GENETIC VARIATION IN VASCULAR ENDOTHELIAL GROWTH FACTOR-A AND LUNG FUNCTION

Online data supplement

Angela Simpson MD , Adnan Custovic MD, Robert Tepper MD, Penelope Graves D.Sc., Debra A. Stern MS, Marcus Jones MD, Jenny Hankinson BSc, John A. Curtin PhD, Jiakai Wu PhD Mario Blekic MD, Blazenka Kljaic Bukvic MD, Neda Aberle MD Susana Marinho MD, Danielle Belgrave MSc, Wayne J. Morgan MD and Fernando D. Martinez MD

DESCRIPTION OF STUDY POPULATIONS, LUNG FUNCTION METHODS AND CLINICAL OUTCOMES USED

All lung function results are expressed as Z scores to allow comparison across the different measures used in the different studies and within studies at different time points. Z scores were derived separately for each population, based on measurements taken within that population in asymptomatic subjects.

All studies were approved by the Research Ethics Committees/Institutional Review boards. Informed consent was obtained from all parents and/or participants, and children gave their assent if appropriate. Only subjects of European ancestry were included.

Manchester Asthma and Allergy Study

The Manchester Asthma and Allergy Study is an unselected, population-based prospective study which follows the development of asthma and other atopic disorders in a cohort of children. The setting is the maternity catchment area of Wythenshawe and Stepping Hill Hospitals, comprising of 50 square miles of South Manchester and Cheshire, UK, a stable mixed urban-rural population. Study was approved by the Local Research Ethics Committee. Informed consent was obtained from all parents.

Screening & Recruitment

All pregnant women were screened for eligibility at 'Booking' antenatal visits (8th-10th week of pregnancy). The study was explained to the parents, and informed consent for initial questionnaires and skin prick testing was obtained. Both parents completed a questionnaire about their and their partner's history of asthma and allergic diseases and smoking habits.

If the pregnant woman's partner was not present at the antenatal clinic visit, an invitation was sent for him to attend an open-access evening clinic for skin prick testing and questionnaire. Once both parents had completed questionnaires and skin prick testing, a full explanation of the proposed future follow-up for the child was given.

Of the 1499 couples who met the inclusion criteria (<10 weeks of pregnancy, maternal age >18 years, questionnaire and skin test data available for both parents), 288 declined to take part in the study. Of 1211 couples who initially agreed to take part, 1185 had a successful full-term pregnancy (>36 weeks gestation) and gave consent to a further follow-up. Of those, 128 were prenatally randomized into an environmental control group, and the remainder were followed as the observational arm of the cohort. The children prenatally randomized to a stringent environmental control measures were excluded from this analysis.

Follow-up

The children have been followed prospectively, and attended review clinics at ages 3, 5, 8 and 11 years (±4 weeks).

Lung Function

At age 3 years, specific airway conductance was measured using whole-body plethysmography (Jaeger, Würzburg, Germany) by a single-step procedure from the simultaneously measured changes of respiratory flow and plethysmographic pressure, omitting the measurement of thoracic gas volume. Measurements were carried out during normal tidal breathing using a modified facemask (Astratech No 2; Astra, Denmark) fitted with a non-compressible mouthpiece. Three measurements of sGaw were performed, and each was calculated from the means of 5 consecutively measured technically acceptable loops (each child performed at least 15 loops). The median of these 3 measurements of effective sGaw was used in the analysis.

At age 5, 8 and 11 dynamic lung volumes and expiratory flow were performed according to ATS Guidelines(1) using a Lilly pneumotachograph system with animated incentive software (Jaeger, Germany). Subjects were asked to inhale to total lung capacity (TLC), then instructed to perform a forced expiration, through a mouthpiece, to residual volume (RV). The test was repeated at intervals of 30 seconds until 3 technically acceptable traces were obtained. Forced expiratory volume in one second (FEV1) and Forced vital capacity (FVC) were recorded. All children were asymptomatic at time of testing. β2-agonists were withheld for at least 4 hours prior to testing.

Definitions of outcomes and exposures Sensitisation and exposure status at age 3 years

Atopic sensitization was ascertained by skin prick testing at age 3, (*D pteronyssinus*, cat, dog, grasses, moulds, milk, egg [Bayer, Elkahrt, IN, USA]). We defined sensitization as a mean weal diameter 3mm greater than negative control to at least one of the allergens tested.

Dust sample collection

Reservoir dust samples were collected in a standardised fashion from the living room floor (LF), the sofa (S), the parental bedroom floor (PF) the parental mattress (PM), the child's floor and the child's mattress. Dust samples were collected by vacuuming a $1m^2$ area using a Medivac dust sampler (airflow 45 l/s; Medivac Plc, Wilmslow, Cheshire, UK) for 2 minutes in a standardised fashion. The sampling head was loaded with a stainless steel mesh filter, to remove particles >300μm diameter, allowing a sample of fine dust to be collected onto a 5 μm pore size vinyl filter (Plastok Associates Ltd, Wirral, UK). Immediately after collection, the dust sample was transferred into a pre-weighed Petri dish and coded. The filled Petri dish was weighed to calculate the mass of fine dust collected. After each sample collection the sampling head was cleaned using 70% isopropyl alcohol. A 100 mg aliquot of the dust was then extracted by rotation with 2 ml borate-buffered saline with 0.1% Tween 20 pH

8.0, at room temperature for 2 hours before being centrifuged for 20 minutes at 2500 rpm at 4oC. The supernatant was stored at -20^{o}C until analysed for allergen content.

Allergen Assays

All samples were assayed for content of major *Dermatophagoides pteronyssinus* allergen Der p 1 and for major cat allergen Fel d 1 using monoclonal antibody (mAb) based ELISAs and for major dog allergen Can f 1 using a monoclonal/polyclonal antibody-based ELISA. Der p 1 ELISA. The technique used was that described by Luczynska et al (2) and the monoclonal antibodies were supplied by Indoor Biotechnologies, courtesy of Dr MD Chapman, University of Virginia, Charlottesville, USA.

Tucson Children's' Respiratory Study (TCRS)

In infancy (Yr1), partial expiratory flow-volume curves were obtained at a mean±SD age of 2.3±1.9 months by the chest-compression technique, and the maximal expiratory flow at functional residual capacity (V'maxFRC) was recorded; technique details have been previously published (3, 4). At the time of the six-year survey (Yr6: 6.0±0.6 yr), partial maximal expiratory flow–volume curves were obtained with voluntary maneuvers as reported earlier (4). Briefly, tidal flow–volume loops were recorded on a computer screen. As the child approached end-tidal inspiration, he or she was encouraged to expel air forcefully, and a partial flow–volume curve was obtained. V'maxFRC was calculated from at least three acceptable expirations; the highest value obtained was used in our analyses. Spirometry was obtained according to American Thoracic Society standards(1) at ages 11 (Yr11: 10.9±0.7 yr), 16 (Yr16: 16.6±0.5 yr) and 22 (Yr22: 21.9±0.7 yr) using a custom built, pneumotachometer-based system running software on a portable computer at age 11 and a portable Schiller Spirovit SP-1 (Schiller AG, Baar, Switzerland) at ages 16 and 22. None of the children had used a bronchodilator within 6 hours of testing. Subsequent to baseline measurements, a fixed dose of 2 puffs of albuterol (180 µg) was administered from a metered-dose inhaler and aerochamber holding device (Monaghan Medical Corp, Plattsburgh, NY) and the post bronchodilator spirometry obtained after 15 minutes. Response to bronchodilator was calculated as 100* (post-BD - pre-BD) / (post-BD + pre-BD). Spirometry measures included the forced vital capacity (FVC, ml), forced expiratory volume in one second (FEV_1, ml), and the forced expiratory flow between 25 and 75% of the FVC ($FEF_{25–75}$, ml/s). The study nurses recorded height, weight, and age at the time of testing.

