

Correction of bronchial challenge data for age and size may affect the results of genetic association studies in children

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Meaningful studies of asthma genetics require careful definition of airway hyperresponsiveness (AHR). In children, several studies have emphasized the need for correction of bronchial challenge data for baseline parameters, such as age, gender, lung function and atopic status, when undertaking airway responsiveness measurements. However, few studies have suggested how this should be performed in practice. This study describes a method for the correction of dose-response slopes (DRS) and PC₂₀ values for baseline parameters in children, and illustrates the effect of such corrections on the association of AHR with the glutathione S-transferase GSTP1 Ile105Val polymorphism in children. Skin prick and methacholine challenge testing, measurement of total serum IgE concentration and GSTP1 genotyping were performed in 145 unrelated British children aged 7–18 years. Correction of bronchial challenge results, expressed as both DRS and PC₂₀ values, for age, gender, baseline lung function and atopic status was performed using linear regression and discriminant analysis, respectively. Adjusting bronchial challenge results for the age and size of the child altered AHR status, defined as a PC₂₀ methacholine < 8 mg/ml, in 37% of children. Correction for baseline parameters also resulted in a significant reduction in mean DRS (original uncorrected DRS 83.6, corrected DRS_c 27.4). This had a marked effect on the results of the association study, unmasking a previously unidentified association between the GSTP1 genotype and AHR in children. Age and size adjustment of bronchial challenge data has a significant effect on AHR status and may influence the results of genetic association studies in children.

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Studying the relationship between genetic variation, environmental factors and specific asthma phenotypes, especially in children, is difficult because there is no standard definition of the disease. Accordingly, most authors have based their definitions of asthma phenotype on a combination of objective and subjective factors (1–4). There is agreement that the asthma phenotype is best assessed using a standardized questionnaire supplemented with measurements of pulmonary function, airway hyperresponsiveness

(AHR), total serum IgE levels and skin prick testing (SPT) (5). However, there remains uncertainty as to how these measurements should be interpreted. This is particularly true in the assessment of airway responsiveness in children. Many studies have stressed the importance of allowing for age, height and baseline lung function when interpreting the results of bronchial challenge tests (6–8). However, few provide specific guidance of how this should be performed. Consequently, most studies of asthma

genetics have failed to correct for age and size differences and have used adult thresholds to define AHR in children.

The results of bronchial challenge tests are influenced by a number of factors including, exposure to environmental triggers, viral infections, random variability and technical imprecision (9, 10). As a result, the expression of AHR varies markedly within an individual over time, with one-third of symptomatic children altering their PC₂₀ to methacholine by four doubling doses over 12 months (11). The interpretation of AHR data is further complicated by a lack of consensus in the definition of a positive response to bronchial challenge testing: threshold PC₂₀s vary from 8 to 32 mg/ml (3, 8, 12, 13). Other authors present dose-response slopes (DRS) (14) or PDs (2, 4), but rarely quote nebulizer outputs, making direct comparisons between studies difficult. Even international guidelines for bronchial challenge testing do not quote clear thresholds below which AHR is certain. Instead an arbitrary cut off PC₂₀ of 4 mg/ml (15) or 8 mg/ml (16) is recommended, with the proviso that PC₂₀s of 1–16 mg/ml should be regarded as borderline and interpreted with reference to the clinical picture.

Without repeated testing, it is difficult to control for the inherent variability of AHR within individuals. However, it may be possible to reduce some of the between-subject variability by correcting bronchial challenge data for the age and size of the subject. Several studies have reported an age-related decline in AHR (17, 18). This may reflect a true reduction in bronchial reactivity, but may also be explained by changes in lung volume, airway configuration, and respiratory mechanics with age (19). These differences alter the effective volume of distribution available to the challenge agent, and, as young children and adults inhale comparable amounts of methacholine during standardized bronchial challenge testing (19), result in the administration of relatively higher doses of methacholine per kg body weight in younger, smaller individuals (19). Several parameters have been shown to be associated with airway responsiveness and may account for the variability in the effective volume of distribution for methacholine. These include age, baseline lung function (variably assessed using combinations of FEV₁, FVC, FEF₂₅₋₇₅ and/or FEV₁/FVC), gender and height (6, 9, 20–22). Atopic status also changes over time (23) and may be associated with alterations in airway reactivity (9).

