; 8: 255-260.
8. Sharma S, Murphy AJ, Soto-Quiros ME, Avila L, Klanderman BJ, Sylvia JS, Celedon JC, Raby BA, Weiss ST. Association of VEGF polymorphisms with childhood asthma, lung function and airway responsiveness. *Eur Respir J* 2009; 33: 1287-1294.
9. Lachheb J, Chelbi H, Ben Dhifallah I, Ammar J, Hamzaoui K, Hamzaoui A. Association of vascular endothelial growth factor polymorphisms with asthma in Tunisian children. *Gene Regul Syst Bio* 2008; 2: 89-96.
10. Abdel-Rahman AM, el-Sahrigy SA, Bakr SI. A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack. *Chest* 2006; 129: 266-271.
11. Hossny E, El-Awady H, Bakr S, Labib A. Vascular endothelial growth factor overexpression in induced sputum of children with bronchial asthma. *Pediatr Allergy Immunol* 2009; 20: 89-96.
12. Lee Chung H, Kim SY, Kim SG. Vascular endothelial growth factor and plasminogen activator inhibitor-1 in children with recurrent early wheeze. *J Allergy Clin Immunol* 2007; 119: 1541-1542.
13. Yoo Y, Choi IS, Byeon JH, Lee SM, La KS, Choi BM, Park SH, Choung JT. Relationships of methacholine and adenosine monophosphate responsiveness with serum vascular endothelial growth factor in children with asthma. *Ann Allergy Asthma Immunol* 2010; 104: 36-41.
14. Kato M, Yamada Y, Maruyama K, Hayashi Y. Serum eosinophil cationic protein and 27 cytokines/chemokines in acute exacerbation of childhood asthma. *Int Arch Allergy Immunol* 2010; 152 Suppl 1: 62-66.
15. Simpson A, Custovic A, Tepper R, Graves P, Stern DA, Jones M, Hankinson J, Curtin JA, Wu J, Blekic M, Bukvic BK, Aberle N, Marinho S, Belgrave D, Morgan WJ, Martinez FD. Genetic variation in vascular endothelial growth factor-a and lung function. *Am J Respir Crit Care Med* 2012; 185: 1197-1204.
16. Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COPSAC): design, rationale, and baseline data from a longitudinal birth cohort study. *Ann Allergy Asthma Immunol* 2004; 93: 381-389.
17. Brunekreef B, Smit J, de Jongste J, Neijens H, Gerritsen J, Postma D, Aalberse R, Koopman L, Kerkhof M, Wilga A, van Strien R. The prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study: design and first results. *Pediatr Allergy Immunol* 2002; 13 Suppl 15: 55-60.

18. Bisgaard H, Bonnelykke K, Sleiman PM, Brasholt M, Chawes B, Kreiner-Moller E, Stage M, Kim C, Tavendale R, Baty F, Phipper CB, Palmer CN, Hakonarsson H. Chromosome 17q21 gene variants are associated with asthma and exacerbations but not atopy in early childhood. *Am J Respir Crit Care Med* 2009; 179: 179-185.
19. The International HapMap Project. *Nature* 2003; 426: 789-796.
20. Loland L, Bisgaard H. Feasibility of repetitive lung function measurements by raised volume rapid thoracoabdominal compression during methacholine challenge in young infants. *Chest* 2008; 133: 115-122.
21. Bisgaard H, Loland L, Holst KK, Phipper CB. Prenatal determinants of neonatal lung function in high-risk newborns. *J Allergy Clin Immunol* 2009; 123: 651-657, 657 e651-654.
22. Loland L, Buchvald FF, Brydensholt Halkjaer L, Anhøj J, Hall GL, Persson T, Grove Krause T, Bisgaard H. Sensitivity of Bronchial Responsiveness Measurements in Young Infants*. *Chest* 2006; 129: 669.
23. Chawes BL, Kreiner-Moller E, Bisgaard H. Upper and lower airway patency are associated in young children. *Chest* 2010; 137: 1332-1337.
24. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. *N Engl J Med* 2006; 354: 1998-2005.
25. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, Brasholt M, Heltberg A, Vissing NH, Thorsen SV, Stage M, Phipper CB. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007; 357: 1487-1495.
26. Bisgaard H, Phipper CB, Bonnelykke K. Endotyping early childhood asthma by quantitative symptom assessment. *J Allergy Clin Immunol* 2011; 127: 1155-1164 e1152.
27. Sorensen M, Bisgaard H, Stage M, Loft S. Biomarkers of exposure to environmental tobacco smoke in infants. *Biomarkers* 2007; 12: 38-46.
28. Bottema RW, Postma DS, Reijmerink NE, Thijs C, Stelma FF, Smit HA, van Schayck CP, Brunekreef B, Koppelman GH, Kerkhof M. Interaction of T-cell and antigen presenting cell co-stimulatory genes in childhood IgE. *Eur Respir J* 2010; 35: 54-63.
29. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; 81: 559-575.
30. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-265.
31. Team R. R: A language and environment for statistical computing. *R Foundation for Statistical Computing Vienna Austria ISBN* 2008; 3.
32. Yuan HY, Chiou JJ, Tseng WH, Liu CH, Liu CK, Lin YJ, Wang HH, Yao A, Chen YT, Hsu CN. FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. *Nucleic Acids Res* 2006; 34: W635-641.
33. Garza-Veloz I, Castruita-De la Rosa C, Cortes-Flores R, Martinez-Gaytan V, Rivera-Munoz JE, Garcia-Mayorga EA, Meza-Lamas E, Rojas-Martinez A, Ortiz-Lopez R, Martinez-Fierro ML. No association between polymorphisms/haplotypes of the vascular endothelial growth factor gene and preeclampsia. *BMC Pregnancy Childbirth* 2011; 11: 35.
34. Watson CJ, Webb NJ, Bottomley MJ, Brenchley PE. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. *Cytokine* 2000; 12: 1232-1235.
35. Talar-Wojnarowska R, Gasiorowska A, Olakowski M, Lekstan A, Lampe P, Smolarz B, Romanowicz-Makowska H, Kulig A, Malecka-Panas E. Vascular endothelial

- growth factor (VEGF) genotype and serum concentration in patients with pancreatic adenocarcinoma and chronic pancreatitis. *J Physiol Pharmacol* 2010; 61: 711-716.
36. Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, Inukai K, Inoue I, Katayama S. A common polymorphism in the 5'-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes. *Diabetes* 2002; 51: 1635-1639.
37. Shahbazi M, Fryer AA, Pravica V, Brogan IJ, Ramsay HM, Hutchinson IV, Harden PN. Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection. *J Am Soc Nephrol* 2002; 13: 260-264.
38. Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F, Marklund SL, Wyns S, Thijs V, Andersson J, van Marion I, Al-Chalabi A, Bornes S, Musson R, Hansen V, Beckman L, Adolfsson R, Pall HS, Prats H, Vermeire S, Rutgeerts P, Katayama S, Awata T, Leigh N, Lang-Lazdunski L, Dewerchin M, Shaw C, Moons L, Vlietinck R, Morrison KE, Robberecht W, Van Broeckhoven C, Collen D, Andersen PM, Carmeliet P. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat Genet* 2003; 34: 383-394.
39. Josko J, Mazurek M. Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. *Med Sci Monit* 2004; 10: RA89-98.
40. Compernelle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D, Carmeliet P. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med.* 2002;8:702-10

