Glutathione s-transferase genotype protects against *in utero* tobacco linked lung function deficits

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Abbreviations

FEF 25-75% - forced expiratory flow at 25-75% of forced vital capacity
FEV_1 –forced expiratory volume in 1 second
FVC – forced vital capacity
GST – glutathione s-transferase
ITS – *in utero* tobacco smoke exposure
PCR – polymerase chain reaction
PIAF – Perth infant asthma follow-up study
ITS – *in utero* tobacco smoke
V'maxFRC – maximum flow at functional residual capacity

Abstract

Rationale

In utero tobacco exposure is associated with reduced infant lung function. Anti-oxidant enzymes from the glutathione-s-transferase (GST) family may protect against these lung function deficits.

Objectives:

(1) Assess the long-term effect of *in utero* smoke exposure on lung function into adulthood.

(2) Assess whether *GSTT1* and *GSTM1* active genotypes have longterm protective effects on lung function.

Methods:

In this longitudinal study, based on a normal population (n=253), lung function was measured during infancy and then at 6, 11, 18 and 24 years. *GSTM1* and *GSTT1* genotype was analysed in a subgroup (n= 179). Lung function was assessed longitudinally from 6 to 24 years (n=144).

Main Results

Exposure to maternal *in utero* tobacco was associated with lower FEV_1 and FVC from 6 to 24 years (mean difference – 3.87% predicted, p=0.021; -3.35% predicted, p=0.035, respectively). Among those homozygous for the *GSTM1* null genotype, *in utero* tobacco exposure was associated with lower FEV_1 and FVC compared with those with no *in utero* tobacco exposure (mean difference -6.2% predicted, p=0.01; -4.7% predicted, p=0.043 respectively). For those with *GSTM1* active genotype, there was no difference in lung function whether exposed to maternal *in utero* tobacco or not. *In utero* tobacco exposure was associated with deficits in lung function among those with both *GSTT1* null and *GSTT1* active genotypes.

Conclusions

GST genotypes may have protective effects against the deficits in lung function associated with *in utero* tobacco exposure. This offers potential preventative targets in anti-oxidant pathways for at-risk infants of smoking mothers.

Abstract Words: 247

Introduction:

The exposure of children's lungs to tobacco smoke is of grave concern as lungs are particularly vulnerable to insult during development. This exposure can occur while still *in utero*, as nicotine and other toxic substances freely pass through the placenta or postnatally in the home environment [1].

Exposure to tobacco smoke antenatally is associated with reduced lung function during infancy and childhood [3 4]. Both ante- and post-natal smoke exposure are linked to an increase in respiratory symptoms throughout childhood and adolescence [2].

We have previously shown that airway function measurements track from infancy into early adulthood indicating an inherent airway structure is laid down early in lung development, while measurements of adult lung size were independent of infant lung function, suggesting lung growth is modifiable by external factors.

Gene-environment interactions modulate the effects of *in utero* smoke (ITS) exposure on respiratory outcomes in childhood and fetal anti-oxidants may play a role in protecting the developing lungs. Activity levels of glutathione s-transferases (GST), a family of enzymes involved in the detoxification of xenobiotics, vary based on genotype, with homozygous deletions of the *GSTT1* and *GSTM1* genes associated with absent function of that particular enzyme [5 6]. Among those exposed to ITS, *GST* active genotypes are linked to higher infant lung function when compared with those with the null genotype, as previously reported in this cohort [7]. *GST* null genotypes are associated with increased risk of childhood asthma compared with those with active genotypes, particularly in the context of passive smoke exposure, suggesting an increased vulnerability to the detrimental effects of tobacco smoke [8-10].

We hypothesised that the negative effect of ITS exposure on lung function would persist into adulthood but that this effect would be lessened for those with higher levels of innate

detoxification enzymes. Our aim was to: (1) assess the long-term effects of both ante and post-natal smoke exposure on lung function, as a measure of lung development; and (2) assess whether *GSTT1* and *GSTM1* active genotypes are protective against the effects of *in utero* tobacco smoke on lung function through into adulthood.

Methods and Materials:

The Perth Infant Asthma Follow study was established in 1987. Over 24 months, 253 subjects were recruited antenatally from an urban maternity hospital in Perth, Western Australia. There was no preselection based on family history of asthma or atopy. Recruitment details have been published previously [11]. Infants were excluded if they were born premature <37 weeks gestation, had any major congenital abnormality or had any significant respiratory illnesses in the first month of life.