Previous studies have used stepwise regression models for the correction of DRS (7). However, correction of PC₂₀ values remains difficult

because of the variability of the thresholds used to define AHR. Furthermore, no study has used all the recognized correction factors in a single model. The aim of this paper is therefore to demonstrate how correction of both DRS and PC₂₀ values can be performed in children. We have used an approach that allows subjects to be categorized into corrected PC₂₀ categories of 0–3.9, 4–7.9, 8–15.9 and ≥16 mg/ml methacholine. To illustrate the potential effect of correction on the results of genetic studies, we also present pre- and postcorrection data in the context of an association study using a functional polymorphism in the gene, glutathione S-transferase GSTP1, previously shown to be associated with AHR in adults (24, 25).

Methods

Subjects

One hundred and forty-five unrelated British children aged 7–18 years were recruited from the North Staffordshire Hospital, local primary schools and GP practices. All children were from families of UK origin (England, Scotland, Wales) for three generations. Subjects had an independently confirmed doctor diagnosis of asthma and a history of wheeze for at least 12 months. Subjects with other cardiac, respiratory or inflammatory diseases were excluded. The local research ethics committee approved the study and all subjects gave informed written consent.

Assessment of atopy and AHR

Atopic status, baseline lung function and airway reactivity were assessed in all subjects using SPT, total serum IgE concentration, spirometry (26) and methacholine challenge testing. Prior to testing, all patients were free of respiratory infections for 6 weeks, had abstained from strenuous exercise for 12 h and withheld bronchodilators, leukotriene receptor antagonists, theophyllines, antihistamines and oral steroids for their duration of action. Inhaled steroids were continued.

SPT was performed against seven aeroallergens using standardized methodology (27). Results were considered valid only if the histamine exceeded the saline response. A positive (atopic) response to SPT was defined as ≥1 wheal of mean diameter ≥3 mm > saline control. Total serum IgE concentration was measured using a standard Immulite assay (Euro/DPC Ltd, Gwynedd, Wales, UK). The between-run coefficient of variation of the assay was 5.1–6.7%. An elevated IgE was defined as ≥100 IU/ml. Overall atopic

status was defined as either a SPT response of ≥ 1 wheal ≥ 3 mm > saline or IgE ≥ 100 IU/ml.

Methacholine challenge testing

The response to the methacholine challenge [tidal breathing method (16)] was expressed as a PC₂₀ and a DRS (28). Where the methacholine challenge was contraindicated (22) or baseline FEV₁ < 70% predicted, a bronchodilator challenge was performed (26). PC₂₀ and DRS slopes were corrected for gender, height, age, atopic status and baseline FEV₁, FVC, FEF₂₅₋₇₅ and FEV₁/FVC (6–9, 29) as outlined in the statistical analysis section.

GSTP1 genotyping

The Ile¹⁰⁵ → Val¹⁰⁵ substitution in GSTP1 was identified using PCR primers to exon 5 as previously described (24, 25). Genotype assignments were confirmed by re-examination of electrophoretic gels by a second observer and by re-assay of 15% of DNA samples.

Statistical analysis

Correction of PC₂₀ category and DRS for baseline parameters was performed using discriminant analysis (30) and linear regression, respectively. Discriminant analysis was performed using the NCSS statistic package (Number Cruncher Statistical Systems, version 6; Kaysville, UH, USA). All other analyses were performed using the Stata statistical package (version 6; State Corporation, College Station, TX, USA).

For DRS correction, data were first transformed to normality using $\ln(\text{DRS} + 2)$ with 2 being added to each value to allow inclusion of those cases with negative DRS values ($\ln \text{DRS}$). Multiple linear regression analysis was then per-

formed including each of the potential baseline variables listed above. Table 1 shows the results of this analysis, including the p-values and coefficients used in the generation of the linear regression equation: corrected $\ln(\text{DRS} + 2) = (\text{atopy} \times 0.686) + (\text{height} \times 0.029) - (\text{gender} \times 0.189) - (\text{age} \times 0.067) + (\text{FEV}_1/\text{FVC} \times 0.043) + (\text{FVC} \times 1.630) - (\text{FEV}_1 \times 1.972) - (\text{FEF}_{25-75} \times 0.512) - 2.946$. This generated a corrected transformed DRS value ($\ln \text{DRS}_c$). Corrected DRS (DRS_c) values were then calculated from the corrected transformed value using $\exp(\ln \text{DRS}_c) - 2$. FVC and FEV₁ are represented as both individual factors and as a ratio as these parameters represent different aspects of lung function and allow comparison with previous data.