FIGURE LEGENDS

Figure 1: The COPSAC study group profile

Figure 2: In the top of the plot the association between SNPs and FEV1, FEV1/FVC ratio, MMEF at age 6 and VEGF levels at 18 months of age in COPSAC2000. Thick horizontal line represents the Bonferroni threshold and the grey thin line the nominal threshold. The middle of the plot describes the gene structure: grey blocks are UTR region and black blocks the translated regions (from <http://genome.ucsc.edu/>, b36 positions). The lower part is the linkage disequilibrium of the single nucleotide polymorphisms in the VEGF gene. The intensity of the shading represents r-squared, and numerical values (percent) were calculated using the Haploview software. Three different haplotype blocks were found using the Gabriel method.[30]

Table 1 – Population descriptives

Characteristics at birth	COPSAC Population n=365	PIAMA Population n=961
Gender (% boys)	49.3	52.2
Study type (%)		
Active intervention	-	24.2
Placebo intervention	-	18.4
Natural history	-	57.5
Allergic mother (%)	83.0	65.9
Allergic father (%)	29.3	31.4
Mother's education (%)		
Low	59.6	18.2
Medium	27.3	42.8
High	13.1	39.0
Mother smoking last trimester pregnancy (%)	15.9	10.3
Breast feeding duration (%)		
Never*	2.2	14.9
< 3 months	8.7	35.3
≥ 3 months	89.1	49.8
Older siblings (%)	38.4	49.0
Daycare attendance first year (%)	57.4	25.3
Cat(s) at home (%)	14.8	29.5
Dog(s) at home (%)	13.1	14.6
Premature birth (%)	6.3	4.6
Rs833052 (A allele frequency, %)	12.0	12.6
Rs699947 (A allele frequency, %)	49.0	51.2
Rs6900017 (T allele frequency, %)	7.5	7.6

*: in COPSAC defined as less than 1 week

Table 2 – Overall discovery, replication and meta-analyzed results for the three top-SNPs

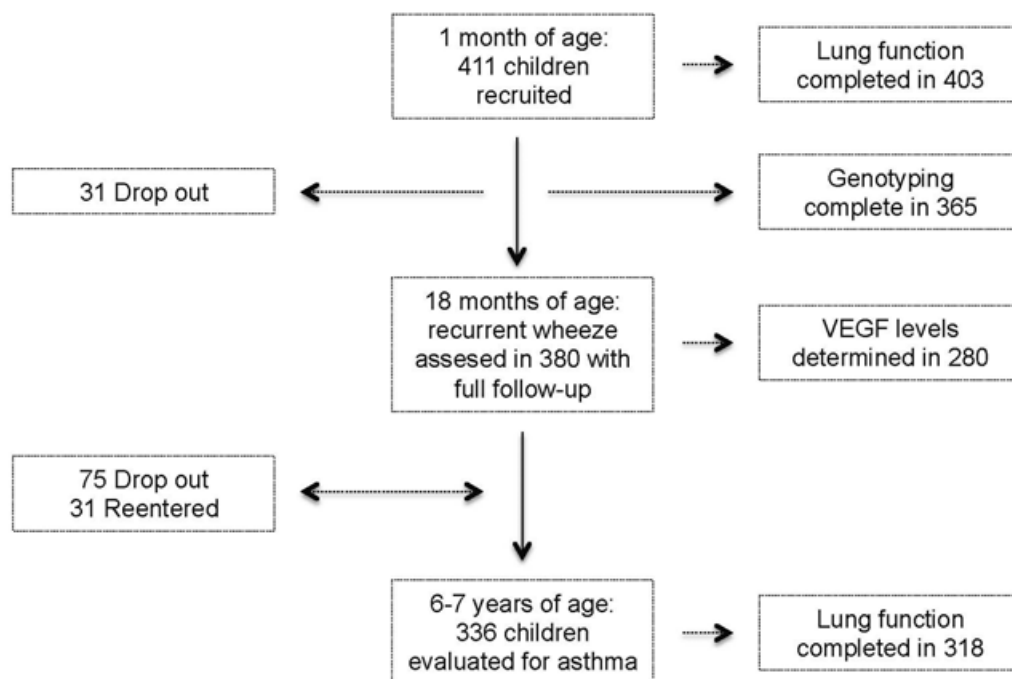
	COPSAC			PIAMA			Meta-analysed			
	Estimate	SE	P-val	Estimate	SE	P-val	Estimate	SE	P-val	Phet
rs833052 (A allele)										
FEV0.5	-1.59	1.48	0.29	-	-	-	-	-	-	-
FEF50	7.40	6.35	0.24	-	-	-	-	-	-	-
PD15*	-0.03	0.08	0.67	-	-	-	-	-	-	-
FEV1#	-0.02	0.02	0.21	-0.02	0.02	0.23	-0.02	0.01	0.11	0.938
FVC#	0.01	0.02	0.66	-	-	-	-	-	-	-
FEV1/FVC	-0.02	7.82E-03	4.09E-03 +	0.001	0.005	0.91	-0.01	0.00	0.16	0.011
MMEF*#	-0.02	0.01	0.06	-	-	-	-	-	-	-

