

Effects of glutathione S-transferase M1, T1 and P1 on lung function in asthmatic families

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Summary

Rationale Previous data have suggested that glutathione-S-transferase (GST) genotypes are important in determining the rate of lung function growth in childhood. This effect was most marked in Caucasian children with asthma.

Objectives We investigated the association of lung function with *GSTM1*, *GSTP1* and *GSTT1* genotypes in Caucasian families with asthma.

Methods Four hundred and eighteen children and 316 parents from 224 Caucasian families were recruited via a child with asthma, the proband. Associations between lung function and GST genotype were determined using multilevel models.

Results There were no observed associations between lung function and *GST* genotype in parents. However, in the children, the *GSTP1* val¹⁰⁵/val¹⁰⁵ and *GSTM1* null genotypes were associated with significantly higher forced expiratory volume in 1 s (FEV₁) and FVC values as percentage of predicted. This effect was not statistically significant in the probands but was marked in their siblings in whom *GSTP1* val¹⁰⁵/val¹⁰⁵ was associated with 9.4% higher FEV₁ and 10.7% higher FVC ($P = 0.005$ and 0.001 , respectively). The *GSTM1* null genotype was associated with a 6.7% higher FEV₁ and 4.1% higher FVC ($P = 0.003$ and 0.063 , respectively). These effects remained significant after correcting for the confounders of individual atopic status, tobacco smoke exposure and familial aggregation of lung function values.

Conclusions *GSTM1* and *GSTP1* genotypes are important determinants of lung function in childhood. The smaller differences seen in probands are predicted by a simple model in which more rapid decline in lung function is seen in these individuals.

Keywords asthma, child, glutathione transferase, respiratory function tests

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Introduction

Combinations of genetic and environmental factors are important in determining lung function throughout childhood. These begin to influence lung function prior to birth, as indicated by associations of adult lung function with exposure to maternal smoking [1] and with gestational age and birth weight [2]. Further effects are seen throughout childhood, with early respiratory infections, environmental tobacco smoke [3] and air pollution [4] all associated with reduced lung function growth. The genetic factors associated with lung function in childhood have been less extensively studied but are clearly important [5–7]. Twin studies give heritability estimates as high as 77% for monozygotic twins [8].

Candidate genes that may determine lung function growth include the glutathione S-transferases (GSTs). Functional polymorphisms in the *GSTM1* and *GSTP1* genes are associated with lung function growth in childhood [9]. These effects are largest in Caucasian children with asthma. GSTs are believed to be critical in the protection cells from reactive oxygen species (ROS) as they can utilize a wide variety of products of oxidative stress as substrates [10]. This may help to minimize inflammation as oxidative stress and the formation of ROS are key components of airway inflammation. The importance of this group of enzymes in human respiratory disease is underlined by associations between *GSTP1* genotype and asthma, airway hyper-responsiveness and acute respiratory illnesses in childhood [11–13].

Several common variants of GSTs exist. *GSTM1*, a member of the μ family located on chromosome 1p13, has a common null allele that results in no protein expression. Almost 50% of European Caucasians are homozygous for this allele [14]. *GSTP1* variants have been extensively studied in relation to asthma and asthma phenotype, in part because

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of its location on chromosome 11q13, a hotspot for asthma-related genes [15, 16]. Approximately 10% of the population are homozygous for an isoleucine → valine substitution at position 105 [14]. *GSTT1* is a member of the θ family located on chromosome 22q11 [17]. Like *GSTM1* it has a variant null genotype resulting in no protein expression with a frequency of approximately 20% in European Caucasians [14].

The Genetics of Asthma collaboration, a multi-centre study aimed at accurately defining and studying the asthma phenotype in children, offered an opportunity to investigate the effects of *GSTM1*, *GSTP1* and *GSTT1* genotypes on lung function in Caucasian families with asthma. We examined mean basal lung function to determine whether genetic variation in *GSTM1*, *GSTP1* or *GSTT1* was associated with basal forced expiratory volume in 1 s (FEV₁), FVC and FEV₁/FVC ratios in children or their parents. We hypothesized that variation in *GSTM1* and *GSTP1* but not *GSTT1* would be associated with significant differences in lung function in children and adults in the study.