To establish corrected PC₂₀ categories, uncorrected PC₂₀ methacholine results were divided into four groups, 0–3.9, 4–7.9, 8–15.9 and ≥ 16 mg/ml. These categories were selected as they best reflect the thresholds used to define AHR in the literature. Discriminant analysis was then used to ‘correct’ for baseline parameters (30). This uses an approach similar to that of multivariate logistic regression for using a set of baseline covariates to predict the probability that a subject will belong to a particular group (e.g. case or control). Discriminant analysis extends this to endpoints with categorical outcomes (four PC₂₀ categories in this case), as opposed to dichotomous endpoints as in logistic regression. The method identifies the combination of variables, which maximizes separation between groups. This is achieved by the use of a linear function of the variables (linear discriminant function) to produce a score for each possible category, which is then converted to a probability of group membership. An individual is then allocated to the category with the highest score. Discriminant analysis is related to the one-way MANOVA test with the reversal of the roles of the variables: the classification variable in MANOVA becomes the dependent variable in discriminant analysis. Table 2 illustrates the scores obtainable by this method.

Chi-squared tests were used to assess associations between the GSTP1 genotype and actual (uncorrected) and corrected PC₂₀. For this purpose, a PC₂₀ threshold of < 8 mg/ml methacholine was used to define AHR. As in previous studies in adults (24,25), the GSTP1 Val¹⁰⁵/Val¹⁰⁵ frequency was compared with the combined frequencies of Ile¹⁰⁵/Val¹⁰⁵ and Ile¹⁰⁵/Ile¹⁰⁵. The difference between means of continuous variables (DRS and DRS_c) according to the dichotomised genotype was assessed using Mann–Whitney U-tests.

Table 1. Multivariate linear regression model of $\ln(\text{DRS} + 2)$ against baseline parameters

Variable	Coefficient	p-value
Atopy	0.686	0.033
Height	0.029	0.228
Gender	−0.189	0.484
Age	−0.067	0.543
FEV ₁ /FVC	0.043	0.485
FVC	1.630	0.273
FEV ₁	−1.972	0.286
FEF ₂₅₋₇₅	−0.512	0.224
Constant	−2.946	—

The model demonstrated an r^2 value of 0.203 and $p = 0.006$.

Table 2. Discriminant analysis of the PC₂₀ category against baseline parameters. PC₂₀ results were divided into four categories: group 1: 0–3.9 mg/ml, group 2: 4–7.9 mg/ml, group 3: 8–15.9 mg/ml and group 4: ≥16 mg/ml methacholine. (a) group means for each variable, (b) coefficients for each category generated by the linear discriminant functions, (c) examples of percentage chance of belonging to a particular group for some patients who changed categories

(a) Group means

Variable	Group			
	1	2	3	4
Age	12.0373	11.75071	11.68	12.45833
Gender	0.63	0.7142857	0.75	0.5
Height	149.622	149.2143	148.5	150.2778
FEV ₁	2.4948	2.743572	2.48	2.581667
FVC	2.9629	3.135	2.835	2.841111
FEF ₂₅₋₇₅	2.6055	3.075714	2.8075	3.255556
FEV ₁ /FVC	84.7606	87.36357	87.62	90.77055
Atopy	0.83	0.7857143	0.75	0.6111111

