GLUTATHION S TRANSFERASE DEFICIENCY AND PASSIVE SMOKING INCREASE CHILDHOOD ASTHMA

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ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS

Subjects

In 1995 and 1996 a cross sectional study was conducted in Munich, western Germany and Dresden, eastern Germany, to assess the prevalence of asthma and allergies in schoolchildren aged 9-11 years as part of the International Study of Asthma and Allergies in Childhood (ISAAC) (E1). Parental questionnaires for self-completion including the ISAAC core questions were distributed through the schools to the parents. Children underwent skin prick testing, pulmonary function testing and bronchial challenge with hyperosmolar saline (4.5%). Blood was obtained for serum IgE measurements and DNA extraction. Total serum IgE was measured in a 50% random sample of all children that had given blood in Dresden and all children with blood available in Munich. In this study only children of German origin who had both DNA and IgE data available were included in the analysis (total N=3099, Munich n=1159, Dresden n=1940). The study methods for all phenotyping procedures have been described in detail elsewhere (E1, E2, E3). Therefore, only methods pertaining to this analysis are given below. Informed written consent was obtained from all parents and all study methods were approved by the ethics committee of the Bavarian Medical Council.

Questionnaire

Self-administered questionnaires included the ISAAC core questions on symptoms of asthma, allergic rhinitis and atopic eczema (E1). Children whose parents reported a doctor's diagnosis of either asthma, recurrent spastic or recurrent asthmatic bronchitis were classified as having asthma. Asthma with a positive skin prick test reaction was defined as

atopic asthma; asthma without a SPT sensitisation was defined as non-atopic asthma. The definition of “wheeze ever” was based on the ISAAC question: "Has your child ever had wheezing or whistling in the chest". Current wheeze was defined as wheezing in the last 12 months. Current asthma was defined as wheezing symptoms during the last 12 months in children with a doctor’s diagnosis of asthma. If wheezing within the last 12 month was induced by exercise this was defined as current wheeze with exercise. Awakening at night within the last 12 months due to wheezing was termed current wheezing at night. Current cough without cold was defined as unproductive coughing within the last 12 months without a cold, sneezing or fever. If a child had visited a doctor or had to take medication due to breathing problems within the last 12 months this was termed current doctor's visit due to asthma and current medication for asthma. The definition of atopic dermatitis and hay fever was based on a parental report of a doctor’s diagnosis of atopic eczema and hay fever, respectively. High socio-economic status was defined as either parent having had at least 12 years of school education.

Current environmental tobacco smoke exposure and exposure to maternal smoking *in utero* was characterized according to responses to the parental questionnaire. Current environmental smoke exposure was classified in 4 categories: none, 0-9, 10-20 and > 20 cigarettes smoked per day by anyone living in the child’s household. *In utero* exposure to maternal smoking was assessed by a positive answer to the question “Did the mother of the child smoke during pregnancy”.

Skin prick test

The sensitivity to six common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass and tree pollen) was assessed using highly standardized extracts and lancets (ALK, Horsholm, Denmark) according to ISAAC phase II protocols. A child was considered sensitized if a wheal reaction of more than 3mm occurred to at least one specific allergen after subtraction of the negative control.

Total serum IgE measurements

Total serum IgE levels were measured using the Insulite system (DPC Biermann, Bad Nauheim, Germany).

Lung function testing and bronchial challenge

In 9–11 year old children lung function was measured with the MasterScope Version 4.1 (Jäger, Würzburg, Germany). A minimum of two baseline spirograms was recorded and the highest of two reproducible (within 5%) measurements of forced expiratory volume in one second (FEV_1) was recorded as baseline FEV_1. Bronchial reactivity was assessed in a random sample of half the study population by changes in FEV_1 after inhalation of nebulized, hyperosmolar (4.5%) saline for increasing periods of time using ultrasound nebulizers (DeVilbiss Sunrise Medical, Langen, Germany). Bronchial challenge was stopped either if the FEV_1 had fallen at least 15% from baseline or if the total inhalation period of 15.5 min had been completed. All children were asked to refrain from all asthma

medications for at least 12 h before lung function testing. Children with a drop in FEV_1 of 15% or more from baseline were classified as having bronchial hyperresponsiveness.