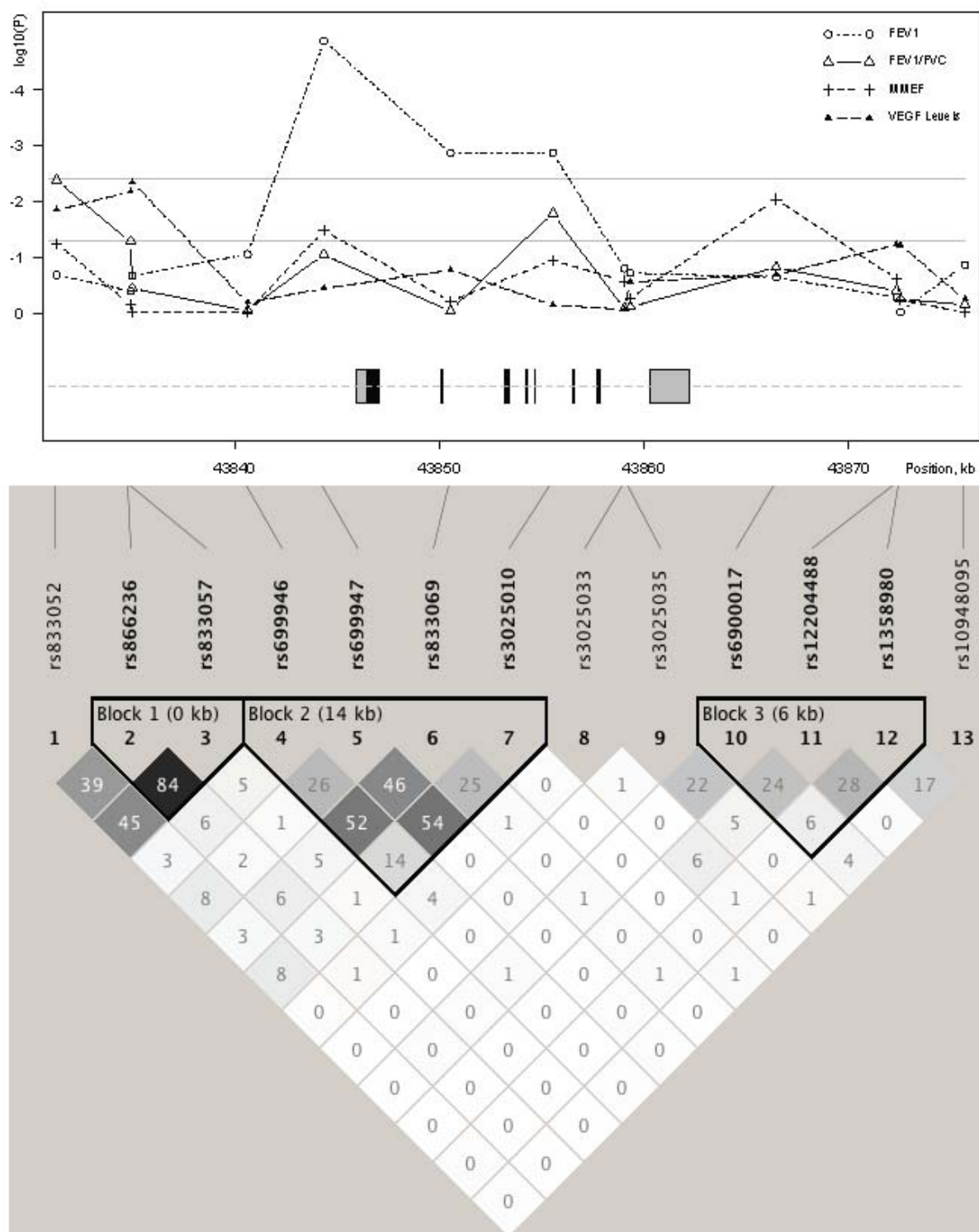
PD20*	-0.10	0.08	0.21		0.04	0.05	0.49	-5.36E-05	0.04	1.00	0.135
rs699947 (A allele)											
FEV0.5	-0.86	0.93	0.36		-	-	-	-	-	-	-
FEF50	0.30	4.01	0.94		-	-	-	-	-	-	-
PD15*	-0.04	0.05	0.43		-	-	-	-	-	-	-
FEV1#	-0.05	0.01	1.31E-05	++	-0.02	0.01	0.07	-0.03	0.01	1.42E-05	++ 0.04
FVC#	-0.03	0.01	0.01	+	-	-	-	-	-	-	-
FEV1/FVC	-8.48E-03	4.98E-03	0.09		0.002	0.003	0.54	-7.91E-04	2.57E-03	0.76	0.07
MMEF*#	-0.02	0.01	0.03	+	-	-	-	-	-	-	-
PD20*	-0.06	0.05	0.20		-0.07	0.03	0.03	-0.07	0.03	0.01	+ 0.93
rs6900017 (T allele)											
FEV0.5	-2.06	1.90	0.28		-	-	-	-	-	-	-
FEF50	-15.24	8.10	0.06		-	-	-	-	-	-	-
PD15*	0.19	0.10	0.05		-	-	-	-	-	-	-
FEV1#	-0.03	0.02	0.23		-0.014	0.019	0.47	-0.02	0.01	0.18	0.64
FVC#	0.00	0.03	0.92		-	-	-	-	-	-	-
FEV1/FVC	-0.01	4.66E-03	0.13		-0.01	0.01	0.07	-0.01	3.68E-03	0.02	+ 0.61
MMEF*#	-0.04	0.02	0.01	+	-	-	-	-	-	-	-
PD20*	0.09	0.11	0.39		0.032	0.064	0.62	0.05	0.06	0.39	0.62

+: nominal significant; ++: significant below the Bonferonni threshold

* Log10-transformed

#Adjusted for height and sex





Online supplementary for

VEGFA variants are associated with pre-school lung function but not neonatal lung function

Eskil Kreiner-Møller, MD^{1,2}; Bo Chawes, MD, PhD^{1,2}; Nadja Hawwa Vissing, MD^{1,2}; Gerard H. Koppelman, MD, PhD³; Dirkje S. Postma, MD, PhD⁴; Jonna Skov Madsen, MD, PhD⁵; Dorte Aalund Olsen, MSc⁵; Florent Baty^{1,2}; Judith M. Vonk, PhD⁶; Marjan Kerkhof, MD, PhD⁶; Patrick Sleiman MSc, PhD⁷; Hakon Hakonarsson, MD, PhD⁷; Li Juel Mortensen^{1,2}; Porntiva Poorisrisak, MD, PhD^{1,2}; Hans Bisgaard, MD, DMSci^{1,2}; Klaus Bønnelykke, MD, PhD^{1,2}

1: COPSAC: Copenhagen Prospective Studies on Asthma in Childhood; Copenhagen University Hospital, Gentofte, Denmark.

2: The Danish Pediatric Asthma Center, Copenhagen University Hospital, Gentofte

3: Department of Pediatric Pulmonology and Pediatric Allergology, Beatrix Children's Hospital, University Medical Center Groningen, GRIAC research institute, University of Groningen, the Netherlands

4: Dept. of Pulmonary Medicine and Tuberculosis; University Medical Center Groningen, The Netherlands

5: Dept. of Clinical Biochemistry, Lillebaelt Hospital, Vejle, Denmark

6: Department of Epidemiology, University Medical Center Groningen, GRIAC research institute, University of Groningen, the Netherlands

7: Center for Applied Genomics and Division of Human Genetics, The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Supplementary methods

Genetic quality control: The data were filtered if minor allele frequency < 0.01 , Hardy Weinberg Equilibrium < 0.0001 , Call rate per individual < 0.98 , Call rate – per SNP < 0.94 . Population stratification was assessed by an identity by state matrix and performed a Multi-Dimensional-Scaling-analysis. Siblings were filtered.

Lung function measurements were obtained at 1-month of age using the raised volume rapid thoracoabdominal compression technique measuring forced expiratory volume in 0.5 seconds (FEV0.5) and forced expiratory flow at 50% of the functional vital capacity (FEF50) during repeated and increasing provocative doses of methacholine. The threshold resulting in a 15 % drop in TcO₂ was used as provocative dose (PD15). [1-3] We did not use the functional vital capacity (FVC) for the neonatal lung function because the FEV0.5 measurement most often reached the FVC (mean FEV0.5= 67.8, mean FVC= 71.0 ml and mean FEV0.5/FVC=0.96).[3]

The FEV0.5 and FEF50 measurements were calibrated according to lifespan, weight and BMI and PD15 to lifespan and weight.[2] Maximum forced expiratory volume during the first second of expiration (FEV1), FVC and maximal mid-expiratory flow (MMEF) was assessed at six years of age by spirometry from up to five technically acceptable maneuvers, in accordance with international criteria for reproducibility[4] using the MasterScope system 754916 spirometer (Erich Jäeger; Würzburg, Germany). If several measurements were performed in the sixth year of life, the mean was used. The spirometry was performed during repeated methacholine measurements measuring a dose resulting in a 20 percent drop in FEV1 (PD20). All measurements were obtained with a computer-animated, volume-driven computer game well known to the children.[5, 6]

Linkage disequilibrium plot in the HapMap data: A Linkage disequilibrium plot were made of the VEGF gene (chromosome 6, 43830931-43877201) using HapMap data[7], CEU population, created in Haploview using the Gabriel method for haplotype block definition.[8] (Figure E1) This was used to compare with the COPSAC linkage disequilibrium plots. A further plot of the linkage disequilibrium structure in the CEU Hapmap population (release 22) of the upstream region focusing on the SNP with the lowest p-value in the analyses of the VEGF plasma levels and the SNPs using the online plotting tool SNAP (<http://www.broadinstitute.org/mpg/snap/ldplot.php>).

Kaplan meier plots: For the three top SNPs (rs699947, rs833052 and rs6900017) Kaplan-Meier plots were plotted using the survival package in R-project for the time to asthma onset. The CA and AA genotype strata for rs833052 were collapsed due to low numbers in the AA group and the CT and TT genotype strata for rs6900017 were collapsed due to low numbers in the TT group.