Methods

Study subjects

Two hundred and twenty-four Caucasian families were recruited via an asthmatic proband (aged 7–18 years) from two centres (North Staffordshire and Sheffield) between January 1999 and December 2001. Inclusion criteria for probands included documented episodes of wheezing in the past year, physician diagnosis of asthma and agreement to participate of the natural parents. Identical twins were not eligible to participate and subjects with cardiac, other respiratory or inflammatory diseases were excluded. The study was approved by the local research ethics committee at each centre and written informed consent was obtained from all participants.

Collection of demographic, questionnaire and lung function data was as described previously [13, 18, 19].

Laboratory methods

GSTP1, *GSTM1* and *GSTT1* genotypes were determined as previously described [11, 13, 14, 20]. Re-assay of 15% of samples selected at random led to no reassignment of genotype. *GSTM1* and *GSTT1* genotyping was unsuccessful in 13 probands and 23 siblings.

Statistical analysis

Our primary analysis was carried out using multilevel models generated with the software MLwiN (Institute of Education version 1.10.0007) [21]. Mixed models, i.e. those containing fixed and random components for the prediction of FEV₁ and FVC, were obtained with genotypes as predictors and corrected for potential confounders (environmental tobacco smoke exposure and atopic status) and allowing the intercept in the regression to be random at the family level. This approach enabled all children to be included in the analysis.

Our secondary analysis used more prosaic statistical techniques but allowed only one child from each family to be considered. Children from each family were identified as

probands or siblings prior to analysis. In families for whom data for more than one sibling were available, one was selected at random for analysis (the 'one sibling' group). Associations of individual *GSTM1*, *GSTT1* and *GSTP1* genotypes and continuous variables (FEV₁, FVC and FEV₁/FVC) were assessed using the Mann–Whitney *U*-test. Multiple linear regression was undertaken to determine the relationships between lung function, genotype combinations and potential confounding factors including parental smoking, smoke exposure during pregnancy and atopic status. These statistical analyses were performed using the Stata statistical package (version 8.0: Stata corporation, College Station, TX, USA).

Results

Of the 224 Caucasian families recruited, 144 were resident in North Staffordshire and 80 in Sheffield. Complete phenotypic data, including lung function, serum IgE and skin prick testing, were available on 222 probands, 159 mothers, 157 fathers and 196 siblings. The characteristics of these groups are presented in Table 1. Probands were younger, more atopic and had lower FEV₁/FVC ratios. All probands and 57.1% of the siblings had asthma. Of children with asthma, severe or moderately severe asthma was more common than mild asthma in probands. As expected, there was no difference in exposure to environmental tobacco smoke between probands and siblings.

GSTM1 and *GSTT1* null genotypes were present in 52.2% and 24.9% of probands, 55.5% and 27.0% of siblings and 50.0% and 20.1% of parents, respectively. *GSTP1* genotype frequencies in parents and children were similar to those reported in control subjects from North Staffordshire [14] and did not significantly deviate from Hardy–Weinberg equilibrium (Table 2).

In the multilevel model we found that *GSTM1* null and *GSTP1* val¹⁰⁵/val¹⁰⁵ genotypes were associated with increases in FEV₁ and FVC but not FEV₁/FVC ratios in all children (Table 3). The associations between increased lung function and *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype remained significant after correction for environmental tobacco smoke exposure and atopic status. Contrary to our initial expectations, these effects were less pronounced or even absent in the probands. However, in the sibling group all the reported associations reached statistical significance. In the siblings the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype was associated with a 9.6% increase in FEV₁ ($P = 0.005$) and a 10.7% increase in FVC ($P = 0.001$). Similarly, in the siblings the *GSTM1* null genotype was associated with a 6.7% increase in FEV₁ ($P = 0.003$) and a 4.1% increase in FVC ($P = 0.063$). These results were only modestly influenced by correction for passive smoking, personal smoking and atopic status within the model and all the reported associations were statistically significant following correction ($P < 0.05$).