Indianapolis

Healthy infants and toddlers were recruited from advertisements in local publications. All subjects were born at a gestational age > 37 weeks, had no cardio-respiratory malformations, and their respiratory history was negative for wheezing, asthma, treatment with asthma medications, or hospitalization for a respiratory illness.

Infants (mean ±SD: 53.6±36.8 weeks, n=30) received 50-75 mg/kg of chloral hydrate orally, and testing was performed while the infant was sleeping in the supine position. Airway function was assessed using the raised volume rapid thoracic compression technique as previously described (5). Forced expiratory flows were initiated from a lung volume at which the airway pressure was 30-cm H_2O and proceeded to residual volume. Jacket compression pressures were progressively increased until no further increases in the flow volume curve were obtained and then three technically acceptable maneuvers were obtained. Forced expiratory flows were measured at 50% and 75% expired volume (FEF_{50} ml/s, FEF_{75} ml/s), between 25% and 75% expired volume (FEF_{25-75}).

Manchester adults

Dynamic lung volumes (forced expiratory volume in one second – FEV1, forced vital capacity – FVC) were measured on a wedge bellow spirometer (Vitalograph, UK) according to the ATS(1) guidelines. Testing was repeated until three technically acceptable traces were obtained. The highest FEV_1 and FVC were recorded. For all airway function measurements adults were asymptomatic at the time of the testing. β2-agonists were withheld for at least 6 hours prior to testing.

Croatian Children with Asthma

This Case-control study was carried out in the setting of the Department of Paediatrics, General Hospital Slavonski Brod, Croatia. The study was approved by the local ethics committee, and informed consent was obtained from all parents (and children when appropriate).Children with asthma aged 6 to 18 years (cases) were recruited into the study from the paediatric asthma clinic if the following criteria were met: (1) physician-diagnosed asthma, (2) asthma symptoms (wheeze, cough, or both) within the previous 12 months, and (3) current use of antiasthma medication. Children of the same age without respiratory symptoms (confirmed by an interviewer-administered questionnaire; controls) were randomly selected from patients with non-respiratory conditions attending the other hospital departments (e.g. fracture clinic); social and environmental variables matched with general population. Cases and controls were not matched by gender and age.

Lung function

Lung function was assessed using spirometer (Jaeger, Germany) to measure dynamic lung volumes, forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and forced expiratory flows (FEF_{25}, $FEF_{50,}$ FEF_{75}) according to the ATS(1).

Table E1 Description of Populations Studied

Description		Indiana	TCRS	MAAS	Manchester adults	Croatian children
Study design		Healthy population	Population- based birth cohort study	Population-based birth cohort study	Some parents of MAAS	Paediatric asthma cases
% with doctor diagnosed asthma		NA	8.8% Active Asthma at age 6 years	11.2% at age 8 years	20.1%	100%
% with atopy		NA	39.0% at age 6 years	30.6% at age 8 years	48.5%	
Infant lung function	n	30	57	NA	NA	NA
lung function measure		raised volume rapid thoracic compression technique (FEF_{50} ml/s, FEF_{75} ml/s, (FEF_{25-75})	maximal expiratory flow at functional residual capacity (V'maxFRC)	NA	NA	NA
Age		mean ±SD: 53.6±36.8 weeks	mean±SD age of 2.3±1.9 months	NA	NA	NA
Pre-school lung function	n	NA	NA	466	NA	NA
lung function measure		NA	NA	sG_{aw}	NA	NA
Age		NA	NA	3 years	NA	NA
Elementary school lung function	n	NA	266	660, 638	NA	NA
lung function measure		NA	partial maximal expiratory flow–volume curves (V'maxFRC)	FEV_1	NA	NA
Age		NA	6 years	5 years, 8 years	NA	NA
Middle school lung function	n	NA	292	656	NA	NA
lung function measure		NA	FEV_1/FVC	FEV_1/FVC	NA	NA
Age		NA	11 years	11 years	NA	NA
Adolescent /adult	n	NA	243, 230	NA	596	403
lung function measure		NA	FEV_1/FVC	NA	FEV_1/FVC	FEV_1/FVC

Age	NA	16 years, 22 years	NA	28-61 yrs	6-18yrs

Genotyping

SNP selection

The most frequent polymorphisms and haplotypes in the VEGF gene are described in the SeattleSNPs' Program for Genomic Applications website ((http://pga.mbt.washington.edu/). The website also describes 25 bins that group closely linked SNPs. To avoid an excessive number of comparisons, we selected 4 such bins (numbers 1, 3, 6 and 7, using the SeatlleSNP's nomenclature) that covered a high proportion of the information content in genetic variation in VEGF. The following SNPs were used as tags for these bins: rs833068 for bin 1; rs833070 for bin 3; rs3025028 and rs10434 for bin 6; and rs2146323 for bin 7. SNPs with high minor allele frequency in two other bins (bins 4 and 11) are in high linkage disequilibrium with SNPs in bin 6 and were not genotyped.

Additional Dense Genotyping

In the MAAS cohort, we then performed dense genotyping of the *VEGF-A* gene. From the 25 bins identified in GVS, we typed an additional 41 SNPs (excluding SNPS with MAF < 5%) making a total of 46 SNPs in MAAS. LD between SNPs is shown in Table E10

Genotyping Methods for the Tucson and Indianapolis samples

The SNPs were genotyped by restriction fragment length polymorphism (RFLP) analysis of PCR products. For determination of the VEGF polymorphism rs2146323, a 169 bp PCR fragment was generated using the primers: 5'-GAGTACATCGTGTGATCTCTG -3' and 5'-CTCTCTCCACCCAGCTCTGTAATTCC- 3'. The underlined base was changed to induce a restriction site. The PCR product was digested with the restriction enzyme Tsp509 I (New England Biolabs, Boston, MA) and the digest was electrophoresed on 3% agarose gels to separate the fragments. Fragments with the 6112C allele are only digested at a control site to produce fragments of 145 and 24 bp, while the A allele was cut into three fragments of 114, 31 and 24 bp. Genotypes for the VEGF SNP rs10434 were determined by amplification of a 292 bp fragment using the primers: 5'-GCTTCAGGACATTGCTGTGCTTTGG -3' and 5'-TGGTTTCAATGGTGTGAGGACA-3'. The product was digested using Hae III. A 98 bp control fragment was digested from a naturally occurring restriction site. If an A allele was present a 194 bp fragment was produced and the G allele digested into 154, 98, and 40 bp fragments.