Supplementary references

- E1. Loland L, Bisgaard H. Feasibility of repetitive lung function measurements by raised volume rapid thoracoabdominal compression during methacholine challenge in young infants. *Chest* 2008; 133: 115-122.
- E2. Bisgaard H, Loland L, Holst KK, Pipper CB. Prenatal determinants of neonatal lung function in high-risk newborns. *J Allergy Clin Immunol* 2009; 123: 651-657, 657 e651-654.
- E3. Loland L, Buchvald FF, Brydesholt Halkjær L, Anhøj J, Hall GL, Persson T, Grove Krause T, Bisgaard H. Sensitivity of Bronchial Responsiveness Measurements in Young Infants*. *Chest* 2006; 129: 669.
- E4. Standardization of Spirometry, 1994 Update. American Thoracic Society. *Am J Respir Crit Care Med* 1995; 152: 1107-1136.
- E5. Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COPSAC): design, rationale, and baseline data from a longitudinal birth cohort study. *Ann Allergy Asthma Immunol* 2004; 93: 381-389.
- E6. Chawes BL, Kreiner-Møller E, Bisgaard H. Upper and lower airway patency are associated in young children. *Chest* 2010; 137: 1332-1337.
- E7. The International HapMap Project. *Nature* 2003; 426: 789-796.
- E8. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-265.

Figure E1

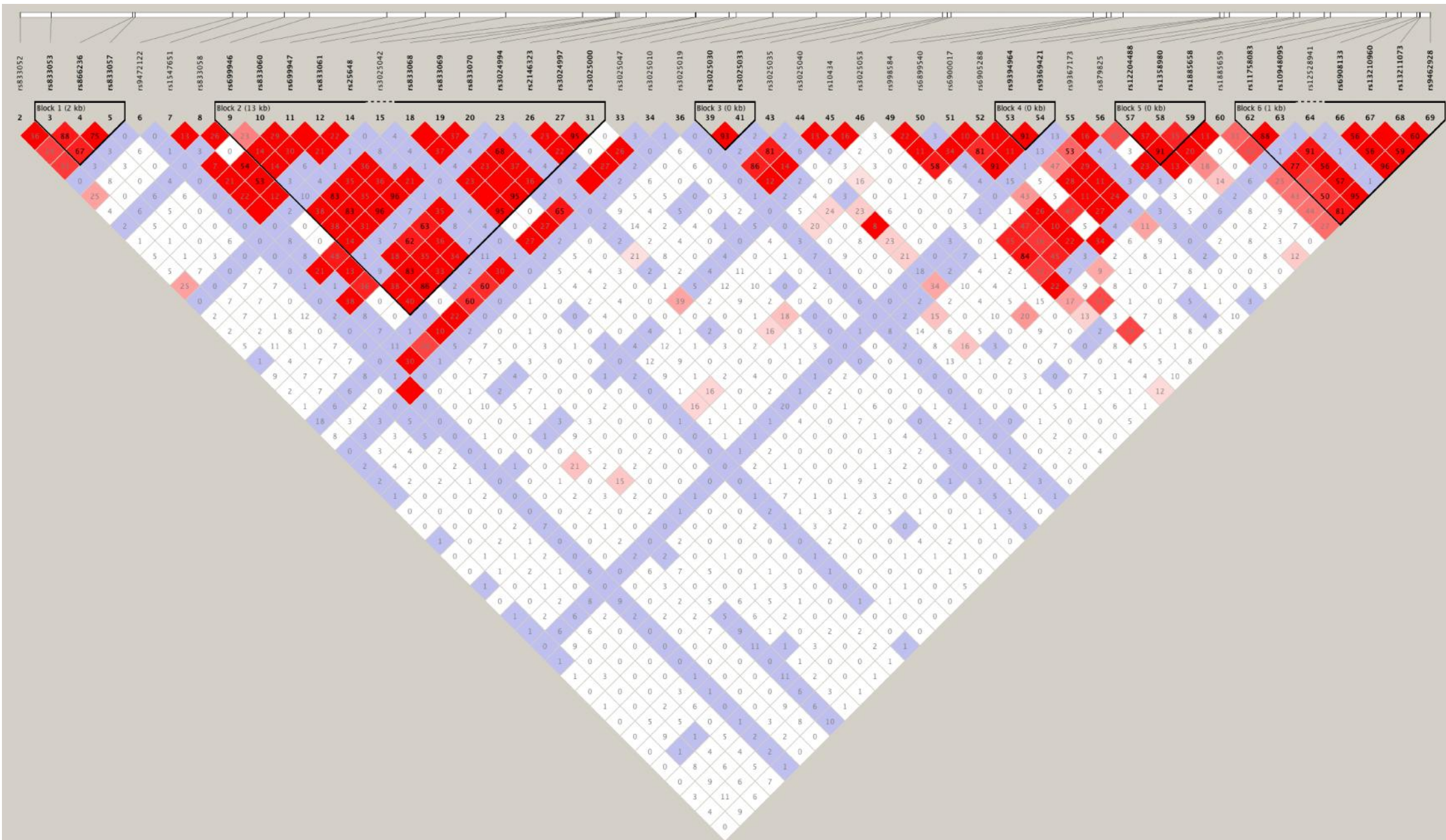
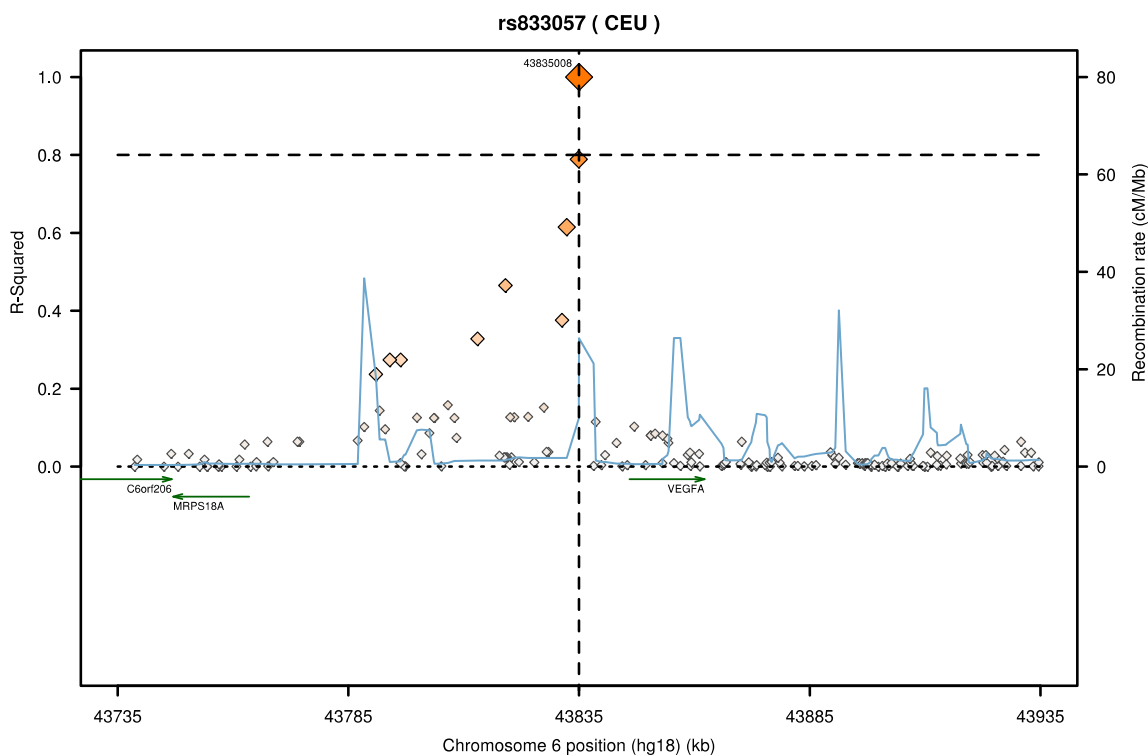
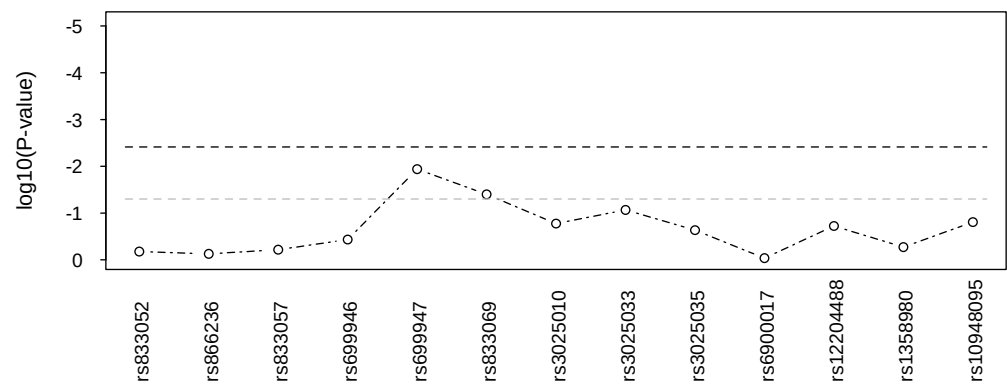