Detailed antenatal smoking history during each trimester of the pregnancy was collected from both parents. Fetal *in utero* tobacco exposure was classified as positive for maternal or paternal exposure if that parent smoked at all during the pregnancy. Subjects could be positive for both maternal and paternal *in utero* tobacco exposure. Subjects were classified as negative for any ITS exposure if neither parent smoked at all during the pregnancy.

Urinary cotinine, a byproduct of nicotine metabolism, was measured in a subgroup of infants at birth (n=85).

Subjects or their parents also completed a questionnaire at each follow-up assessment[12]. This included questions on history of physician-diagnosed asthma and tobacco smoke exposure in the household. “Postnatal smoke exposure only” was classified as a positive response to post-

natal smoke exposure at either 1, 6 or 11 years of age and no history of maternal *in utero* smoke exposure. “Incomplete postnatal data” was recorded if there was less than 2 postnatal assessments.

The participants performed lung function testing at regular intervals from infancy through to young adulthood at 1 month, 6 months, 12 months, 6, 11, 18 and 24 years of age.

The rapid thoraco-abdominal compression technique during tidal breathing of sedated infants was used during infant lung function testing [13]. The interaction between *in utero* tobacco exposure and GST genotype on infant lung function in this cohort has been published previously [7].

Spirometry was performed at each assessment from 6 to 24 years[14]. FEV_1, FVC, FEF25-75% and FEV_1/FVC were recorded and converted into percent predicted scores based on sex, age, height and ethnicity using GLI reference values[15].

GST genotyping analysis was performed on blood samples taken at either the 6 or 11 year assessments. *GSTT1* and *GSTM1* deletion polymorphisms were identified by polymerase chain reaction (PCR) methods as previously described in detail [7 16]. A subset of specimens had genotype results confirmed by PCR with a second set of primers [7]. *CYP1A1* primers were used as a positive control for each PCR performed. Those who were homozygous for the *GST* null genes were classified as *GST* null and those who were either heterozygous or homozygous for non-null *GST* genotype was classified as *GST* active.

The study was approved by the Western Australian Child and Adolescent Health Service Human Research Ethics Committee (2054EP). Parents, or subjects when appropriate (aged > 18 years), signed informed consent forms for each assessment.

Statistical analysis:

Comparisons between the participants seen at each assessment was analysed by Pearson's chi-square for categorical variables and independent t-test for continuous variables.

Mean difference in cotinine levels between infants whose mothers reported smoking at recruitment and infants whose mothers reported not smoking at recruitment were measured using the Mann Whitney U test.

In the longitudinal analysis, the link between tobacco smoke exposure or *GST* genotype and lung function from 6 to 24 years were assessed by generalised estimating equations. These equations adjust for inherent covariance in each subject [17.]

Mean lung function results (FEV_1, FVC, FEF25-75% and FEV_1/FVC% predicted) from the 6, 11, 18 and 24 year assessments were the longitudinal outcome variables for each participant. Maternal ITS, paternal ITS and postnatal tobacco exposure only were all assessed separately and compared with no *in utero* smoke exposure.

In order to assess the effect of *GST* polymorphisms on lung function in the context of *in utero* tobacco exposure, we then split the group into *GST* genotype i.e firstly *GSTT1* null versus *GSTT1* active and then *GSTM1* null and *GSTM1* active and applied the generalised estimating equations. Mean longitudinal lung function (% predicted) for those exposed and not exposed to maternal ITS in each subgroup were included as outcome variables.

Two-sided p value <0.05 determined statistical significance.

This study had a power of 0.813 to reject the null hypothesis of no significant difference in FEV_1 between *GSTT1* null and *GSTT1* non-null genotype groups based on 411 assessments, if the real difference between groups was 5% predicted, with standard deviation of 12% predicted. The Type I error probability associated with this test of this null hypothesis is 0.05.

Analyses were performed using SPSS Statistics for Windows, version 24.0. (2016, Armonk, NY: IBM Corp).

Results:

General

Of the original 253 subjects recruited, smoking history during the pregnancy was collected from 252 mothers and 240 fathers. Eighty five subjects (34%) reported exposure to maternal smoking during the pregnancy and ninety-four (39%) reported exposure to paternal smoking during the pregnancy. A further 47 subjects out of 129 with no maternal ITS exposure and at least 2 postnatal assessments, reported exposure to post-natal tobacco smoke in the home, with 82 subjects reporting no pre or post natal smoke exposure and incomplete post-natal exposure data on the remaining 38 subjects.