Genotyping assays for two SNPs located very close together used PCR product generated from the same primers: 5' -GTGTCATTTCCTATATAGACATGTCACACTTGAGG-3' and 5'-CACAGCACCCGAACATACTC-3'. For rs833068 the 164 bp PCR product was digested using Tsp45 I. If the A allele was present only a control site was digested to produce 142 and 22 bp fragments. The G allele produced fragments of 124, 22, and 18 bp. The rs833070 polymorphism was genotyped by digesting the same PCR product with Sml I and the T allele was cut only at a control site into 135 and 29 bp fragments. The C allele was digested into three fragments of 113, 29, and 22 bp. PCR reactions were carried out in a total volume of 15 ul containing approximately 20 ng genomic DNA, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.5 mM MgCl2, 200 μM of each deoxynucleotide triphosphate, 30 ng of each primer, and 0.375 unit of Taq DNA polymerase.

As quality controls we ran 2 negative controls and 3 positive controls on each 96-well plate.

Genotyping Methods for the Manchester and Croatian populations

Genomic DNA was extracted from blood using phenol-chlorophorm method. Genotyping was performed using Sequenom® MassARRAY® iPLEX™ Gold system, combining a primer extension reaction chemistry with MALDI-TOF mass spectrometry(6-13). Genotyping calls were made with MassARRAY® Typer Analyser realtime software.SNP detection was performed using the Sequenom Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) data acquisition platform and iPLEX assay. PCR primers and probes were designed using the Sequenom iPLEX software provided by the manufacturer.

PCR reaction consisted of 2.850μl Nano-pure water, 0.625μl (10x stock) polymerase chain reaction (PCR) Buffer, 0.325μl MgCl2 (final conc 1.625mM), 0.1μl dNTP mix (500uM), 1μl Primer mix (500nM of each), Genomic DNA (20ng, dried down), 0.1μl Hotstar Taq (Qiagen, Germany) (0.5U/reaction) in a final reaction volume of 5μl. PCR amplification conditions were as follows; 94ºC for 15 minutes, 45 cycles of 94ºC for 20 seconds, 56ºC for 30 seconds, 72º C for 1 minute and a final extension step of 72º C for 3 minutes.The PCR product was cleaned using a Shrimp alkaline Phosphatase (SAP) mixture to dephosphorylate the unincorporated dNTPs. SAP treatment mixes consisted of the following; 1.530μL Nano-pure water, 0.170μl 10x SAP buffer, 0.300μl SAP enzyme (1U/μl) in a total reaction volume of 2μl.

The probes were split into low and high mass and were used at 0.625uM and 1.25uM respectively. The 200-short-cycle SBE-PCR program used two cycling loops, one of five cycles that sits inside a loop of 40 cycles. These two loops resulted in a 200 cycle program. The sample was denatured at 94ºC. Strands were annealed at 52ºC for 5 seconds and extended at 80ºC for 5 seconds. The annealing and extension cycle was repeated four more times for a total of five cycles and then looped back to a 94ºC denaturing step for 5 seconds, thus entering the 5 cycle annealing and extension loop

again. The five annealing and extension steps with the single denaturing step were repeated an additional 39 times for a total of 40. A final extension was performed at 72ºC for three minutes. SBE-PCR products were de-salted using 6mg of cleaning resin according to the manufacturer's instructions (Sequenom, Hamburg, Germany).

A nano-dispenser was used to dispense de-salted SBE-PCR reaction products onto a 384-element Spectro CHIP bioarray (Sequenom, Hamburg, Germany). Each well contained 15nl of purified SBE-PCR product. Samples were then analysed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

Western Blotting

None of the children and adults from Manchester whose samples were used for this part of the study was related.

The relative ratios of plasma VEGF-A165b to panVEGF-A165 protein levels were obtained using western blotting. Primary antibody specific for VEGFA165a and pan-VEGFA are tested in two separate runs of Western blotting (Fig E2)

Plasma samples (4µl) were mixed with 60µl ddH2O and 16µl 5x sample buffer and boiled for 1 min. The protein samples were resolved on 10% SDS PAGE and transferred to a nitrocellulose membrane. Equal transfer of protein were examined by Ponceau red stain (Fig.E3). After blocking with 1% milk (w/v), 0.15M NaCl, 0.1% Tween 20(w/v) for 4h in room temperature, membranes were exposed to the VEGF-A165b antibody (1:1000, ABcam: ab14994) at 4^{o}C overnight followed by incubation with a horseradish peroxidise-conjugated rabbit anti-mouse antibody (1:10000, a9044, Sigma). Signals were detected with ECL plus (PRN2132, GE healthcare) in a BioRad Universal Hood II. The intensity of the immunoreactive bands at 46kDa were analysed with Quantity One software and normalised to one common reference sample.

The membranes were stripped by incubating in 0.1M glycine, pH2 for 30 min with vigorous shaking. A complete stripping was optimized and examined by re-probe the membrane with the secondary antibody and ECL. After incubating in a blocking buffer for 4h, the membranes were reprobed with panVEGF-A165 primary antibody (1:1000, 512901 Biolegend) at 4^{o}C overnight and the band intensities for Pan-VEGFA protein were generated, quantified and normalised as described for VEGF-A165b.

In each blot, a random serum sample was used as a reference sample (Ref in FigE4a and E4b). Every sample from that blot is expressed as a relative ratio to that reference sample so that the background effect and length of ECL exposure between blots could be normalized.

The intensity of the VEGFA165b and Pan-VEGFA165 dimer were expressed as a relative ratio to the reference sample. The results were then expresses as a ratio in arbitrary units of VEGF-A165b : panVEGF-A165.

Investigation of Coverage of VEGF-A region in GWAS

DNA samples were genotyping on an Illumina 610 quad chip; genotypes were called using the Illumina GenCall application following the manufacturer's instructions. Quality control criteria for samples included: 97% call rate, exclusion of samples with an outlier autosomal heterozygosity (scree-plot visualization) and gender validation. Quality control criteria for SNPs included a 95% call rate, HWE > 5.9X10-7, minor allele frequency > 0.005.

Genotypes were imputed with IMPUTE version 2.1.2 with 1000 genomes and HapMap Phase 3 reference genotypes using the recommended parameters to ~6 million SNPs. In the 31KB region containing VEGF-A, imputed SNPs were converted from probabilistic to called genotypes when the probability of correct call was >= 0.9. All imputed SNPs were used regardless of imputation quality. LD (r2) was calculated between rs3025028 and the Illumina genotyped as well as the called imputed SNPs.