Figure E2



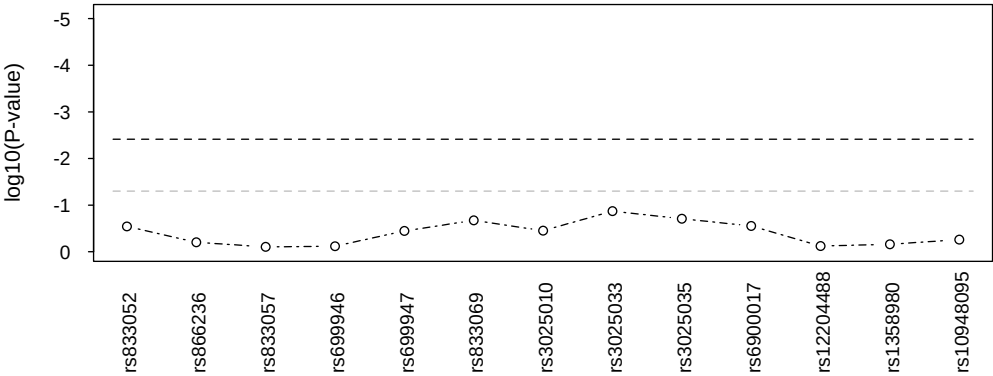
Data from the CEU Hapmap population (release 22). Blue line indicate recombination rate as indicated on the right hand y-axis. The correlation with the query SNP rs833057 is indicated for each SNP in this region based on localization on the left-hand axis and the color of the SNP (amount of red). X-axis represents the physical distance.

Figure E3



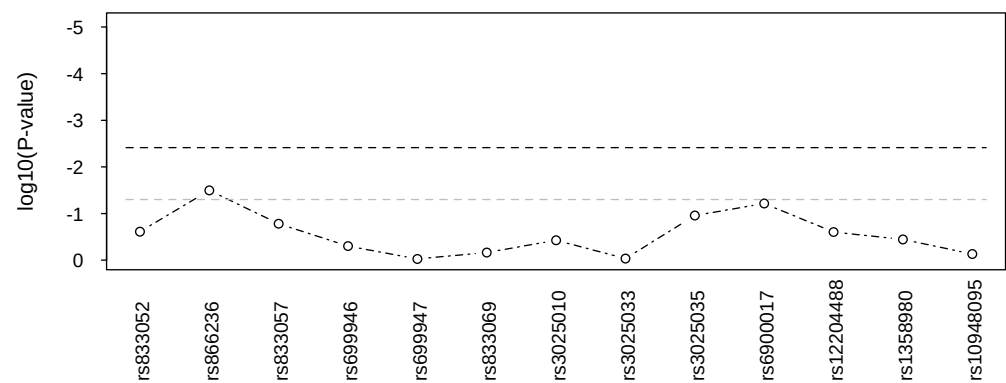
Association between single nucleotide polymorphisms and FVC at six years of age. Additive genetic model. Dashed line represents the Bonferroni threshold ($P < 3.80 \times 10^{-3}$) and the grey dashed line the nominal threshold ($P < 0.05$).

Figure E4



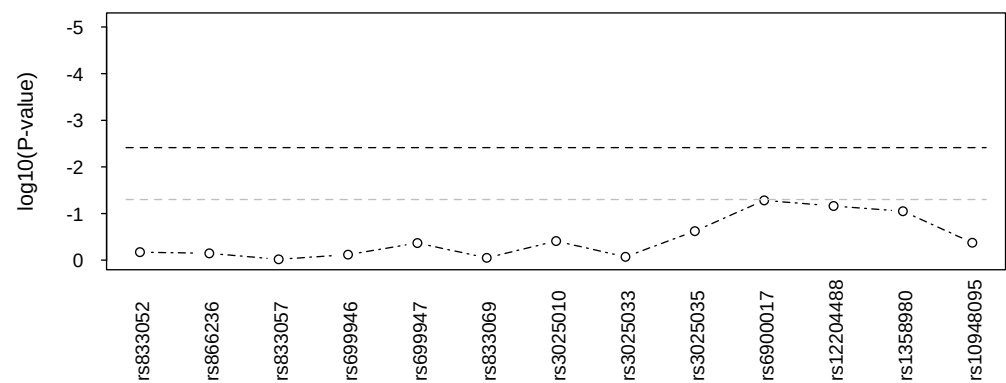
Association between single nucleotide polymorphisms and FEV0.5 at 1 month of age. Additive genetic model. Dashed line represents the Bonferroni threshold ($P < 3.80 \times 10^{-3}$) and the grey dashed line the nominal threshold ($P < 0.05$).

Figure E5



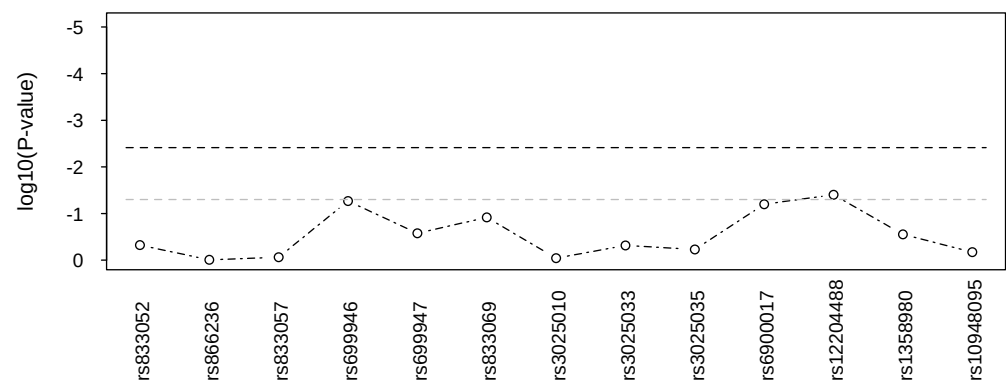
Association between single nucleotide polymorphisms and FEF50 at 1 month of age. Additive genetic model. Dashed line represents the Bonferroni threshold ($P < 3.80 \times 10^{-3}$) and the grey dashed line the nominal threshold ($P < 0.05$).