In our confirmatory analysis, using the Mann–Whitney *U*-test and one child from each family, the associations between lung function and genotype also only reached statistical significance in the sibling group (Table 4). We compared the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype against the *GSTP1* val¹⁰⁵/ile¹⁰⁵

Table 1. Demographics of the study population

	Probands	Siblings	Mothers	Fathers
Number	222	196	159	157
Mean age in years $\pm$ SD	12.4 $\pm$ 3.9	14.3 $\pm$ 5.3	41.1 $\pm$ 6.2	44.1 $\pm$ 7.0
Male gender (%)	133 (59.9)	103 (52.5)	0 (0)	157 (100)
Atopic (%)	185 (83.3)	139 (70.9)	101 (63.5)	111 (71.2)
SPT positive (%)	162 (73.0)	111 (56.6)	77 (48.4)	98 (62.4)
IgE $\geq$ 100 IU/mL (%)	146 (65.8)	99 (50.5)	50 (31.4)	60 (38.2)
Current asthma (%)	222 (100)	112 (57.1)	65 (40.9)	69 (43.9)
Mild or intermittent asthma (%)	127 (57.2)	79 (40.3)	41 (25.8)	48 (30.6)
Moderate or severe asthma (%)	95 (42.7)	33 (16.8)	24 (15.1)	20 (12.7)
Smoking history (ever) (%)	8 (3.6)	12 (6.1)	41 (25.8)	53 (33.8)
Environmental tobacco smoke (%)	86 (38.7)	90 (45.9)	72 (45.3)	69 (43.9)
FEV ₁ as % predicted $\pm$ SD	98.5 $\pm$ 16.5	99.9 $\pm$ 15.1	97.8 $\pm$ 17.0	96.0 $\pm$ 20.5
FVC as % predicted $\pm$ SD	101.1 $\pm$ 14.4	101.4 $\pm$ 14.7	101.6 $\pm$ 14.3	97.3 $\pm$ 17.5
FEV ₁ /FVC as % predicted $\pm$ SD	97.9 $\pm$ 10.2	101.1 $\pm$ 8.8	100.6 $\pm$ 11.3	100.8 $\pm$ 9.6

SPT, skin prick test; IgE, immunoglobulin E; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Table 2. Allele and genotype frequencies for *GSTM1*, *GSTP1* and *GSTT1* among the study population

	Probands	Siblings	Parents
Genotypes	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
<i>GSTP1</i>			
Ile ¹⁰⁵ /Ile ¹⁰⁵	103 (46.4)	99 (50.5)	139 (44.0)
Ile ¹⁰⁵ /Val ¹⁰⁵	92 (41.4)	75 (38.3)	144 (45.6)
Val ¹⁰⁵ /Val ¹⁰⁵	27 (12.2)	22 (11.2)	33 (10.4)
<i>GSTM1</i>			
Null	109 (52.2)	96 (55.5)	145 (50.0)
Positive	100 (47.8)	77 (44.5)	145 (50.0)
<i>GSTT1</i>			
Null	52 (24.9)	47 (27.0)	56 (20.1)
Positive	157 (75.1)	127 (73.0)	222 (79.9)

and *GSTP1* ile¹⁰⁵/ile¹⁰⁵ genotypes throughout as the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype alone has been shown to influence lung function [9] and risk of asthma [11, 12, 22]. Siblings with the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotypes had FEV₁ and FVC values (as percentage of predicted) 8.6–9.9% higher than those with the *GSTP1* ile¹⁰⁵/val¹⁰⁵ or *GSTP1* ile¹⁰⁵/ile¹⁰⁵ genotypes, and the *GSTM1* null genotype was associated with a smaller but statistically significant increase of 6.2% in FEV₁. These effect sizes are of a similar magnitude to those predicted in the multilevel model. In contrast, there were no clear associations between lung function and any GST genotype in the parents (lowest *P*-value = 0.129).