Lung function testing was performed on 110 subjects at 6 years, 183 at 11 years, 141 at 18 years and 118 subjects at 24 years. Comparison of subject characteristics at each assessment have previously been published[18]. The only significant difference between the original cohort and those seen at follow up was less parental ITS exposure in those seen at later follow ups (54% ITS exposure in original cohort; 45% of those seen at 18 years; 43% of those seen at 24 years). *GSTT1* and *GSTM1* genotyping was performed on 179 subjects and the frequency of each genotype is presented in table 1. There were 144 subjects with 443 assessments included in the longitudinal analysis of smoke exposure, and 128 subjects with 411 assessments in the longitudinal analysis of *GST* genotype and smoke exposure.

Cotinine levels and maternal smoke exposure

Neonatal cotinine levels were higher amongst infants whose mothers reported smoking at recruitment during pregnancy (n=25; mean 75.3ng/ml creatinine, SD 57.7), than those whose mothers reported no smoking at recruitment (n=60; mean 9.3ng/ml creatinine, SD 30.5), p<0.001.

In utero tobacco smoke exposure and lung function

Exposure to maternal ITS was associated with significantly lower FEV_1 and FVC from 6 to 24 years of age (mean difference – 3.87% predicted, p=0.021, and -3.35% predicted, p=0.035, respectively) Figure 1; supplementary table 1. Exposure to maternal ITS was not associated with a difference in either FEV_1/FVC or FEF25-75%. Neither exposure to paternal ITS nor postnatal tobacco exposure only were associated with significant changes in lung function from 6 to 24 years. Therefore, further reference to ITS refers to maternal ITS exposure only, independent of post-natal smoke exposure.

GST polymorphisms and *in utero* tobacco smoke exposure

There was no difference in lung function from 6 to 24 years of age between the *GST* null and active genotype groups (supplementary table 2).

Among those with the *GSTM1* active genotype, maternal ITS exposure was not associated with any significant difference in lung function from 6 and 24 years of age. Among those with the *GSTM1* null genotype, ITS exposure was associated with a lower FEV_1 and FVC compared with those with no ITS exposure (mean difference FEV_1 -6.2% predicted, p=0.01; mean difference FVC -4.7% predicted, p=0.043) (table 2; figure 2)

Among those with the *GSTT1* active genotype, FEV_1 and FVC were lower in those exposed to maternal ITS compared with no maternal ITS exposure (mean difference FEV_1= -4.05% predicted, p=0.034; FVC= -3.7% predicted, p=0.037 respectively). Among those with the *GSTT1* null genotype, FEV_1 and FEF25-75% were lower in those exposed to ITS, compared with no ITS (mean difference FEV_1 = - 10.29% predicted, p=0.021; mean difference FEF25-75% = -15.2% predicted, p=0.008). (table 2; figure 2). However, only three subjects with exposure to maternal ITS had the *GSTT1* null genotype and all three also had the *GSTM1* null genotype.

Discussion:

This longitudinal, birth-cohort study of lung function confirms that maternal ITS exposure is linked to lower lung function from infancy to early adulthood, and establishes an important new finding: the major effect of *in utero* smoke exposure is on lung size rather than airway size or function. This conclusion stems from the finding that the deficits in future respiratory function were specifically in FEV_1 and FVC and not FEF25-75% or FEV_1/FVC. A previous study from this cohort revealed that variables reflecting airway function, V'maxFRC in infancy and FEF 25-75% and FEV_1/FVC thereafter, track from infancy into early adulthood. Together these findings provide compelling evidence suggesting that the foundations of airway structure are laid down during antenatal development, persist throughout childhood and are relatively resistant to environmental insults. In contrast, lung size in childhood, as measured by FEV_1 and FVC, had no correlation with infant airway function, but did correlate with tobacco smoke exposure, implying that lung size (perhaps as a reflection of alveolar number) is vulnerable to external, environmental factors.