Statistical methods

In the Tucson and Manchester samples, each airway function measure was adjusted for covariates using multiple regressions, and the standardized residuals (as z-scores) were used as the outcome measures. In the Tucson sample, infant airway function was adjusted for length and sex; airway function at age 6 was adjusted for height, weight and sex at ages 11, 16 and 22 years was adjusted for height, weight, sex, and age. In the Manchester sample, sGaw was loge-transformed prior to analysis and adjusted for significant covariates identified in our previous studies (14), namely height, sex, allergen sensitization and level of exposure to sensitizing allergen. At ages 5 and 8 years, FEV_1 was adjusted for height and sex. In the Indianapolis sample, values from the best curve (highest product of FVC and FEF_{25-75}) were expressed as Z-scores from a reference population evaluated in our laboratory (5). The fit of the three genetic models (log-additive, recessive, or dominant) was evaluated based on the lowest value for Akaike's information criteria. Kruskall-Wallis rank test or T-test with unequal variances was used to assess differences between groups. P-values ≤ 0.05 were considered significant. Eta-squared was used to describe the variance explained in the dependent variable by genotype while controlling for other predictors. The original design of this study was to attempt replication of nominally significant results obtained in the Tucson Children's Respiratory Study, Manchester Asthma and Allergy Study and a study of infants in Indianapolis in the studies of adults from Manchester (UK) and asthmatic children from Croatia; therefore, no correction was made

for multiple comparisons. In the protein analysis, we used the normalized values of VEGF-A_{165b} and panVEGF-A_{165} to calculate a ratio. Data could not be normalized so non-parametric tests were used. Statistical analyses were carried out using SPSS for Windows, version 18.0 and STATA version 9.0.

Results

Table E2 Call rate and the Hardy-Weinberg Equilibrium p value for each SNP in each population

	MAAS		TCRS		Indiana		Manchester Adults		Croatian Children	
SNP	Call rate %	HWE p value	Call rate %	HWE p value	Call rate %	HWE p value	Call rate %	HWE p value	Call rate %	HWE p value
rs833068	96.0	0.65	97.5	0.43	100.0	0.49				
rs833070	93.2	0.64	96.9	0.50	100.0	0.24				
rs2146323	97.4	0.83	96.3	0.40	96.7	0.50				
rs3025028	93.7	0.94	96.3	0.49	100.0	0.48	93.8	0.10	97.0	0.67
rs10434	97.2	0.89	93.2	0.44	96.7	0.32	99.5	0.89	96.0	0.52

Table E3 Call rate and the Hardy-Weinberg Equilibrium p value for 46 SNPs genotyped in MAAS (available as an excel file which is accessible from this issue's table of content online at www.atsjournals.org).

The fit of the three genetic models (log-additive, recessive, dominant) was evaluated based on the lowest value for Akaike's information criteria (AIC). For each model, tested in each cohort for each measure of lung function the AICs are summarized in Tables E3 and E4

Table E4 AIC values rs3025028

Lung function measure	AIC CC vs. CG vs. GG	AIC CC+CG vs. GG	AIC CC vs. CG+GG
sGaw 3 years MAAS	1112.7	**1111.1**	1115.1
FEV_1 5 years MAAS	1503.8	1504.8	**1502.3**
FEV_1 8 years MAAS	1308.6	1311.7	**1307.3**
V'maxFRC 6 years TCRS	**721.95**	724.52	723.05
FEV_1/FVC Ratio 11 years TCRS	796.79	796.78	**796.78**
FEV_1/FVC Ratio 16 years TCRS	673.41	**672.71**	676.12
FEV_1/FVC Ratio 22 years TCRS	**630.55**	634.35	632.61
FEV_1/FVC ratio Manchester adults	1575.8	1577.6	**1574.4**
FEV_1/FVC ratio Croatian children	1102.4	1102.9	**1100.9**

Table E5 AIC values rs10434

Lung function measure	AIC AA vs. AG vs. GG	AIC AA + AG vs. GG	AIC AA vs. AG+GG
sGaw 3 years MAAS	1154.7	**1153.2**	1156.3
FEV_1 5 years MAAS	1560.1	1560.7	**1558.1**

FEV_1 8 years MAAS	1391.3	1391.8	**1389.5**
V'maxFRC 6 years TCRS	**706.82**	707.89	709.81
FEV_1/FVC Ratio 11 years TCRS	782.62	782.66	**782.58**
FEV_1/FVC Ratio 16 years TCRS	648.53	**648.01**	652.08
FEV_1/FVC Ratio 22 years TCRS	**599.04**	601.89	602.07
FEV_1/FVC ratio Manchester adults	1669.8	1674.9	**1667.8**
FEV_1/FVC ratio Croatian children	1082.4	1083.5	**1081.3**

Table E6 Genotype frequencies for *VEGF-A* SNPs in five populations

VEGF-A SNPs		Tucson		Indianapolis		Manchester children		Manchester adults		Croatia	
		n	%	n	%	n	%	n	%	n	%
rs833068	AA	35	10.3	5	16.7	81	10.4				
	AG	140	41.3	14	46.7	337	43.3				
	GG	164	48.4	11	36.7	360	46.3				
rs833070	CC	76	22.6	8	26.7	215	27.4				
	CT	166	49.3	18	60.0	379	48.3				
	TT	95	28.2	4	13.3	190	24.2				
rs2146323	AA	44	13.1	3	10.3	97	12.2				
	AC	147	43.9	13	44.8	356	44.8				

	CC	144	43.0	13	44.8	342	43.0				
rs3025028	CC	77	23.0	6	20.0	159	21.2	133	23.0	72	17.6
	CG	170	50.7	14	46.7	372	49.7	278	48.0	205	50.1
	GG	88	26.3	10	33.3	218	29.1	168	29.0	132	32.3
Rs10434	AA	78	24.1	5	17.2	178	22.7	142	23.5	69	17.3
	AG	165	50.9	17	58.6	393	50.1	303	50.2	206	51.8
	GG	81	25.0	7	24.1	213	27.2	158	26.2	123	30.9

Table E7 – Associations between SNPs in *VEGF-A* and lung function in infancy

		Z-scores of adjusted airway function										
VEGF-A SNPs		TCRS V'maxFRC Age 2.3 months				Indiana Age 3 months FEF_{25-75}				Indiana Age 3 months FEF_{50}		
		N	mean	95% CI	p	N	mean	95% CI	p	mean	95% CI	p
rs833068	AA	5	-0.22	-0.70, 0.27		5	-0.57	-2.03, 0.90		-0.41	-2.03, 1.21	
	AG	27	0.07	-0.30, 0.44		14	-0.60	-1.33, 0.12		-0.55	-1.31, 0.21	
	GG	24	0.03	-0.47, 0.53	0.85	11	-1.00	-1.94, -0.05	0.4	-0.69	-1.61, 0.21	0.7
rs833070	CC	15	-0.01	-0.44, 0.43		8	-0.35	-1.15, 0.44		-0.14	-1.03, 0.74	
	CT	30	-0.12	-0.53, 0.30		18	-0.94	-1.63, -0.25		-0.83	-1.51, -0.14	

	TT	12	0.30	-0.35, 0.96	0.49	4	-0.63	-2.94, 1.69	0.9	-0.35	-2.50, 1.81	0.8
rs2146323	AA	6	-0.19	-1.17, 0.79		3	-0.80	-5.10, 3.51		-0.58	-4.46, 3.30	
	AC	19	-0.41	-0.96, 0.15		13	-0.56	-1.11, -0.01		-0.50	-1.05, 0.05	
	CC	30	0.19	-0.11, 0.49	0.10	13	-0.63	-1.36, 0.10	0.9	-0.41	-1.23, 0.41	0.8
rs3025028	CC	14	0.38	-0.08, 0.85		6	0.13	-0.27, 0.52		0.39	-0.15, 0.93	
	CG	25	-0.28	-0.71, 0.15		14	-0.80	-1.41, -0.18		-0.82	-1.44, -0.21	
	GG	13	0.01	-0.52, 0.55	0.12	10	-1.19	-2.38, -0.00	0.13	-0.83	-2.02, 0.37	0.12
rs10434	AA	14	0.33	-0.13, 0.80		5	0.18	-0.31, 0.67		0.40	-0.32, 1.12	
	AG	28	-0.21	-0.66, 0.23		17	-0.77	-1.29, -0.25		-0.69	-1.23, -0.15	
	GG	12	-0.01	-0.60, 0.57	0.27	7	-0.81	-2.15, 0.52	0.21	-0.54	-1.93, 0.84	0.17