In common with previous reports, *GSTM1* and *GSTP1* genes appear to be operating through independent biologic pathways [9]. In models generated through multiple regression increases in FEV₁ and FVC in siblings associated with the *GSTM1* null and *GSTP1* val¹⁰⁵/val¹⁰⁵ genotypes were approximately additive (Table 5). Moreover, the magnitude and direction of the associations between genotype and lung function within the proband group were highly consistent with those predicted by the multilevel model (i.e. *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype is associated with a 3.5% increase in FEV₁ in the regression model and a 3.8% increase in the multilevel model).

Discussion

We found that children with the *GSTM1* null genotype and those that were homozygous for the *GSTP1* val¹⁰⁵ variant allele had higher lung function than children with the more common alleles at the two loci. This resulted in statistically significant associations between FEV₁ and FVC (expressed as percentage of predicted) and the *GSTP1* and *GSTM1* genotypes in the siblings but *not* the probands recruited to our study.

The lack of any association between the *GSTT1* genotype and lung function is an important negative finding. This gene is not expressed within the lung [23] and although it may be important in other tissues our data suggest that this gene has little or no part in determination of lung function. This is consistent with previous reports [9].

Based on reports of lung function growth we had expected to find a reduction in lung function in individuals with the *GSTM1* null and the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype. Moreover, we had expected these effects to be largest in children with asthma. However, our results showed a clear and statistically significant effect opposite to that initially predicted. Lack of clear associations between adult lung function and *GSTM1* or *GSTP1* genotype were also difficult to explain. Crucially, our initial hypothesis assumed that lung function would be the same in both groups during early childhood.

Critical examination of the data led us to a revised hypothesis which takes account of all the available information. We propose that *GSTP1* val¹⁰⁵/val¹⁰⁵ and *GSTM1* null genotypes are associated with slower lung function growth, but that this represents a 'normalisation' of lung function for these groups of individuals who start early childhood with *higher* lung function values (Fig. 1). It has already been shown that this return towards the mean is quicker for children with asthma (identified as probands). Hence, associations between lung function and GST genotype are likely to be weakest in this group.

The size of the perceived association between genotype and lung function in this study is clinically significant. Siblings

Table 3. Effect of GST genotype on percentage predicted lung function in multi-level model

	All children <i>n</i> = 418	Probands <i>n</i> = 222	Siblings <i>n</i> = 196
<i>FEV₁</i>			
<i>GSTP1</i> val ¹⁰⁵ /val ¹⁰⁵	+6.60 ± 2.50	+4.46 ± 3.38	+9.64 ± 3.41
<i>P</i> -value different	0.008	0.190	0.005
Corrected effect*	+6.73 ± 2.50	+3.82 ± 3.42	+10.71 ± 3.51
<i>P</i> -value different	0.007	0.263	0.002
<i>GSTM1</i> null	+2.64 ± 1.66	+0.11 ± 2.27	+6.68 ± 2.29
<i>P</i> -value different	0.112	0.960	0.003
Corrected effect*	+2.47 ± 1.66	+0.17 ± 2.26	+6.43 ± 2.32
<i>P</i> -value different	0.136	0.936	0.005
<i>GSTT1</i> null	+0.08 ± 1.86	+1.70 ± 2.54	−1.23 ± 2.57
<i>P</i> -value different	0.968	0.503	0.631
Corrected effect*	+0.38 ± 1.86	+1.80 ± 2.50	−2.35 ± 2.65
<i>P</i> -value different	0.842	0.472	0.374
<i>FVC</i>			
<i>GSTP1</i> val ¹⁰⁵ /val ¹⁰⁵	+5.90 ± 2.28	+1.99 ± 2.96	+10.73 ± 3.32
<i>P</i> -value different	0.010	0.503	0.001
Corrected effect*	+5.61 ± 2.31	+2.55 ± 3.02	+9.81 ± 3.44
<i>P</i> -value different	0.015	0.401	0.004
<i>GSTM1</i> null	+2.01 ± 1.52	+0.09 ± 1.99	+4.14 ± 2.21
<i>P</i> -value different	0.187	0.960	0.063
Corrected effect*	+2.20 ± 1.53	+0.47 ± 2.00	+4.52 ± 2.22
<i>P</i> -value different	0.150	0.810	0.041
<i>GSTT1</i> null	−0.63 ± 1.68	+0.16 ± 2.21	−0.87 ± 2.48
<i>P</i> -value different	0.704	0.944	0.726
Corrected effect*	−0.91 ± 1.71	+0.29 ± 2.20	−1.82 ± 2.56
<i>P</i> -value different	0.596	0.897	0.478