A further important finding was evidence suggesting that the presence of active glutathione s-transferase enzymes provide longterm protection from the damage caused by maternal ITS exposure. In particular, the functional *GSTM1* active genotype appears to have this protective effect. Maternal ITS was only associated with deficits in lung function up to adulthood for those with the *GSTM1* null genotype and not for those with the *GSTM1* active genotype. The functional *GSTT1* active genotype did not appear to share this degree of protective effect, as those with this genotype still had significant deficits in lung function if exposed to maternal ITS compared with those with no ITS exposure. However, the difference between those exposed and not exposed was smaller than among the *GSTT1* null genotype group suggesting that *GSTT1* active may still provide a degree of protection, although not enough to fully overcome the damage associated with ITS exposure. *GSTT1* may be of more importance in protection very early on, as in a previous study from this cohort, *GSTT1* active genotype was associated with higher lung function during infancy mong those exposed to maternal ITS than the *GSTT1* null group [7].

Glutathione s-transferase is an enzyme which catalyses the reaction between glutathione and electrophilic xenobiotics and reactive oxygen species, making it crucial to the body's detoxification processes[19]. There are eight classes of cytosolic GST in humans, each with several subclasses, which vary in their structure and substrate specificity [20]. Although nicotine is not metabolised by the GST enzymes, many of the other toxic substances within cigarettes are [21 22]. Homozygous *GSTM1* and *GSTT1* null polymorphisms have been associated with the development of asthma in childhood, thought to be due to an increase in oxidative stress, although they are not related to asthma severity (turner new paper). Adjusting for confounding variables during childhood such as tobacco exposure and environmental pollution is difficult and may be the reason results from previous studies have not been convincing [23]. *GSTM1* and *GSTT1* null genotypes have both been found to be strong predictors of COPD in adult

females[24]. The *GSTM1* null genotype is also associated with reduced lung function growth in children, while the *GSTT1* null genotype is associated with accelerated lung function decline in adult males[25 26]. However, these studies did not specifically investigate those exposed to tobacco smoke *in utero*, a critical time during lung development and we did not find a link between *GST* genotype and lung function among those not exposed to ITS.

The *GSTT1* null genotype is rarer than the *GSTM1* null genotype, affecting only 15% of the population. The subject numbers with both GSM1 null genotype and maternal ITS exposure, n=3, make definitive statements about this group difficult. However, the length of follow up from infancy through into early adulthood in this study is an important advantage, as this spans the entire post-natal lung growth phase, to the peak in lung function in early adulthood.

During the *in utero* period of organogenesis, the fetus is exposed to the same levels of nicotine and other toxic substances as found in the actively-smoking mother, as these toxic substances pass freely through the placenta. Cotinine, a by-product of nicotine metabolism, can be measured in umbilical cord blood of newborn infants whose mothers smoked during pregnancy[1]. Although the tobacco is not inhaled into the lungs of the fetus, the serum exposure to these toxins is associated with reduced lung function when measured in the first few days of life, even before any post-natal tobacco smoke expsoure[27]. Given the negative associations between tobacco exposure while pregnant and infant outcomes, pregnant mothers may be reluctant to admit smoking to study researchers. However we collected objective evidence of tobacco exposure, with neonatal urinary cotinine levels, which confirm the reliability of the parent reported smoking data.

Interestingly, there was no link between either paternal ITS exposure or postnatal tobacco smoke exposure alone, on lung function throughout childhood, suggesting the most

significant impact of tobacco exposure on lung function is a dose response effect during the *in utero* developmental phase.

Children with two hits, a toxic exposure and a genetic vulnerability, are at risk for the largest deficits in lung growth during lung development. Our data suggests there is a protective benefit in having higher anti-oxidant levels during *in utero* development and this warrants further exploration. Finding a way to protect the lungs during critical periods of development, for example with anti-oxidant supplementation or boosting the fetal glutathione pathway, and avoiding long-term detrimental consequences could potentially be an important target in minimising chronic respiratory morbidity for the children of smoking mothers.