Figure E6



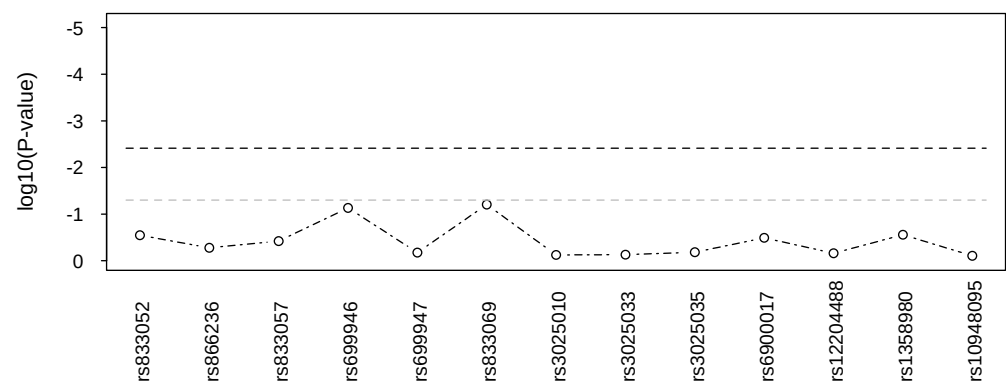
Association between single nucleotide polymorphisms and PD15 at 1 month of age. Additive genetic model. Dashed line represents the Bonferroni threshold ($P < 3.80 \times 10^{-3}$) and the grey dashed line the nominal threshold ($P < 0.05$).

Figure E7



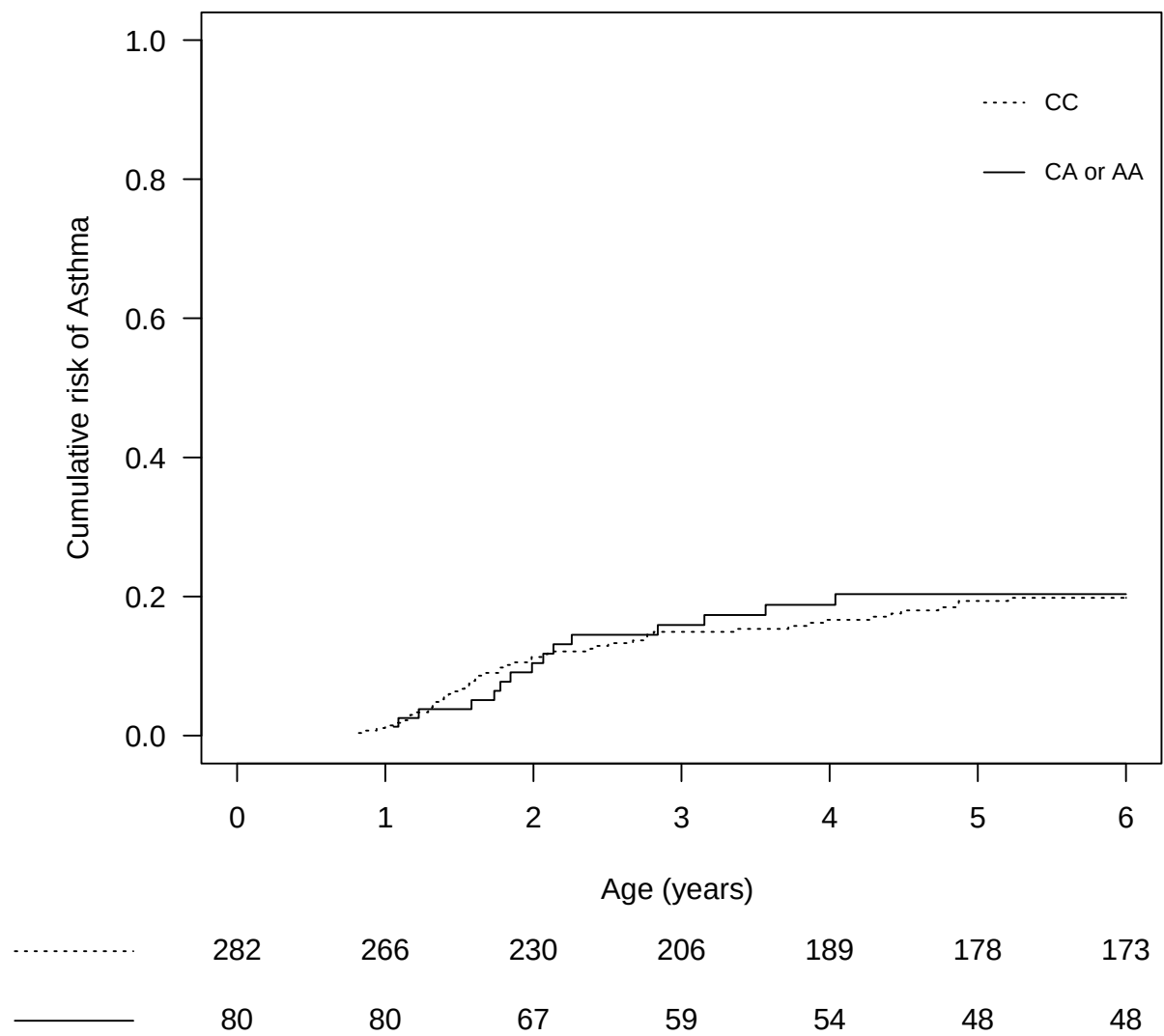
Association between single nucleotide polymorphisms and recurrent troublesome lung symptoms at 18 months of age. Additive genetic model. Dashed line represents the Bonferroni threshold ($P < 3.80 \times 10^{-3}$) and the grey dashed line the nominal threshold ($P < 0.05$).