GST, glutathione S-transferase; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Lung function expressed as increment of percentage predicted value associated with genotype ± standard error, i.e. *GSTP1* val¹⁰⁵/val¹⁰⁵ is associated with a 10.73% higher FVC than other genotypes.

*Corrected value indicates magnitude of effect ± standard error following correction for tobacco smoke exposure and atopic status.

with a *GSTM1* null and *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype have an almost 15% higher FEV₁ and FVC than children with other genotypes. Although the relationship of lung function to structure is variable, this would suggest that individual *GSTM1* and *GSTP1* genotypes are important determinants of early lung development, while previously reported associations between GST genotype and lung function growth suggest a role in lung protection or repair. This type of dual genetic function has also been seen with the *ADAM33* gene – suggesting roles in both lung development and repair [24, 25]. Like *ADAM33*, the *GSTP1* and *GSTM1* genes are expressed extensively in fetal lung tissue during a critical stage in lung development [26, 27].

There are several important limitations of our study. Firstly, it is impossible to correct for all the environmental and individual factors that potentially influence lung function measurements; some of these were not recorded and others remain unknown. However, multilevel models incorporating individual atopic status, smoking and environmental tobacco smoke exposure showed a persistence of the reported genetic associations. Although both gender and asthma status can also influence lung function, the associations between *GSTP1* and *GSTM1* genotypes and lung function in the sibling group remained significant following further correction for these variables in multilevel models (*P* = 0.001 and 0.002 for FEV₁ and *P* = 0.002 and 0.014 for FVC). Moreover, the genetic effects are best described by an additive model, suggesting a

degree of biological independence. Secondly, our data are cross-sectional and yet lung function normally is subject to increases throughout childhood. Consideration of lung function as a percentage of predicted value for height and gender helps to standardize the values for the size of the child, although this also has its limitations. Consequently, we also analysed the data using raw (uncorrected) lung function values. These demonstrated reduced but broadly similar values for the observed associations, although the associations with *GSTM1* failed to achieve statistical significance. This is unsurprising given the wide age-range of the patients studied and its impact on the range of uncorrected lung function values. Finally, we studied a highly selected population of asthmatic children. There is a potential for bias introduced by the tendency of families with more severe asthma to be participants in genetic studies. Nonetheless, our objective asthma severity score revealed a wide range of asthma severities in subjects with over half the probands having mild or intermittent asthma. The relative frequency of mild asthma within our cohort is confirmed by the near-normal lung function values seen in the children taking part in the study. However, the number of children with abnormal lung function (FEV₁ < 80% predicted) is similar to that seen in other European genetic studies (15.8% of children in the EGEA study compared with 12.2% of children in our cohort) [28].

The likely biological importance of *GSTP1* in determining lung development has been recently confirmed in animal