(b) Linear discriminant functions

Variable	Group			
	1	2	3	4
Age	−8.652426	−8.869679	−8.763931	−8.434978
Gender	6.056806	6.461645	6.879414	6.266876
Height	4.996488	4.934072	5.033234	5.016874
FEV ₁	−594.3022	−587.743	−593.835	−598.5781
FVC	478.0004	473.9513	477.0356	479.4053
FEF ₂₅₋₇₅	−26.91110	−26.61573	−26.62486	−25.19528
FEV ₁ /FVC	22.54233	22.40397	22.57077	22.62117
Atopy	−11.09297	−10.88161	−11.52332	−12.33346
Constant	−1206.114	−1188.413	−1212.088	−1216.231

(c) Predicted percent chance of belonging to each group

Patient ID	Initial group	Predicted group	Group 1 (%)	Group 2 (%)	Group 3 (%)	Group 4 (%)
1	1	2	27.1	48.5	21.4	3.0
2	1	3	16.1	12.6	49.6	21.7
3	1	4	29.2	9.9	26.7	34.2
4	2	1	41.1	28.1	21.6	9.2
5	2	3	19.0	15.5	35.9	29.6
6	2	4	21.1	13.8	13.7	51.5
7	3	1	34.2	22.8	31.3	11.6
8	3	4	18.5	18.1	29.6	33.7
9	4	1	41.0	14.6	29.3	15.0
10	4	2	7.5	44.5	21.4	26.6

Results

One hundred and forty-five children (mean age ± SD, 11.8 ± 2.7, 64% male) were studied. Their mean ± SD baseline FEV₁ was 97.1 ± 18.1% as predicted (31). Despite one child refusing IgE testing, it was possible to determine atopic status in all children. One hundred and twenty-two (84%) children completed methacholine challenge testing. These children were used to assess the effects of correcting AHR data for baseline parameters on phenotype assignment. Predicted PC₂₀

categories and DRS slopes could be generated for all subjects. Using a cut-off of PC₂₀ < 8 mg/ml, 103/122 (84.4%) children exhibited AHR to methacholine prior to correction.

Effect of correcting AHR status for age, gender, height, atopic status and baseline lung function on phenotype assignment

The literature suggests that age influences bronchial challenge results, primarily by its influence on baseline lung function (FEV₁, FVC, FEF₂₅₋₇₅ and FEV₁/FVC). Height, gender and atopic

Table 3. Number of children in each PC₂₀ category before and after correction for baseline lung function, age, height, gender and atopic status

Actual PC ₂₀ category (mg/ml)	Corrected PC ₂₀ category (mg/ml)				Total
	0–3.99	4–7.99	8–15.99	≥16	
0–3.99	36	2	1	2	41
4–7.99	17	8	0	2	27
8–15.99	22	2	2	6	32
≥16	14	2	0	6	22
Total	89	14	3	16	122

Numbers in bold represent children who did not change PC₂₀ category after correction for baseline parameters.

status may also be important (7, 8, 29). In our population, a significant correlation was seen between age and baseline *FEV*₁, *FVC* and *FEF*_{25–75} ($r = 0.79, 0.84$ and 0.91 , respectively, $p < 0.001$ in all cases). PC₂₀ and DRS results were therefore corrected for age, height, gender, atopic status, baseline *FEV*₁, *FVC*, *FEF*_{25–75} and *FEV*₁/*FVC*.

Correcting actual PC₂₀ results for baseline parameters using discriminant analysis, followed by categorization as for uncorrected PC₂₀, resulted in 70/122 children changing PC₂₀ category. Most of these, 57/70 (81%), moved into a less severe category (Table 3). This correction changed the number of children defined as AHR positive (PC₂₀ methacholine < 8 mg/ml) from 103/122 (84.4%) to 68/122 (55.7%) after correction.

As expected, the mean values of the uncorrected and corrected transformed values ($\ln$ DRS and $\ln$ DRS_c) had the same means. However, the mean values of the untransformed DRS and DRS_c were not equal (see Methods section), though their geometric means were the same. Indeed, correction of DRS for baseline parameters using linear regression resulted in a marked difference between untransformed mean DRS (83.6) and the DRS_c (27.4) slope.

Associations between the GSTP1 genotype and AHR

In addition to those who completed methacholine challenge testing, AHR status could be assigned to a further 22 children (12 based on a positive response to bronchodilator, and 10 on a $\geq 20\%$ fall in FEV₁ after the saline step and a history of current asthma). These data were not corrected for baseline parameters and the children were assigned to the AHR positive group throughout. The association between AHR, corrected AHR (AHR_c) and genotype could therefore be assessed in 144/145 children.