Figure E8



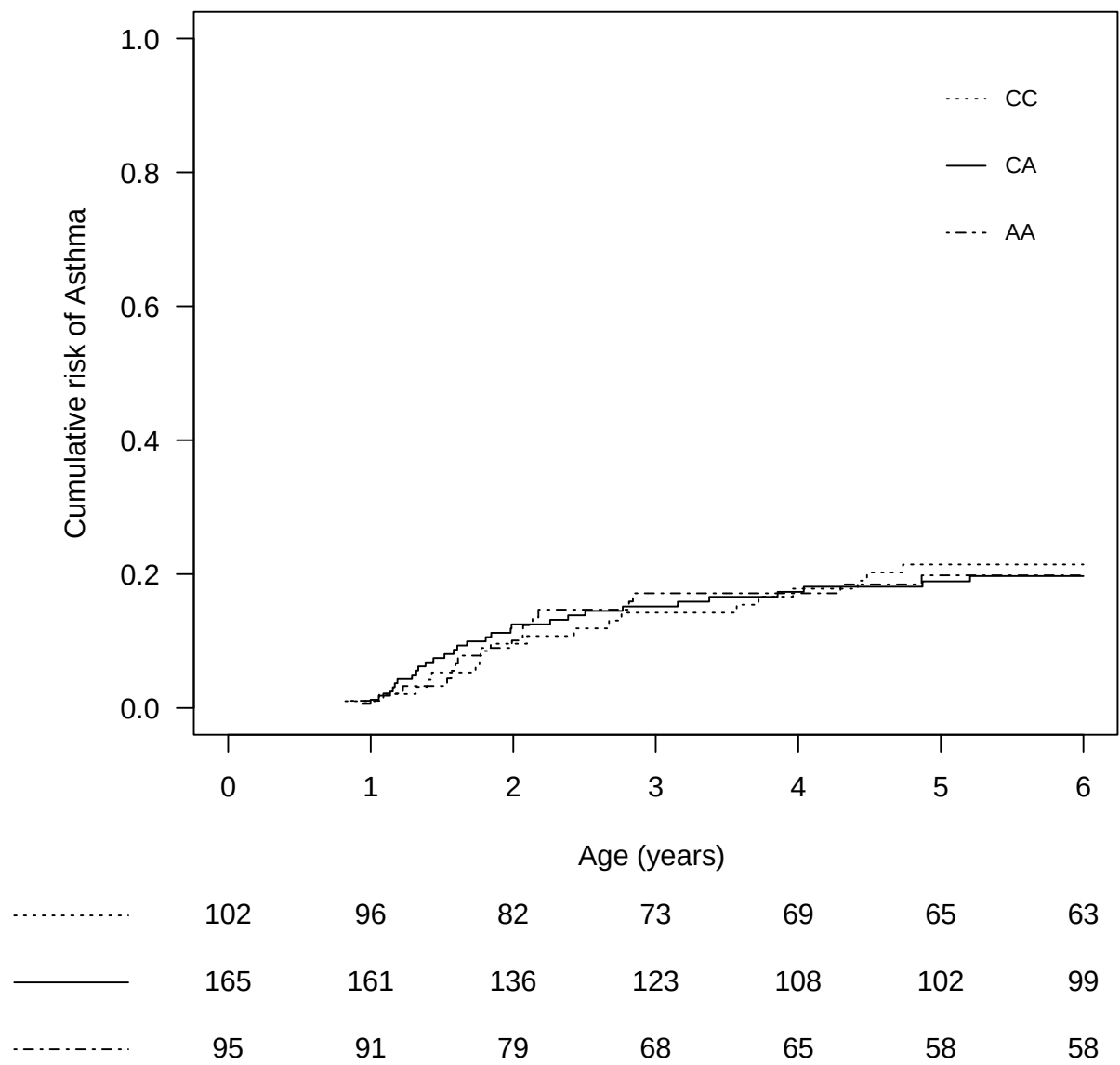
Association between single nucleotide polymorphisms and asthma at age 6-7. Additive genetic model. Dashed line represents the Bonferroni threshold ($P < 3.80 \times 10^{-3}$) and the grey dashed line the nominal threshold ($P < 0.05$).

Figure E9



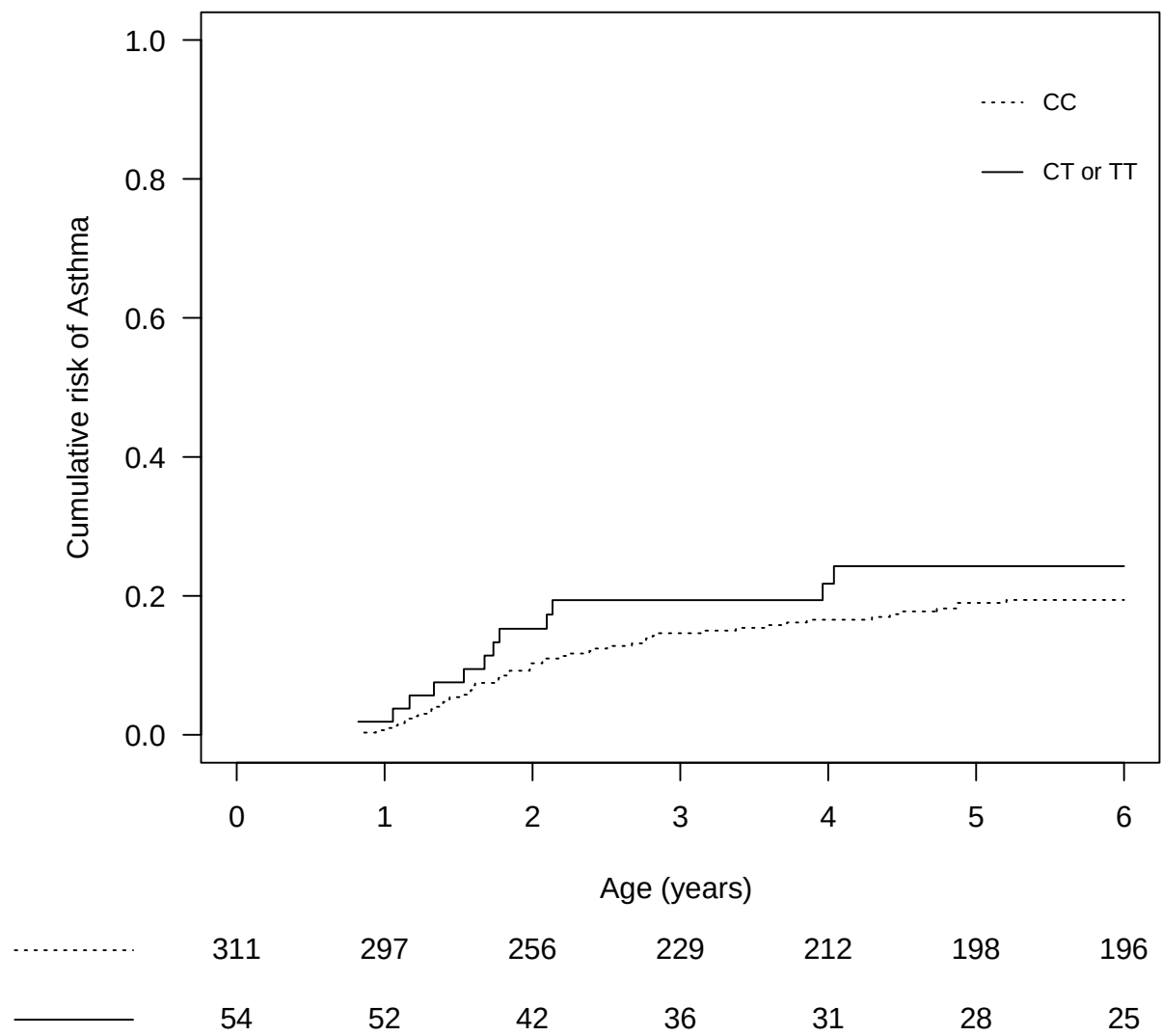
Kaplan-Meier plot of the risk for asthma stratified on rs833052 genotypes. Below the plot is printed the number at risk.

Figure E10



Kaplan-Meier plot of the risk for asthma stratified on rs699947 genotypes. Below the plot is printed the number at risk.

Figure E11



Kaplan-Meier plot of the risk for asthma stratified on rs6900017 genotypes. Below the plot is printed the number at risk.

