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Short title: GST variants and asthma outcomes in children

ABSTRACT

Our hypothesis was that children with mutations in genes coding for Glutathione S-transferases (GST) have worse asthma outcomes compared to children with active type genotype. Data were collected in five populations. The rs1695 single nucleotide polymorphism (*GSTP1*) was determined in all cohorts (3692 children) and *GSTM1* and *GSTT1* null genotype were determined in three (2362 children). *GSTT1* null (but not other genotypes) was associated with a minor increased risk for asthma attack and there were no significant associations between *GST* genotypes and asthma severity. Interactions between *GST* genotypes and SHS exposure or asthma severity with the study outcomes were non-significant. We find no convincing evidence that the GST genotypes studied are related to asthma outcomes.

INTRODUCTION

The Glutathione S-transferases (GST) are a family of enzymes which are important for the antioxidative defences of many organs in the body including the respiratory system[1],[2]. Asthma is a very common chronic respiratory condition characterised by increased oxidative stress[3,4], and in adults plasma GST concentration are elevated in asthma[5]. The oxidative burden is further increased during an acute attack of asthma[6], and this may indicate an inadequacy of host antioxidative properties.

There are polymorphisms in the genes coding for M, P and T members of the GST family which are common and may be relevant to asthma and asthma outcomes. Approximately 50% of the population is null for *GSTM1* (i.e. individuals with the null variant have no gene product), 20% is null for *GSTT1* and 15% is homozygous for the Ile-Val single nucleotide polymorphism (rs1695)[7]. The GST variants may be relevant to asthma causation[8-10] and the mechanism may involve interactions with oxidative exposures including antenatal[11,12] or postnatal[13,14] second hand smoke exposure and air exposures[15-17].

There is some evidence that GST genotype may be associated with altered risk for asthma attacks and severity of asthma is poorly understood, and that interactions with inhaled exposures may also be present[7,18]. Among asthmatic children there is evidence of interaction between GST genotype and second hand smoke (SHS) for reduced lung function[19] and between GST genotypes and ozone exposure for wheeze[20]. One study of 341 families found an association between a GST genotype and increased asthma severity score (unpublished data presented in a systematic review[8]) but this was not confirmed in two small case-control studies [21,22]. No study has linked GST genotypes to asthma attacks.

The nature of the relationship between GST genotypes and asthma outcomes is subject to methodological issues including publication bias, false positive reporting, small sample size and

multiple testing and the role of genotypes in the genes coding for GST results in disease modification is still uncertain[7]. The Pharmacogenomics In Childhood Asthma (PiCA) consortium has come together to address these methodological issues[23-25], and our aim was to use data from a number of patient populations within the PiCA consortium and to explore interactions between GST genotypes and asthma outcomes. Our hypothesis is that children with variants in the genes coding for GST are at increased odds for attacks and for more severe asthma within a population of children with asthma. In recognition of previous work[13,14], an additional hypothesis was that any association between GST genotype and asthma outcomes would be modified by exposure to second hand smoke.

SUBJECTS AND METHOD

Cohorts. Four cohorts of children with asthma provided individual patient data (IPD) and summary statistics were provided from a fifth cohort. The four cohorts were BREATHE (no acronym), recruited from primary and secondary care in North East Scotland[19]; Pharmacogenetics of Asthma medication in Children: Medication with Anti-inflammatory effects (PACMAN), recruited from children attending community pharmacies in the Netherlands[26]; the Paediatric Asthma Gene Environment Study (PAGES), recruited from primary and secondary care across Scotland[27]; and in the Swedish population birth cohort BAMSE (Swedish abbreviation for Children, Allergy, Milieu, Stockholm, Epidemiology), asthmatic children were included[28]. Summary statistics were provided from a fifth study, the Genes-environment and Admixture in Latino Americans study (GALA II), consisting of children diagnosed with asthma in the US with four grandparents of Latino ancestry[29]. More details of the inclusion criteria for the five cohorts are presented in the supplement.