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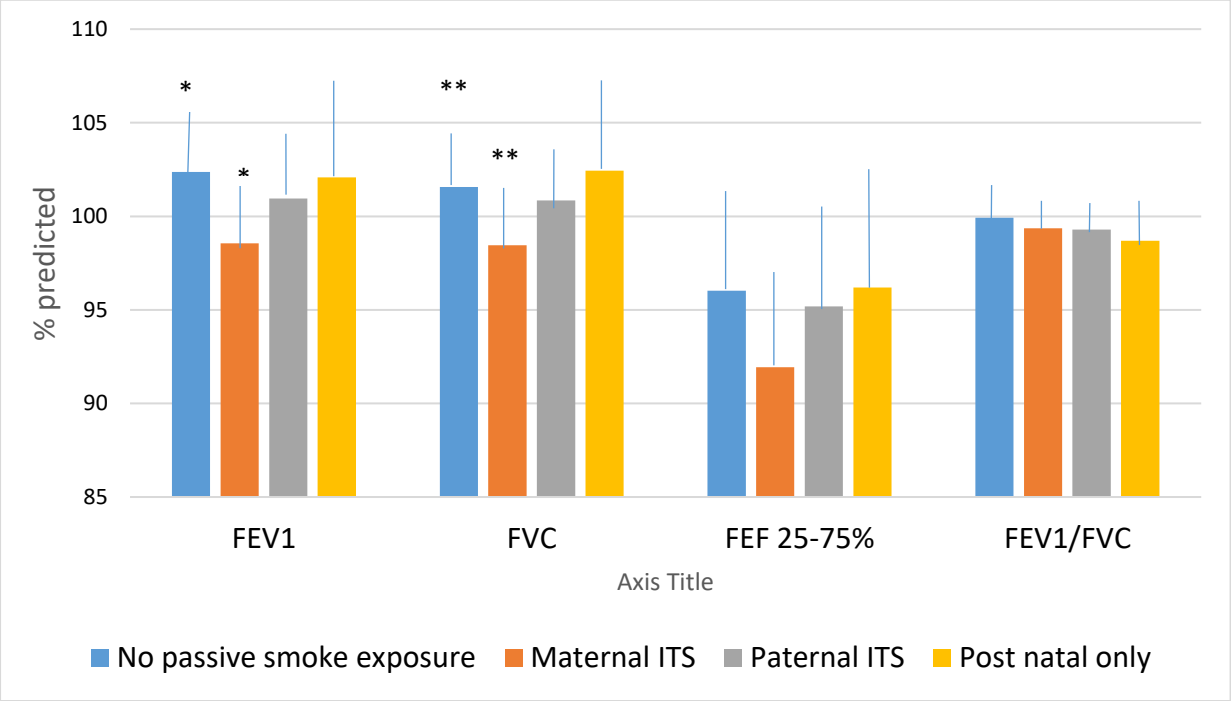
Table 1. Prevalence of *GST* genotypes (n=179)

		N
	GSTM1 –	105 (59%)
	GSTM1 +	74 (41%)
	GSTT1 –	27 (15%)
	GSTT1 +	152 (85%)
	GSTM1 - and *GSTT1*-	11 (6%)

– = homozygous null genotype; + = heterozygous or homozygous non-null genotype.

Figure 1. Mean lung function from 6-24 years by tobacco smoke exposure. Number of subjects=144; number of assessments = 443



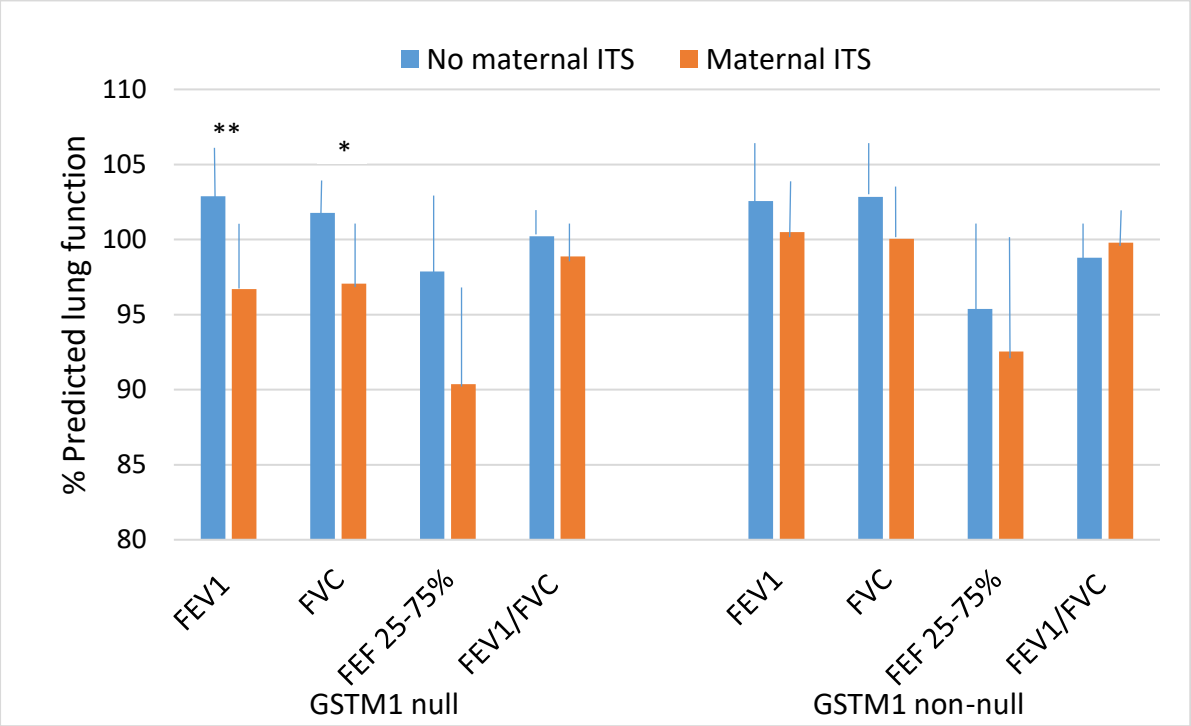
Lung function from 6-24 years. % predicted based on GLI reference values. Generalised estimating equations. Error bars depicting 95% confidence interval.
*p=0.021; **p=0.035