Table E1

Genotype distributions in the COPSAC cohort

SNP	Minor allele	Major allele	Minor allele frequency	Ma:Ma	Ma:Mi	Mi:Mi	N, no missing	N	Call Rate
rs833052	A	C	0.12	282	73	7	362	365	0.99
rs866236	T	C	0.23	219	127	19	365	365	1.00
rs833057	G	T	0.20	234	114	17	365	365	1.00
rs699946	G	A	0.21	224	127	14	365	365	1.00
rs699947	A	C	0.49	102	165	95	362	365	0.99
rs833069	G	A	0.33	162	166	37	365	365	1.00
rs3025010	C	T	0.36	152	163	50	365	365	1.00
rs3025033	G	A	0.17	251	103	11	365	365	1.00
rs3025035	T	C	0.06	322	42	1	365	365	1.00
rs6900017	T	C	0.08	311	53	1	365	365	1.00
rs12204488	T	C	0.23	215	130	20	365	365	1.00
rs1358980	C	T	0.50	97	174	94	365	365	1.00
rs10948095	C	T	0.40	138	159	64	361	365	0.99

Table E2

Analysis of main outcomes and top three SNPs in three different linear regression analyses: overall (A), stratified for asthma (B) and adding asthma*SNP as interaction term to the overall analysis (C).

+: P-value<0.05; ++:P-value< 3.80e-03 (Bonferroni threshold). FEV1, FVC and MMEF were further adjusted for height and sex. MMEF was log10 transformed.

Outcome	SNP	Asthma - level	Beta	SE	P value
FEV1	rs833052	A: overall	-0.0221	0.0174	2.06E-01
		B: asthma no	-0.0236	0.0195	2.28E-01
		B: asthma yes	-0.0043	0.0385	9.11E-01
		C: interaction term	0.0192	0.0459	6.77E-01
	rs699947	A: overall	-0.0478	0.0108	1.31E-05 ++
		B: asthma no	-0.0506	0.0118	2.68E-05 ++
		B: asthma yes	-0.0349	0.0266	1.97E-01
		C: interaction term	0.0171	0.0309	5.79E-01
	rs6900017	A: overall	-0.0283	0.0235	2.29E-01
		B: asthma no	-0.0262	0.0276	3.43E-01
		B: asthma yes	-0.0219	0.0432	6.14E-01
		C: interaction term	0.0034	0.0547	9.50E-01
FEV1/FVC	rs833052	A: overall	-0.0226	0.0078	4.09E-03 +
		B: asthma no	-0.0230	0.0088	9.12E-03 +
		B: asthma yes	-0.0141	0.0173	4.20E-01
		C: interaction term	0.0090	0.0207	6.64E-01
	rs699947	A: overall	-0.0078	0.0053	1.43E-01
		B: asthma no	-0.0080	0.0059	1.73E-01
		B: asthma yes	-0.0037	0.0123	7.65E-01
		C: interaction term	0.0043	0.0155	7.80E-01
	rs6900017	A: overall	-0.0125	0.0109	2.56E-01
		B: asthma no	0.0001	0.0131	9.91E-01
		B: asthma yes	-0.0549	0.0179	3.83E-03 ++
		C: interaction term	-0.0550	0.0263	3.73E-02 +
FVC	rs833052	A: overall	0.0090	0.0207	6.65E-01
		B: asthma no	0.0110	0.0236	6.42E-01
		B: asthma yes	0.0050	0.0419	9.06E-01
		C: interaction term	-0.0061	0.0549	9.11E-01
	rs699947	A: overall	-0.0334	0.0131	1.15E-02 +
		B: asthma no	-0.0350	0.0147	1.78E-02 +
		B: asthma yes	-0.0311	0.0290	2.91E-01
		C: interaction term	0.0063	0.0377	8.67E-01
	rs6900017	A: overall	-0.0029	0.0279	9.19E-01
		B: asthma no	-0.0153	0.0334	6.48E-01
		B: asthma yes	0.0432	0.0467	3.60E-01
		C: interaction term	0.0569	0.0653	3.84E-01
MMEF	rs833052	A: overall	-0.0241	0.0126	5.66E-02
		B: asthma no	-0.0206	0.0136	1.31E-01
		B: asthma yes	-0.0307	0.0326	3.52E-01
		C: interaction term	-0.0084	0.0328	7.98E-01
	rs699947	A: overall	-0.0172	0.0080	3.33E-02 +
		B: asthma no	-0.0194	0.0084	2.27E-02 +
		B: asthma yes	-0.0029	0.0233	9.03E-01
		C: interaction term	0.0160	0.0227	4.81E-01
	rs6900017	A: overall	-0.0443	0.0169	9.17E-03 +
		B: asthma no	-0.0221	0.0192	2.52E-01
		B: asthma yes	-0.1014	0.0333	4.16E-03 +
		C: interaction term	-0.0778	0.0385	4.44E-02 +

Table E3

Analysis of main outcomes and top three SNPs in three different linear regression analysis: overall (A), adjusted for smoking exposure (B) and adding smoking exposure*SNP as interaction term to the overall analysis (C). +: P-value<0.05; ++:P-value< 3.80e-03 (Bonferroni threshold). FEV1, FVC and MMEF were further adjusted for height and sex. MMEF was log10 transformed.

Outcome	SNP	Analyses	Beta	SE	P value
FEV1	rs833052	A: overall	-0.0221	0.0174	2.06E-01
		B: adjusted for smoking	-0.0288	0.0181	1.12E-01
		C: smoking interaktion term	-0.0117	0.0055	3.63E-02 ⁺
	rs699947	A: overall	-0.0478	0.0108	1.31E-05 ⁺⁺
		B: adjusted for smoking	-0.0489	0.0110	1.28E-05 ⁺⁺
		C: smoking interaktion term	0.0005	0.0016	7.81E-01
	rs6900017	A: overall	-0.0283	0.0235	2.29E-01
		B: adjusted for smoking	-0.0213	0.0242	3.80E-01
		C: smoking interaktion term	0.0008	0.0033	8.05E-01
FEV1/FVC	rs833052	A: overall	-0.0226	0.0078	4.09E-03 ⁺
		B: adjusted for smoking	-0.0256	0.0081	1.74E-03 ⁺⁺
		C: smoking interaktion term	-0.0042	0.0025	9.43E-02
	rs699947	A: overall	-0.0078	0.0053	1.43E-01
		B: adjusted for smoking	-0.0076	0.0055	1.62E-01
		C: smoking interaktion term	0.0007	0.0008	3.74E-01
	rs6900017	A: overall	-0.0125	0.0109	2.56E-01
		B: adjusted for smoking	-0.0135	0.0113	2.32E-01
		C: smoking interaktion term	-0.0023	0.0016	1.52E-01
FVC	rs833052	A: overall	0.0090	0.0207	6.65E-01
		B: adjusted for smoking	0.0051	0.0217	8.16E-01
		C: smoking interaktion term	-0.0075	0.0067	2.67E-01
	rs699947	A: overall	-0.0334	0.0131	1.15E-02 ⁺
		B: adjusted for smoking	-0.0358	0.0135	8.69E-03 ⁺
		C: smoking interaktion term	-0.0005	0.0020	7.96E-01
	rs6900017	A: overall	-0.0029	0.0279	9.19E-01
		B: adjusted for smoking	0.0052	0.0290	8.58E-01
		C: smoking interaktion term	0.0044	0.0040	2.70E-01
MMEF	rs833052	A: overall	-0.0241	0.0126	5.66E-02
		B: adjusted for smoking	-0.0280	0.0131	3.31E-02 ⁺
		C: smoking interaktion term	-0.0098	0.0040	1.49E-02 ⁺
	rs699947	A: overall	-0.0172	0.0080	3.33E-02 ⁺
		B: adjusted for smoking	-0.0157	0.0082	5.69E-02
		C: smoking interaktion term	-0.0002	0.0012	8.68E-01
	rs6900017	A: overall	-0.0443	0.0169	9.17E-03 ⁺
		B: adjusted for smoking	-0.0415	0.0174	1.79E-02 ⁺
		C: smoking interaktion term	0.0000	0.0024	9.96E-01