V'maxFRC adjusted for length and sex

FEF_{25-75} and FEF_{50} adjusted for length and expressed as Z-scores from a reference population evaluated in our laboratory

P value is for ANOVA model

Table E8. Airway function by *VEGF* SNPs at age 11 years MAAS and TCRS

			Z-scores of adjusted airway function*											
VEGF SNPs			MAAS Age 11yr FEV_1				MAAS Age 11 yr FEV_1/FVC				TCRS Age 11 yr FEV_1/FVC			
			n	mean	95%CI	p	n	mean	95%CI	p	n	mean	95%CI	p
rs833068	AA		70	-0.14	-0.30, 0.02		70	0.09	-0.15, 0.32		26	0.38	0.08, 0.67	
	AG		272	-0.04	-0.13, 0.04		272	-0.01	-0.13, 0.12		117	-0.07	-0.25, 0.10	
	GG		287	-0.08	-0.16, 0.00	0.81	286	-0.07	-0.19, 0.04	0.22	143	0.01	-0.16, 0.19	0.12
rs833070	CC		164	-0.06	-0.16, 0.04		164	-0.02	-0.18, 0.13		58	0.16	-0.09, 0.41	
	CT		292	-0.04	-0.13, 0.03		292	0.02	-0.10, 0.14		145	-0.07	-0.24, 0.09	
	TT		149	-0.10	-0.21, 0.01	0.58	149	-0.14	-0.30, 0.13	0.35	82	0.02	-0.21, 0.24	0.32
rs2146323	AA		76	-0.10	-0.25, 0.06		76	-0.04	-0.28, 0.19		38	-0.01	-0.32, 0.30	
	AC		265	-0.06	-0.14, 0.03		264	-0.01	-0.14, 0.11		129	-0.06	-0.25, 0.12	
	CC		277	-0..04	-0.12, 0..04	0.57	277	-0.03	-0.15, 0.09	0.99	114	0.07	-0.11, 0.25	0.59
rs3025028	CC		135	0.00	-0.12,		135	-0.06	-0.24,		70	--	-0.25,	

					0.11				0.11			0.004	0.24	
	CG		298	-0.08	-0.15, 0.00		297	-0.01	-0.12, 0.11		139	-0.03	-0.20, 0.143	
	GG		168	-0.07	-0.18, 0.03	0.42	168	0.05	-0.10, 0.21	0.32	70	0.004	-0.25, 0.24	0.98
rs10434	AA		154	-0.01	-0.12, 0.10		154	-0.08	-0.24, 0.09		70	-0.05	-0.29, 0.20	
	AG		320	-0.07	-0.14, 0.01		319	-0.01	-0.12, 0.10		136	-0.005	-0.17, 0.16	
	GG		159	-0.08	-0.19, 0.03	0.36	159	-0.02	-0.18, 0.14	0.62	66	-0.009	-0.27, 0.25	0.96

*TCRS: Adjusted for height and sex

*MAAS: Adjusted for height, age, sex

Table E9. Result of Westerns measuring ratio of plasma VEGF-A_{165b} to panVEGF-A_{165} in relation to genotype for rs3025028.

		Ratio of plasma VEGF-A_{165b} to panVEGF-A_{165}											
VEGF-A SNP		MAAS				Manchester adults				Croatian Asthmatic Children			
		N	mean	95% CI	p	N	mean	95% CI	p	n	mean	95% CI	p
rs3025028	CC	22	1.39	1.02-1.96		16	1.69	1.26-2.27		30	1.31	1.11-1.54	
	GG	23	0.73	0.54-0.97	0.004	13	0.93	0.71-1.22	0.004	31	1.01	0.86-1.17	0.02

Table E10 MAAS dense genotyping and lung function (available as an excel file which is accessible from this issue's table of content online at www.atsjournals.org).

Table E11 LD between rs3025028 and 45 other SNPs in MAAS (available as an excel file which is accessible from this issue's table of content online at www.atsjournals.org).