Table 4. Associations of lung function by genotype in families

	Probands <i>n</i> = 222	One sibling <i>n</i> = 135
<i>FEV</i> ₁		
<i>GSTP1</i> val ¹⁰⁵ /val ¹⁰⁵	102.4 ± 12.7	107.3 ± 11.2
<i>GSTP1</i> other	97.9 ± 16.9	98.7 ± 15.4
<i>P</i> -value different	0.201	0.013
<i>GSTM1</i> null	98.4 ± 18.6	102.4 ± 14.6
<i>GSTM1</i> present	98.4 ± 13.6	96.2 ± 15.8
<i>P</i> -value different	0.594	0.012
<i>GSTT1</i> null	97.5 ± 18.0	100.0 ± 13.5
<i>GSTT1</i> present	99.2 ± 15.2	101.3 ± 15.5
<i>P</i> -value different	0.568	0.703
<i>FVC</i>		
<i>GSTP1</i> val ¹⁰⁵ /val ¹⁰⁵	102.9 ± 10.6	110.0 ± 14.2
<i>GSTP1</i> other	100.9 ± 14.9	100.1 ± 15.0
<i>P</i> -value different	0.396	0.015
<i>GSTM1</i> null	101.1 ± 15.5	103.4 ± 13.7
<i>GSTM1</i> present	101.3 ± 13.1	98.3 ± 16.6
<i>P</i> -value different	0.730	0.075
<i>GSTT1</i> null	101.3 ± 13.5	100.7 ± 12.3
<i>GSTT1</i> present	101.4 ± 14.0	102.6 ± 15.5
<i>P</i> -value different	0.930	0.508

GST, glutathione-S-transferase; *FEV*₁, forced expiratory volume in 1 s; *FVC*, forced vital capacity.

All lung function values expressed as percentage of predicted value ± standard deviation.

studies. A murine model for studying *GSTP1* exists, the *GSTP1* null mouse [29]. While most of the organ weights of the *GSTP1* null mouse are slightly but not significantly heavier than those of non-transgenic controls, the lungs are more than twice the weight of matched controls [30]. In animal models therefore the *deletion* of a gene pivotal to

Table 5. Relationship between GST genetic variation, tobacco smoke exposure, atopic status and lung function: multiple linear regression

<i>FEV</i> ₁	Probands <i>n</i> = 222		One sibling <i>n</i> = 135	
		<i>P</i> -value		<i>P</i> -value
<i>GSTP1</i> val ¹⁰⁵ /val ¹⁰⁵	+3.53 ± 3.50	0.314	+7.96 ± 3.87	0.042
<i>GSTM1</i> null	+0.38 ± 2.32	0.871	+6.53 ± 2.62	0.013
<i>GSTT1</i> null	+1.58 ± 2.67	0.555	−1.11 ± 3.07	0.717
Passive smoking*	−2.26 ± 2.42	0.353	−2.73 ± 2.91	0.349
Active smoking	−5.65 ± 7.04	0.423	+0.46 ± 5.43	0.933
Atopic status	−7.19 ± 3.09	0.021	−4.57 ± 3.08	0.140
<i>FVC</i>				
<i>GSTP1</i> val ¹⁰⁵ /val ¹⁰⁵	+1.81 ± 3.06	0.554	+8.40 ± 3.75	0.027
<i>GSTM1</i> null	−0.18 ± 2.03	0.931	+6.27 ± 2.53	0.015
<i>GSTT1</i> null	+0.18 ± 2.33	0.940	−2.31 ± 2.96	0.437
Passive smoking*	+0.55 ± 2.12	0.797	−4.58 ± 2.81	0.106
Active smoking	−7.55 ± 6.15	0.221	+7.66 ± 5.25	0.147
Atopic status†	−1.47 ± 2.70	0.585	−0.12 ± 2.97	0.969

GST, glutathione-S-transferase; *FEV*₁, forced expiratory volume in 1 s; *FVC*, forced vital capacity.

Effects of each variable on lung function expressed as increment of percentage of predicted value associated with genotype ± standard error.

*At least one current smoker living in the household.

†Atopy defined as a positive response to SPT (≥1 weal ≥3 mm greater than saline response to a panel of seven aeroallergens) or a total serum IgE ≥100 IU/mL.