No association was identified between the GSTP1 genotype and uncorrected AHR (Table 4). There was also no association between genotype and AHR severity as defined by uncorrected DRS (mean DRS Val¹⁰⁵/Val¹⁰⁵ vs. Ile¹⁰⁵/Ile¹⁰⁵ or Ile¹⁰⁵/Val¹⁰⁵, 96.0 vs. 81.6, $p = 0.35$). However, when PC₂₀ results were corrected for baseline lung function, height, age, gender and atopic status, a significant association was seen between the GSTP1 genotype and AHR_c status with Val¹⁰⁵/Val¹⁰⁵ demonstrating a protective effect (Table 4). Similarly, when DRS results were corrected for baseline parameters Val¹⁰⁵/Val¹⁰⁵ was associated with a significantly lower mean DRS (mean DRS_c Val¹⁰⁵/Val¹⁰⁵ vs. Ile¹⁰⁵/Ile¹⁰⁵ or Ile¹⁰⁵/Val¹⁰⁵, 20.1 vs. 32.7, $p = 0.032$).

Discussion

This is the first study to explore the association of AHR in children with polymorphism in GSTP1. The results show that, as in adults, GSTP1 Val¹⁰⁵/Val¹⁰⁵ is associated with an approximately threefold reduction in the risk of AHR. However, this association is only apparent after correction of AHR data for the child's age, height, gender, atopic status and baseline lung function. This illustrates that, when interpreting the results of bronchial challenge tests in children, correcting for baseline parameters may

Table 4. Association between the GSTP1 genotype and AHR

	GSTP1 genotype			Odds ratio (95% CI)	p-value
	Ile/Ile	Ile/Val	Val/Val		
Uncorrected AHR					
No AHR	11 (57.9%)	5 (26.3%)	3 (15.8%)	0.78 (0.22–2.77)	$p = 0.720$
AHR	59 (47.2%)	50 (40.0%)	16 (12.8%)		
Corrected AHR _c					
No AHR _c	27 (50.0%)	16 (29.6%)	11 (20.4%)	0.38 (0.14–0.98)	$p = 0.046$
AHR _c	43 (47.2%)	40 (44.0%)	8 (8.8%)		

AHR defined as PC₂₀ < 8 mg/ml. Odds ratios and p-values are based on comparisons of Val/Val with Ile/Val and Ile/Ile genotype. 95% CI = 95% confidence intervals.

have a significant effect on the results of genetic association studies.

This study confirms that correction for baseline parameters makes a marked difference to the number of children assigned AHR positive status. This is not surprising as body size, breathing pattern and baseline lung function are all important in determining a child's response to bronchial challenge testing (6–9, 19, 20). Gender may also be important but this remains controversial (6, 7, 9, 29). The use of challenge protocols designed for adults, results in children being exposed to relatively higher doses of methacholine per kg body weight than their adult counterparts. AHR data from adults and children, and from children of different ages and sizes, is therefore not directly comparable. For this reason, correction of AHR data for age, height, gender, atopic status and baseline lung function has been widely advocated in the pediatric literature (6–9, 19, 20). However, no study to date has indicated how this type of correction should be performed, particularly for PC₂₀. Accordingly we have illustrated a possible approach here.

In our study, a significant correlation was seen between DRS and the baseline FEF_{25-75} and FEV_1/FVC ratio in children. Controlling for baseline lung function, gender, height and atopic status resulted in up to 37% of children changing their final AHR assignment (5/122 to a more severe and 40/122 to a less severe PC₂₀ category; Table 3). This compared with 17% of children changing AHR status when the PC₂₀ threshold was altered by two doubling doses, from 4 to 16 mg/ml (data not shown). Correcting for baseline parameters also had a significant implication for the association of the GSTP1 genotype with AHR. Although the degree to which this correction affects phenotype designation will depend on the population studied, our results emphasize the importance of considering age and size when using AHR as an outcome variable in genetic association studies in children.