Genotyping. The genotypes of interest were the *GSTM1*, *GSTT1* null genotype and homozygous genotype for the substitution of isoleucine with valine at the 105 position of the gene coding for *GSTP1* (rs1695 single nucleotide polymorphism). For PACMAN, BREATHE and PAGES, saliva for DNA extraction was collected using the Oragene system (DNA Genotek ref. OG-250) and specimens were processed following the manufacturer's protocol. *GSTM1 and GSTT1* null mutations were determined by polymerase chain reaction and *GSTP1* polymorphism by an allelic discrimination assay as previously described[6]. For GALA II, DNA was extracted from whole blood before an array (Axiom® LAT1, World Array 4, Affymetrix, Santa Clara, CA) was used to determine genome wide-genotype data. In BAMSE, *GSTP1* was genotyped by matrix-assisted laser desorption/ionization– time of flight (MALDI-TOF) mass spectrometry (Sequenom)[28].

Definition of outcomes. In PACMAN, the definition of attacks was reported use of oral prednisolone and/or asthma-related ER visits in the previous 12 months. For GALA II, the definition of attack was at least one of the following in the previous 12 months: oral prednisolone treatment, receiving emergency care or admission to hospital for asthma symptoms. BREATHE and PAGES used the same attack definition, which was at least one of the following in the previous six months: receipt of oral corticosteroid treatment, absence from school or hospital admission for asthma. For BAMSE an attack was defined as an asthma related emergency room and/or asthma-related hospital visit in the previous 12 months.

Asthma severity was determined by treatment step as described in the British Thoracic Society/Scottish Intercollegiate Guidelines Network [BTS/SIGN] treatment step[30]. There were very few children on step 5 treatment and a composite step 4/5 group was created.

Children with at least one parent who smoked were categorised as smoke exposed. Smoking exposure was validated by salivary cotinine in 139 participants of the PAGES cohort where median (standard error of mean) cotinine for children with no, one and two resident smokers was 0.5 ng/ml (0.04), 0.58 ng/ml (0.16) and 1.39 (0.95) respectively, Kruskal Wallace test $p < 0.001$[27].

Statistical analysis and power calculation. IPD analysis was undertaken for the PACMAN, BREATHE, PAGES and BAMSE cohorts. Age (measured in years), gender (female coded as 1 and male coded as 0), exposure to second hand smoke (SHS - coded 1 for presence of or 0 for absence of as per definitions above), cohort of origin (PACMAN coded as 1, BREATHE coded as 2 and PAGES coded as 3). Genotypes for *GSTM1* and *GSTT1* were coded 1=null and 0=active type. For *GSTP1*, the homozygous valine genotype was coded 1 while the heterozygous and homozygous isoleucine rs1695 genotypes were combined and coded 0. To explore the effect of second hand smoke (SHS) on the relationship between genetotype and attack, where associations between outcomes and genotype were significant, an interaction term between the genotype and SHS was calculated.

A logistic regression model was used to investigate the factors affecting risk of attacks. A multinomial regression model was used to investigate the relationship between BTS/SIGN treatment step (with BTS/SIGN treatment step 1 as the reference group).

To utilise all applicable genotype data within the PiCA consortium, meta-analysis of *GSTP1* data from IPD analysis of the BREATHE, PACMAN, PAGES and BAMSE populations with the GALA II population was performed using a random effects inverse variance–weighted model for the attacks outcome, where the individual study ORs are weighted by their confidence intervals. The I^2 statistic was calculated to examine the heterogeneity in the population. This meta-analysis was implemented using the GWAMA software[31]. Details of the power calculation (table 1) and further details of the statistical analysis are presented in the supplement.

RESULTS

Study subjects

Table 1 presents details of the 3,692 individuals included in the analysis. Details of all three GST variants were available in 2197 individuals, including 983 from BREATHE, 719 from PACMAN and 495

from PAGES. Additionally, *GSTP1* details were available in 165 BAMSE and 1,330 GALA II participants.