Genotyping

Genomic DNA was extracted from leucocytes using a standard salting out method (E4). To test simultaneously for the presence or absence of GSTM1 and GSTT1 genes, a modified allele specific PCR assay established by Chen et al. was used (E5). Primers for the amplification of GSTM1 (E6), GSTT1 (E7) and an unrelated genomic control sequence (5'-TGC AAT TGT GAG GAT TTC ACA G-3' and 5'-CCT GAA CAT GAT CTG TGG ATC-3') were obtained from Metabion (Martinsried, Germany). PCR reactions were carried out in a total volume of 25 µl containing 2.5 µl PCR buffer (Puffer S, Peqlab, Erlangen, Germany), 200 µM each dNTP (Peqlab), 0.4 µM of each GSTM1 primer, 0.4 µM of each GSTT1 primer, 0.25 µM of each control primer, 1 U Taq DNA polymerase (Promega, Madison, Wisconsin, USA) and 40 ng DNA. After initial denaturation (96°C, 2 min), thirty-five cycles of denaturation (96°C, 40 sec), annealing (56°C, 40 sec) and elongation (72°C, 50 sec), and then final extension at 72°C for 5 min were performed. PCR products were run on agarose gels and visualized with ethidium bromide staining and ultraviolet illumination. The visualization of a band for GSTT1 or GSTM1 was considered as an indication for the presence of GSTT1 or GSTM1 alleles in the sample. The absence of a GSTT1 or GSTM1 band in the presence of the control genomic template was interpreted as a lack of GSTT1 or GSTM1 alleles in the sample. For quality control, a random selection of 5% of all samples was genotyped twice to assure genotyping accuracy.

Statistical analysis

Chi-square statistics as well as Cochran-Armitage trend tests were used to compare qualitative traits between groups. Total serum IgE levels were summarized descriptively with geometric means, and respective 95% confidence intervals. For binary outcomes, logistic regression models for gene-environment interactions were used to estimate the combined effect of either GSTM1 or GSTT1 with smoking exposition in pregnancy and at present, adjusting for the effects of age, sex, city and family history (asthma, atopic rhinitis, or atopic dermatitis). This analysis used the Botto-Khoury approach which summarises the data in a two-by-four table, enabling the evaluation of independent and joint roles of genotype and exposure on disease risk. The relative risk estimates for each factor alone and for the joint exposure are comparable, due to the use of a common reference group. Additionally, the departure from the multiplicative model of interaction was derived from the table (E8). Similarly for continuous outcomes (lung function variables), a two-way analysis of covariance (ANCOVA) was carried out to evaluate possible gene-environment interactions, also adjusting for the possible confounders mentioned above.

With several outcomes, two genotypes, and two smoking exposures, the problem of multiple testing needed to be addressed. The Benjamini-Liu method controls the false discovery rate (FDR), a criterion described in Reiner at al. [18], within a pre-defined family of tests. We used the Benjamini-Liu multiple test correction with families for asthma, wheeze, treatment, and lung function, for each smoking exposure and each genotype: within each family, the false discovery rate was controlled to either 1% or 5% (Figures 1a & 1b). All calculations were carried out using the SAS software package (version 8.2).

Table E1 Prevalence of asthma, asthma symptoms and atopic diseases by city and GSTM1 and GSTT1 genotypes

	Munich & Dresden (n=3054)			
	GSTM1 (–)	GSTM1 (+)	GSTT1 (–)	GSTT1 (+)
Asthma diagnosis	9.6 (148)	8.2 (120)	8.1 (42)	9.1 (226)
Current asthma	4.6 (72)	4.3 (64)	4.0 (21)	4.6 (115)
BHR	17.1 (119)	17.8 (111)	19.0 (42)	17.1 (188)
Wheeze ever	21.9 (338)	20.5 (299)	23.1 (120)	20.8 (517)
Current wheeze	8.7 (135)	7.8 (113)	8.7 (45)	8.2 (203)
Current wheeze with exercise	6.8 (106)	6.5 (94)	6.4 (33)	6.7 (167)
Current cough without cold	17.2 (268)	17.6 (258)	18.9 (99)	17.1 (427)
Atopic dermatitis	16.6 (252)	19.7 (282)	17.8 (90)	18.2 (444)
Hay fever	8.6 (133)	9.6 (139)	9.8 (50)	9.0 (222)
SPT reactivity	25.8 (393)	25.8 (370)	27.4 (142)	25.5 (621)

Table E2 Odds ratios and 95% confidence intervals for the combined effect of ETS exposure (in utero or ≥20 cig) and GSTM1 deficiency adjusted for age, sex, family history of asthma and ETS exposure.