The use of multiple linear regression for correction of DRS values has previously been used (7), though percentage predicted, rather than absolute, values for FVC and FEF_{25-75} were used in their models. These values are themselves corrected for age, gender and height and may therefore potentially distort the regression equation generated. We have also opted not to use a stepwise approach as described by Ownby et al. (7) as this will select different significant variables depending on the population under study. By correcting for all baseline variables, irrespective of their degree of significance, our approach is applicable to any

population. If a variable plays a minor role, or is nonsignificant, its effect on the corrected values will be minimal, as seen in Table 1.

No study has previously corrected PC₂₀ values for baseline parameters. Several methods are available for this depending on the number of categories chosen. For example, if a single cut-off is used, binary multiple logistic regression can be used to generate a regression equation, as for DRS. However, as there is no consensus as to which PC₂₀ threshold best defines AHR, we have adopted a multiple threshold approach. Consequently, the discriminant analysis technique allows the borderline values of 4–16 mg/ml methacholine to be considered separately.

In relation to the GSTP1 data, the risk of population stratification was reduced by the recruitment of three-generation British families from North Staffordshire, an area with a stable patient base with relatively little population movement. However, the numbers, especially of nonatopic and non-AHR children, were relatively small. The AHR data may be criticised because data obtained using different methods (bronchodilator responsiveness and methacholine challenge) were included in the same association analyses. This allowed more subjects to be included but, although there is correlation between bronchodilator responsiveness and the response to methacholine challenge (32), they may assess different phenotypic characteristics. Similar trends were, however, seen between the GSTP1 genotype and both DRS and AHR (PC₂₀ and bronchodilator results combined). Nor did exclusion of PC₂₀ data from children in whom bronchodilator challenge was performed change the overall data trend [odds ratio (95% CI) = 0.43 (0.16–1.19), $p = 0.106$]. The AHR results may also have been influenced by the continuation of inhaled steroids, but in North Staffordshire this was a requirement for ethical approval.

Allelic variants of GSTP1 demonstrate different catalytic efficiencies due to changes in the active site (33,34). In addition to the wild-type, *GSTP1**A allele, three further alleles, *GSTP1**B, *GSTP1**C and *GSTP1**D have been identified. *GSTP1**B contains an A-G transition, resulting in an Ile¹⁰⁵–Val¹⁰⁵ (I105V) substitution while *GSTP1**D contains a C-T transition giving rise to an Ala¹¹⁴–Val¹¹⁴ (A114V) change. *GSTP1**C contains both these transitions. Val¹⁰⁵ enzymes have a sevenfold higher catalytic efficiency for diol epoxides of polycyclic aromatic hydrocarbons (PAH), than Ile¹⁰⁵ isoenzymes. In contrast, the Val¹⁰⁵ enzyme is threefold less effective using 1-chloro-2,4-dinitrobenzene as a substrate (35,36). There is no evidence of a functional

effect of the A114V substitution alone (*GSTP1*D*), though it is suggested that it augments the increased PAH activity of the I105V substitution (*GSTP1*C*) (36). However, we were unable to identify any additional effect from the A114V substitution in previous studies on asthma phenotypes in adults (37) and accordingly, focus only on the Ile¹⁰⁵-Val¹⁰⁵ substitution.

The results for AHR and atopy are slightly weaker than, but similar to our previous data in adults (24, 25). The current study shows that the Val¹⁰⁵/Val¹⁰⁵ genotype confers a threefold reduction in risk in both AHR and atopy in children, compared with five-, four- and 10-fold reductions in risk of AHR, SPT positivity and IgE ≥ 100 IU/ml in adults (24). Despite this, the association has now been confirmed in three independent populations, adding confidence to the validity of the association between the *GSTP1* genotype and asthma phenotypes.

In conclusion, we have shown that the *GSTP1* Val¹⁰⁵/Val¹⁰⁵ genotype is associated with a reduced risk of AHR in children. However, this association is only present after correction of bronchial challenge data for baseline lung function, age, gender, height and atopic status. This suggests that the interpretation of bronchial challenge data may be highly significant in ensuring appropriate conclusions are drawn from studies of asthma genetics in children. In light of this, we present an approach to allow correction of such AHR data, which will be applicable for any population.

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