Factors associated with an asthma attack

Out of 3,692 patients, 1,178 patients (36%) experienced at least one asthma attack as per the definitions described. Individuals who were *GSTT*1 null had an increased odds ratio (OR) for an asthma attack (OR 1.20 [1.03, 1.59], p=0.03), independent of age, second hand smoke exposure and asthma severity (supplement table 2). There was no association between *GSTM1* null (supplement table 2) or being homozygous for Val for rs1695 (supplement table 3) and risk for asthma attack. The interaction term between *GSTT1* and exposure to SHS approached significance (OR - 1.53 [0.98, 2.38], p=0.06). When analysed separately, individuals null for *GSTT1* were at increased risk for an asthma attack when also exposed to SHS (1.60 [1.06, 2.43] p=0.02 whereas those with active GSTT1 were not at increased risk (1.15 [0.88, 1.51] p=0.30)

There was no association between GSTP1 genotype and risk for asthma attack in the meta-analysis The meta-analysed OR [95% CI] was 0.99 [0.81, 1.21]. An I^2 value of 0 showed no heterogeneity was present in the population. Results from each individual cohort are presented in supplement table 2.

Factors associated with severity

BTS/SIGN treatment step information was available for 3,382 patients, of which 512 (15%), 1,767 (52%), 792 (24%) and 311 (9%) were being prescribed medications that categorised them into BTS/SIGN treatment steps 1, 2, 3 and 4/5 respectively. In the univariate analysis, there was a trend for *GSTM1* null genotype to be potentially associated with the odds of severity (p=0.08), this was driven by significantly decreased odds of BTS/SIGN treatment step 2 (OR 0.79 [0.64, 0.98] p=0.043, see supplement table 4) but this association was clearly non-significant when adjusted for age and exposure to second hand smoke. The interaction between *GSTM1* and SHS exposure showed no

evidence of association with severity (p=0.90). Neither *GSTT1* (p=0.63) or *GSTP1* (p=0.43) genotype showed evidence of association with severity.

DISCUSSION

There is a plausible mechanism whereby variations in the genes coding for GST could modify asthma outcomes in the context of an environment containing an increased oxidative burden (such as SHS exposure), but current understanding is restricted by methodological factors including heterogeneity between populations, power and multiple testing[7,18]. Our study has addressed many of these methodological issues by focussing on populations of children with asthma, demonstrating adequate sample size and by undertaking IPD analysis.

The first finding was that in a multivariable model, *GSTT1* null genotype was associated with an increased risk of attack, however this was of small magnitude and borderline significance. The second notable finding was that children null for *GSTM1* had a borderline significant reduction (and biologically implausible) OR for step 2 treatment in the multinomial model but in not the multivariable model. Our third and final finding was that despite SHS exposure being associated with both outcomes, there were no statistically significant interactions with *GSTT1* for asthma attacks and *GSTM1* for asthma severity. The well-recognised heterogeneity of asthma is thought to be due to disease-modification by within-population variations in genetic and environmental factors[32], but in our study the GST variants studied were not consistently linked to asthma outcomes.

There are a number of reasons why there may be no association between the GST variants studied and asthma outcomes. First, there may be no association and factors other than GST are more important to lung antioxidative properties, and publication bias may have led to the literature

predominantly containing studies which find an association (either with increased or occasionally reduced[33,34] risk for asthma outcomes) whereas studies which find no association are not submitted or accepted for publication. Second, whilst a power calculation indicates that the population was of adequate size to detect an OR of 1.3 with >92% power for the variants studied, there may be a smaller effect size associated with an interaction with smoking. Third, even though the variants studied here have been linked with asthma causation and some asthma outcomes in other studies, we may have not studied the most relevant GST variants. Finally, no standard definition of an asthma attack was applied across the five populations studied and this may have weakened any relationship between the genotypes studies and asthma attack, however despite this weakness an association was determined for *GSTT1*.

Whilst variants in the genes coding for GST are thought to be important to disease causation and modification, the underlying mechanism(s) are still not fully understood and our study suggests that the relevance of the GST variants studied to asthma attacks and severity in children is either small or none at all. Plasma GST concentrations are not different between individuals carrying active type or mutations of *GSTM1, GTSP1* and *GSTT1*[5,35] but concentrations within the lung correlate with rs1695 status[2] suggesting that genetic polymorphisms are important to GST activity within the lung. It is possible that the reduction in GST activity related to mutations of the three genes studied is either not sufficient to have clinical relevance for children with asthma and/or the populations studies did not experience a burden of oxidative stress sufficient to make the reduced GST activity important.