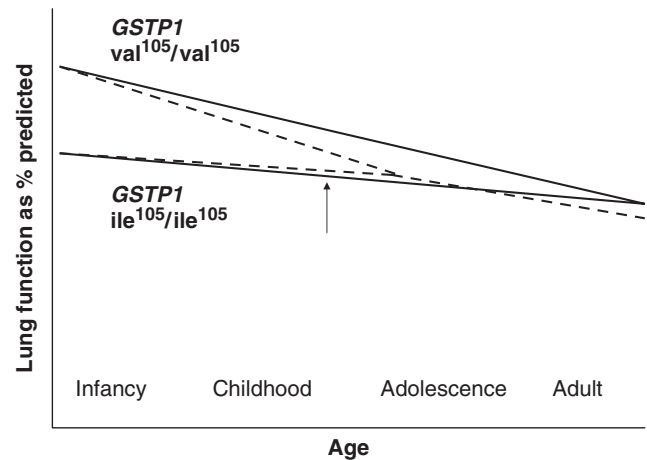


Fig. 1. Proposed model of glutathione-S-transferase (GST) genetic variation on lung function and growth. Compared with other genotypes lung function starts higher but declines more rapidly for individuals with the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype, becoming roughly equal by adult life. Decline in lung function is more rapid in asthmatic children (dashed line) resulting in smaller differences in lung function between genotypes in late childhood (arrow).

oxidative stress seems to increase lung size. While this event is unexpected it highlights the complexity of the biological systems regulating oxidative stress. Moreover, this observation lends a degree of biological plausibility to the surprising observation that a *GSTM1* null genotype is associated with increased lung function in childhood.

Further follow-up of the initial Genetics of the Asthma cohort will be necessary in order to test the hypothesis generated by these observations. Nonetheless, these data reiterate the importance of GST genes and hence indirectly oxidative stress as potential determinants of childhood lung function.

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Appendix 1. Additional statistical notes

Analysis of data from family studies has to take into account the correlation of measurements of lung function between individuals within each family. This precludes the use of standard regression techniques which assume independence of the observations. There are at least two possible statistical approaches to this problem, each with potentially important limitations. We chose to apply both of these approaches to our data.

In the first, analysis was carried out using multilevel models using the software MLwiN (Institute of Education version 1.10.0007) [A1]. This type of data is known as hierarchical or nested data and in this case measurements are nested within two levels namely at the family and the individual level. Mixed models, i.e. those containing fixed and random components for the prediction of FEV₁ and FVC, were obtained with genotypes as predictors and corrected for potential confounders (environmental and personal tobacco smoke exposure and atopic status) and allowing the intercept in the regression to be random at the family level. This approach has the advantage of allowing all children to be included in the analysis. This effect on sample size has obvious implications for the statistical power of our study.

The second applicable methodology adopted more prosaic statistical techniques but allowed only one child from each family to be considered. As previous reports had suggested a larger effect in asthmatic children, children from each family were identified as probands or siblings prior to analysis. In families for whom data for more than one sibling were available, one was selected at random for analysis. A random number list was generated using the Stata statistical software and assigned to each child. In families with more than two children, the sibling with the highest random number was

included in the analysis. This is referred to as the 'one sibling' group in Table 4. Associations of individual *GSTM1*, *GSTT1* and *GSTP1* genotypes and continuous variables (FEV_1 , FVC and FEV_1/FVC) were assessed using the Mann–Whitney *U*-test. We compared the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype against the *GSTP1* val¹⁰⁵/ile¹⁰⁵ and *GSTP1* ile¹⁰⁵/ile¹⁰⁵ genotypes throughout as the *GSTP1* val¹⁰⁵/val¹⁰⁵ genotype alone has been shown to influence lung function [A2] and risk of asthma [A3–A5].

Multiple linear regression was undertaken to determine the relationships between lung function, genotype combinations and potential confounding factors including parental smoking, smoke exposure during pregnancy and atopic status. This approach had the advantage of relative simplicity, but as it reduces the number of subjects available for study it reduces the accuracy of the estimates of the regression parameters. However, it did allow lung function data for each genotype to be expressed as percentage predicted values and allowed an internal validation of methodologies by comparison of the results for the proband group. Thus, we were able to directly compare the results for the probands of multiple regression (Table 5) and the multilevel model (Table 3). These are highly

consistent. These statistical analyses were performed using the Stata statistical package (version 8.0: Stata corporation).

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