	In utero ETS GSTM1+	**In utero ETS GSTM1−**	**ETS (≥20 cig) GSTM1+**	**ETS (≥20 cig) GSTM1−**
Outcomes				
Asthma				
asthma diagnosis	1.14 (0.52-2.50)	1.54 (0.82-2.89)	2.11 (0.66-6.79)	2.09 (0.70-6.19)
current asthma	0.80 (0.23-2.73)	1.36 (0.57-3.26)	2.94 (0.61-14.05)	5.48 (1.62-18.55)**
Atopic asthma	0.72 (0.21-2.43)	1.67 (0.77-3.65)	0.88 (0.10-7.35)	2.96 (0.80-10.89)
non-atopic asthma	1.67 (0.61-4.53)	1.33 (0.49-3.60)	3.42 (0.89-13.20)	0.98 (0.12-8.02)
Wheezing and asthma severity				
wheeze ever	0.90 (0.50-1.61)	1.28 (0.80-2.05)	1.81 (0.75-4.41)	2.81 (1.31-6.04)**
current wheeze	1.01 (0.41-2.48)	1.64 (0.84-3.18)	2.03 (0.55-7.54)	4.74 (1.79-12.57)**
current wheeze with exercise	0.71 (0.24-2.08)	1.43 (0.9-2.93)	5.48 (1.82-16.54)**	6.46 (2.44-17.05)**
speech limiting wheeze	1.49 (0.42-5.32)	3.58 (1.52-8.43)**	6.98 (1.32-36.84)*	8.93 (2.08-38.42)**
current wheeze at night	1.13 (0.33-3.93)	1.71 (0.67-4.37)	4.22 (0.86-20.63)	5.28 (1.33-21.01)*
current cough without cold	0.66 (0.33-1.32)	1.52 (0.95-2.43)	1.04 (0.34-3.18)	2.76 (1.23-6.17)*
Treatment				
current doctor's visit due to asthma	1.18 (0.51-2.75)	1.02 (0.06-3.38)	1.08 (0.27-4.25)	1.62 (0.51-5.20)
current medication for asthma	0.48 (0.14-1.60)	1.15 (0.54-2.47)	1.89 (0.51-7.06)	3.50 (1.21-10.10)*

* $p \leq 0.05$
** $p \leq 0.01$

Table E3 Odds ratios and 95% Confidence intervals for the combined effect of ETS exposure (in utero or ≥20 cig) and GSTT1 deficiency adjusted for age, sex, family history of asthma and ETS exposure.

	In utero ETS GSTT1+	**In utero ETS GSTT1−**	**ETS (≥20 cig) GSTT1+**	**ETS (≥20 cig) GSTT1−**
Outcomes				
Asthma				
asthma diagnosis	1.10 (0.63-1.91)	1.57 (0.58-4.28)	1.32 (0.51-3.43)	4.87 (1.07-22.23)*
current asthma	1.13 (0.53-2.41)	0.49 (0.06-3.77)	3.25 (1.14-9.27)*	4.10 (0.43-39.04)
atopic asthma	0.89 (0.41-1.93)	1.98 (0.64-6.11)	0.69 (0.15-3.29)	7.33 (1.39-38.71)*
non-atopic asthma	1.34 (0.63-2.88)	0.83 (0.11-6.30)	2.30 (0.71-7.40)	0.00 (-)
Wheezing and asthma severity				
wheeze ever	1.04 (0.69-1.56)	1.13 (0.49-2.62)	1.77 (0.92-3.42)	4.37 (1.17-16.39)*
current wheeze	1.26 (0.70-2.26)	0.98 (0.28-3.39)	2.61 (1.09-6.21)*	3.30 (0.61-17.91)
current wheeze with exercise	1.15 (0.61-2.15)	0.34 (0.04-2.60)	4.33 (1.92-9.75)**	4.59 (0.86-24.40)
speech limiting wheeze	2.94 (1.37-6.28)**	1.06 (0.14-8.31)	7.78 (2.45-24.76)**	0.00 (-)
current wheeze at night	1.44 (0.66-3.11)	0.00 (-)	3.04 (0.96-9.65)	6.87 (0.69-68.32)
current cough without cold	1.23 (0.81-1.88)	0.90 (0.34-2.40)	1.50 (0.71-3.20)	6.72 (1.77-25.52)**
Treatment				
current doctor's visit due to asthma	1.10 (0.60-2.02)	0.35 (0.05-2.65)	1.07 (0.39-2.94)	0.77 (0.08-7.12)
current medication for asthma	0.83 (0.42-1.62)	0.30 (0.04-2.29)	2.10 (0.85-5.19)	1.29 (0.14-11.68)

* $p \leq 0.05$
** $p \leq 0.01$

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