There are no studies, which are directly comparable to our findings. Rs 1695[13] and GSTM1 null[14] genotypes have been associated with increased risk for asthma diagnosis, but we find no increased risk for adverse asthma outcomes for these variants within asthmatic populations. Three studies have identified associations between homozygous val genotype rs 1695[15,17,20] and increased risk for asthma symptoms in association with ozone exposure, and we did not record ozone exposure in

our study. A third study of 43 children with asthma found that there was no difference daily peak flow measurements between those null for *GSTT1* or *GSTM1* in the context of air pollution exposure[36], and our results are consistent with these findings. A previous report from the BREATHE cohort has described reduced lung function among older children with asthma with were null for *GSTM1* and homozygous for valine for *GSTP1* and who were exposed to second hand smoke[19]; we do not report lung function outcomes in the present paper and no direct comparison can be made.

There are a number of factors which should be considered when interpreting these results. The strengths of our study include the relatively large sample size and the inclusion of more than one population. One potential limitation is that we did not adjust for ethnicity and our cohorts included children of European and Hispanic ethnicity and this introduced a small difference in the distribution of *GSTP1* genotype between populations (table 1), however in the meta analysis there was no heterogeneity in the relationship between this genotype and the outcomes studied and in the IPD for *GSTT1* and *GSTM1* the cohort was included as a covariate which will mitigate against any differences in ethnicity between populations affecting the relationship between genotype and outcome.. Second, we have focussed on only three genotypes and many genes, e.g. *NQO1*, *HMOX*, may be relevant to host antioxidative status either individually or in combination[18]. Third, SHS exposure was by report in four cohorts and thus may be unreliable, but SHS exposure was validated by salivary cotinine in the PAGES cohort[27]. Fourth, we have not related GST variants to objective markers of asthma such as lung function. Finally, in our study the exposure of interest was SHS and we did not consider other exposures, e.g. ozone[20], nitrogen dioxide[37], vitamin C[38], which might have modified the effect of SHS in genetically-susceptible individuals.

In summary, we find no evidence for there being an important association between the GST variants we have studied and adverse asthma outcomes in children. We believe that the associations between the *GSTT1* null variant and asthma attacks and *GSTM1* null variant and asthma severity are

false positive findings. Our findings are based on a large number of children from more than one

population, meaning that our findings may be generalisable across similar populations[39].

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Table 1. Details of the participants from the five cohorts whose data were used in this study. NA=not available

		BREATHE n=1459	PACMAN n=996	PAGES n=970	GALA II n=1330	BAMSE n=165
% Male sex (n)		60% (592)	64% (458)	59% (284)	58% (772)	60.6% (100)
Mean age in years (SD)		9.6 (3.8)	8.4 (2.4)	9.5 (3.8)	11.9 (2.7)	8.3 (0.45)
% exposed to second hand smoke at home (n)		36% (337)	10% (69)	23% (107)	21% (283)	21.8% (36)
% with recent attack (n)		44% (430)	9% (59)	60% (261)	66% (884)	10% (17)
GSTM1 status	% Active type	52% (508)	49% (351)	45% (223)	NA	NA
	% Null	48% (475)	51% (368)	55% (272)	NA	NA
GSTT1 status	% Active Type	79% (777)	78% (564)	79% (393)	NA	NA
	% Null	21% (206)	22% (155)	21% (102)	NA	NA
GSTP1 status	Ile/Ile	40% (395)	40% (287)	45% (222)	34% (453)	46% (76)
	Ile/Val	47% (459)	46% (329)	42% (209)	46% (615)	46.7% (77)
	Val/Val	13% (129)	14% (103)	13% (64)	20% (262)	7.3% (12)
Number of GST mutants	0	34% (331)	33% (240)	33% (163)	NA	NA
	1	51% (504)	48% (346)	47% (232)	NA	NA
	2	14% (138)	17% (119)	19% (94)	NA	NA
	3	1% (10)	2% (14)	1% (6)	NA	NA
BTS/SIGN treatment step	No treatment or 1	16% (147/945)	14% (93/674)	8% (35/456)	49% (649/1330)	40.0%(66)
	2	55% (523/945)	62% (418/674)	25% (115/456)	21% (283/1330)	59.3%(98)
	3	16% (147/945)	18% (123/674)	55% (251/456)	9% (123/1330)	0.6% (1)
	4 or 5	14% (128/945)	6% (40/674)	12% (55/456)	21% (275/1330)	0