Figure E1: VEGF isoforms exon structure with highlighted antibody binding sites

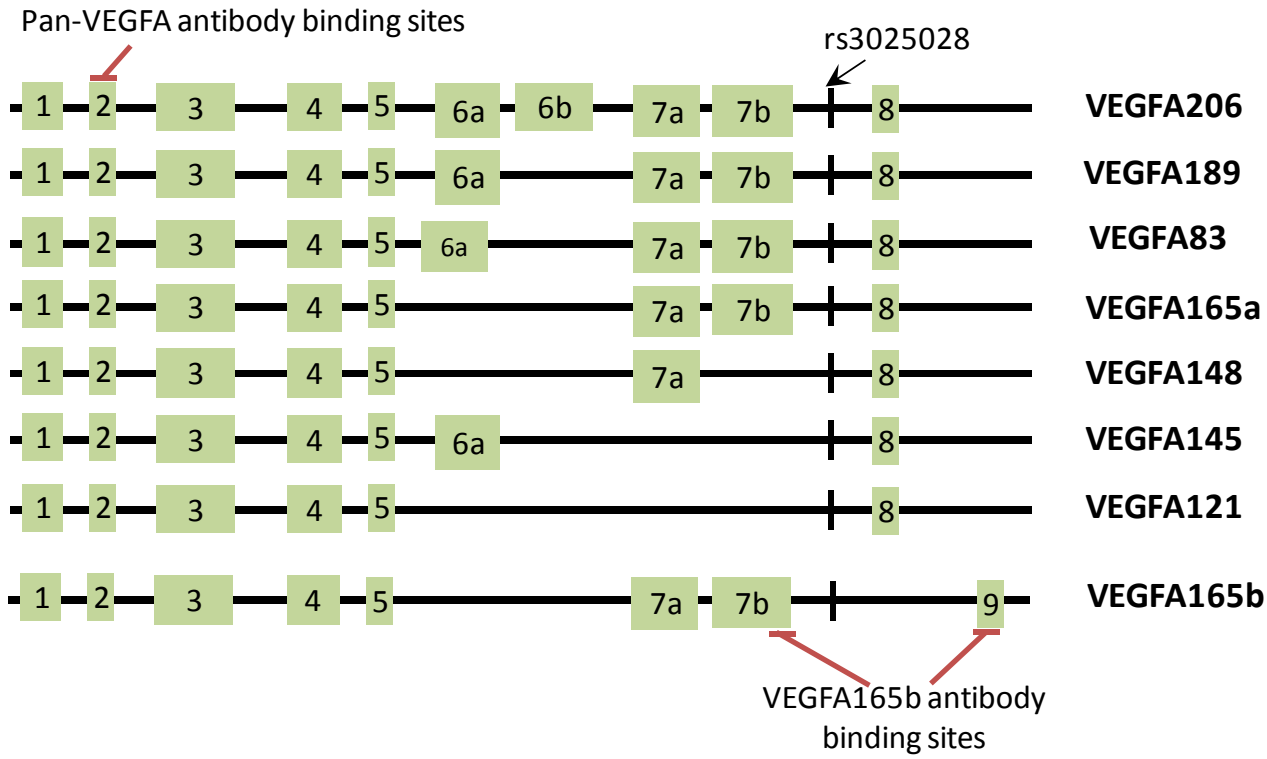
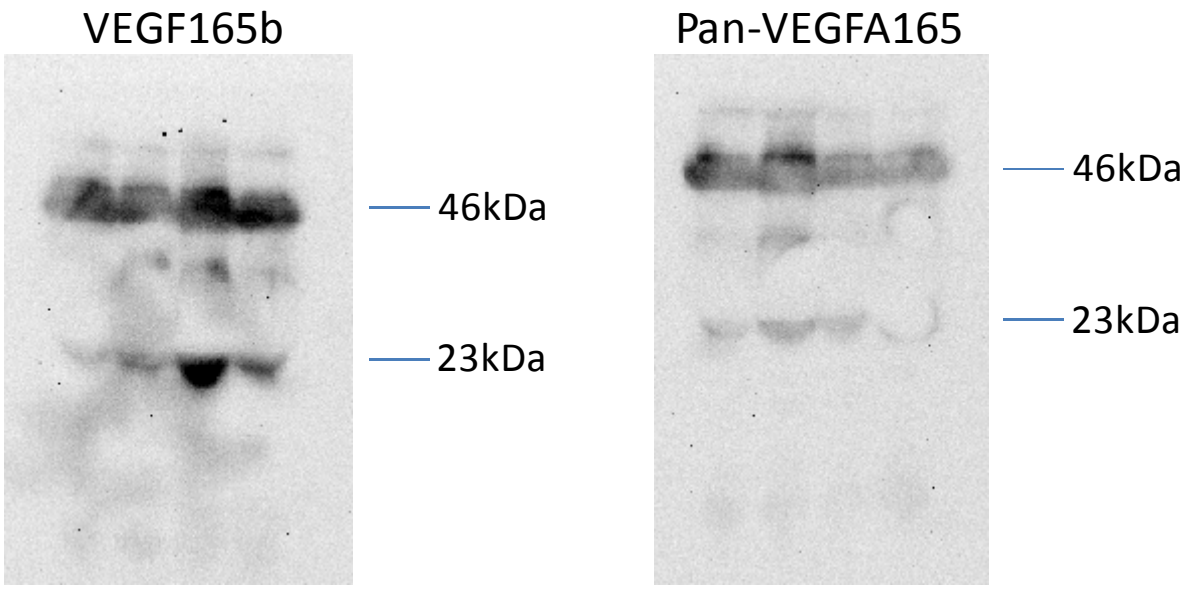


Illustration of the exon structure of all currently described active isoform and inhibitory isoform (VEGF165b) (not to scale) (Picture modified from (15)). Exon 6a and 6b and exon 7a and 7b encodes for heparin binding sites, this allow them binds to heparin and other ECM components. Isoforms such as VEGFA121, VEGFA145 and VEGFA148 don't have the heparin binding ability and only present in the secreted form, VEGFA165a and VEGF165b can be both heparin bound and secreted. Isoforms larger than VEGFA165 only exist in the heparin bound form. The only difference between VEGFA165a and VEGFA165b is the 6 amino acids at the COOH terminal sequence of the protein which are encoded by exon 8 and exon 9 respectively.

Fig.E2 VEGFA165b and pan-VEGFA antibody test on two separate runs of western blotting.



Fig,E3 Ponceau red stain after transfer protein from SDS-PAGE gel to nitrocellulose membrane

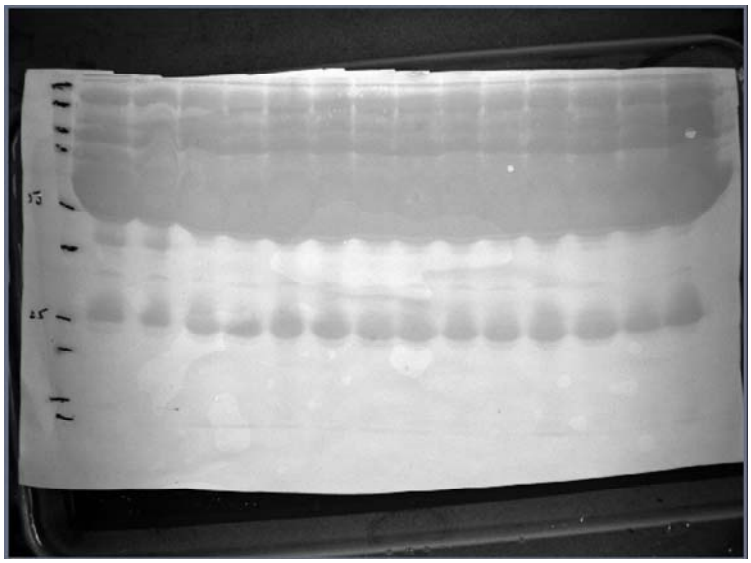


Fig.E4a representative blot of VEGFA165b, The densitometry values of the samples were normalised to one common reference sample (Ref) so that the quantified value is comparable between blots.

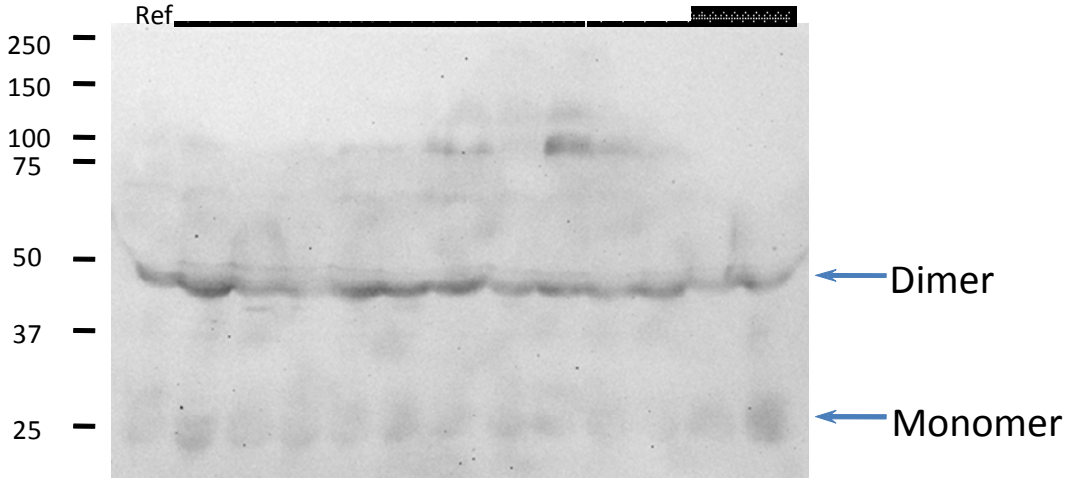


Fig. E4b representative blot of Pan-VEGFA

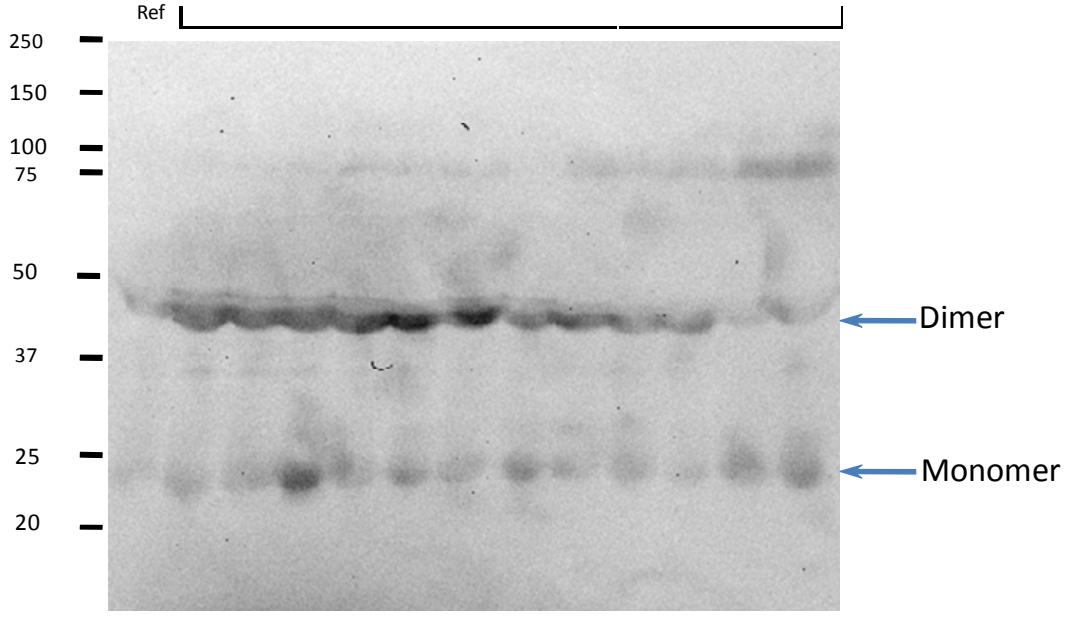


Figure E5. Linkage disequilibrium (r^2) for *VEGF* SNPs. LD was estimated using Visual Genotype 2 (http://gvs.gs.washington.edu/GVS/).

.

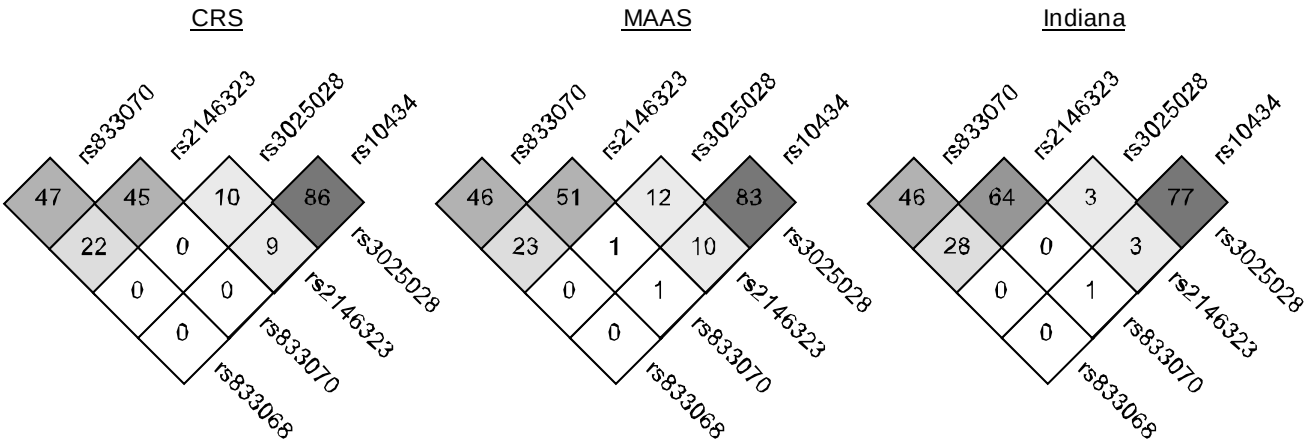
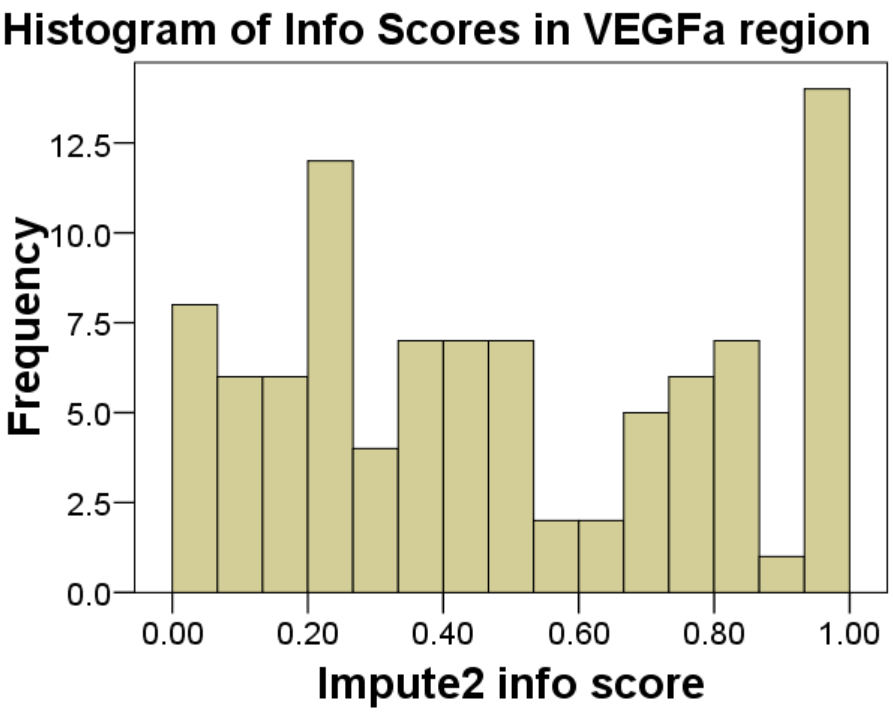


Figure E6

Histograms of Impute 2 info scores for individual SNPs. The info score is defined as a measure of the observed statistical information associated with the allele frequency estimate. This measure has a maximum value of 1 indicating perfect information. Panel a is the distribution of info scores of imputed SNPs from the *VEGF-A*, region (31kb). The majority of the SNPs are poorly imputed. Panel b is the distribution of info scores of imputed SNPs from a 5MB region that contains *VEGF-A*. Here the majority of SNPs are imputed well.

a)



b)

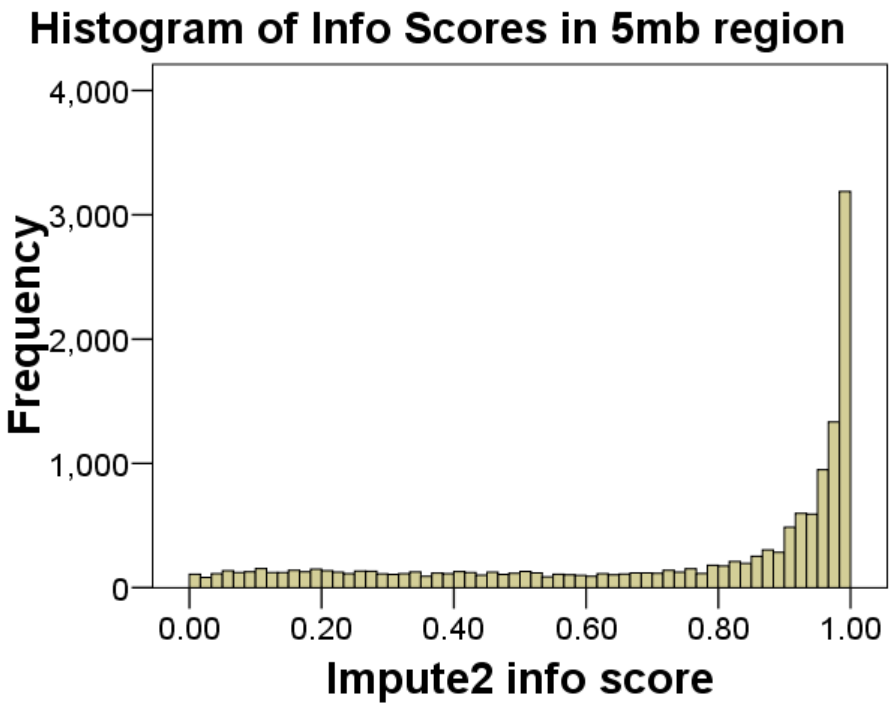
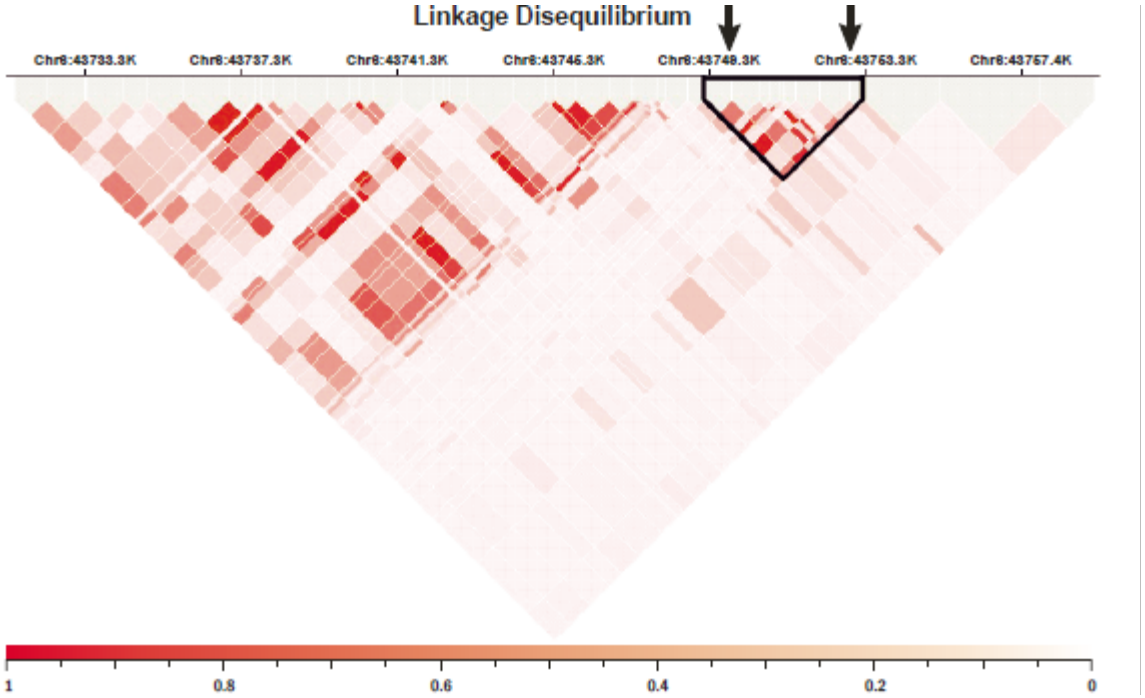

Histogram of Info Scores in 5mb region
Frequency
4,000
3,000
2,000
1,000
0
0.00
0.20
0.40
0.60
0.80
1.00
Impute2 info score

Figure E7. Plot of linkage disequilibrium of 46 densely typed SNPs in MAAS. Arrows indicate rs3025028 and rs 10434



References

1. Standardization of spirometry, 1994 update. American thoracic society. *Am J Respir Crit Care Med* 1995;152:1107-1136.

2. Luczynska CM, Arruda LK, Platts-Mills TA, Miller JD, Lopez M, Chapman MD. A two-site monoclonal antibody elisa for the quantification of the major dermatophagoides spp. Allergens, der p i and der f i. *J Immunol Methods* 1989;118:227-235.

3. Tepper RS, Morgan WJ, Cota K, Wright A, Taussig LM. Physiologic growth and development of the lung during the first year of life. *Am Rev Respir Dis* 1986;134:513-519.

4. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ, The Group Health Medical Associates. Asthma and wheezing in the first six years of life. *New England Journal of Medicine* 1995;332:133-138.

5. Jones M, Castile R, Davis S, Kisling J, Filbrun D, Flucke R, Goldstein A, Emsley C, Ambrosius W, Tepper RS. Forced expiratory flows and volumes in infants. Normative data and lung growth. *Am J Respir Crit Care Med* 2000;161:353-359.

6. Bray MS, Boerwinkle E, Doris PA. High throughput multiplex snp genotyping with maldi tof mass spectrometry: Practice, problems and promise. *Human Mutation* 2001;17:296-304.

7. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE. Multiplex pcr: Optimization and application in diagnostic virology. *Clin Microbiol Rev* 2000;13:559-570.

8. Ross P, Hall L, Smirnov I, Haff L. High level multiplex genotyping by maldi-tof mass spectrometry. *Nat Biotechnol* 1998;16:1347-1351.

9. Sauer S, Gut IG. Genotyping single-nucleotide polymorphisms by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;782:73-87.

10. Stanssens P, Zabeau M, Meersseman G, Remes G, Gansemans Y, Storm N, Hartmer R, Honisch C, Rodi CP, Bocker S, et al. High-throughput maldi-tof discovery of genomic sequence polymorphisms. *Genome Res* 2004;14:126-133.

11. Sun X, Ding H, Hung K, Guo B. A new maldi-tof based mini-sequencing assay for genotyping of snps. *Nucleic Acids Res* 2000;28:E68.

12. Tost J, Gut IG. Genotyping single nucleotide polymorphisms by mass spectrometry. *Mass Spectrom Rev* 2002;21:388-418.

13. Yang H, Wang H, Wang J, Cai Y, Zhou G, He F, Qian X. Multiplex single-nucleotide polymorphism genotyping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Biochem* 2003;314:54-62.

14. Lowe LA, Woodcock A, Murray CS, Morris J, Simpson A, Custovic A. Lung function at age 3 years: Effect of pet ownership and exposure to indoor allergens. *Arch Pediatr Adolesc Med* 2004;158:996-1001.