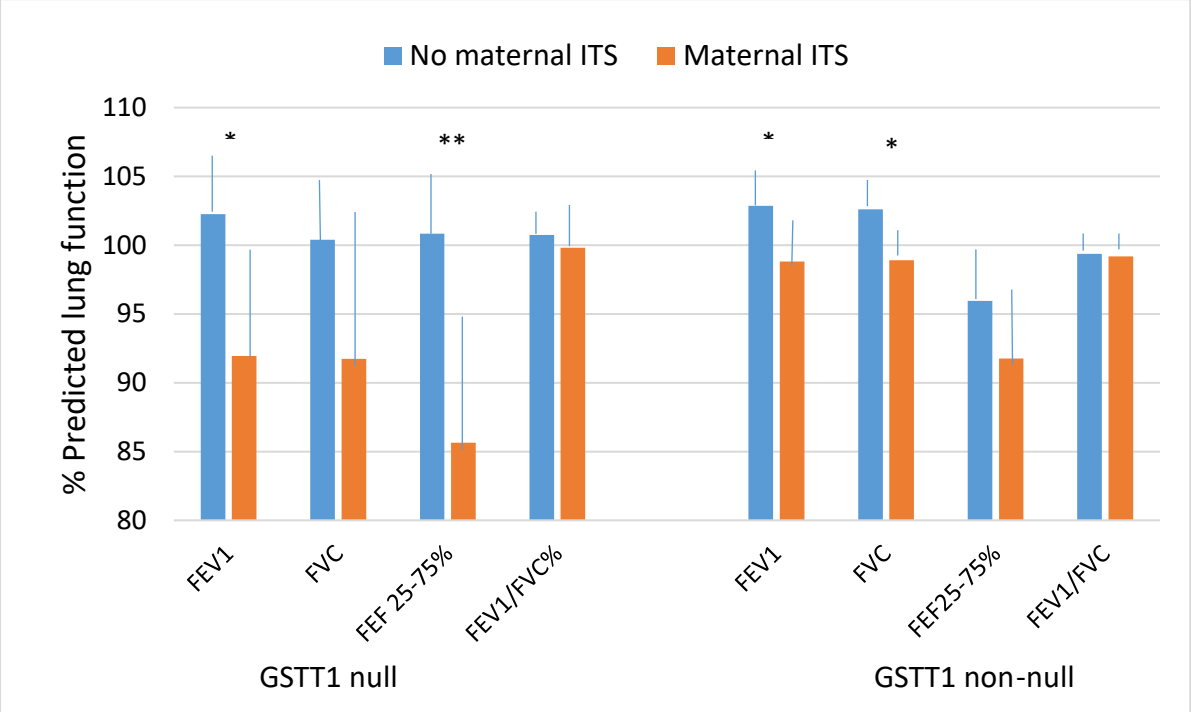
Table 2. Association between maternal *in utero* tobacco smoke exposure and lung function from 6-24 years, grouped by *GST* genotype.

		FEV_1 % pred (95% CI)	**FVC % pred (95% CI)**	**FEF 25-75% pred**	**FEV_1/FVC % pred (95% CI)**
GSTM1 null	No ITS (n=52)	**102.88 (100.02 -105.73)**	**101.77 (99.04 – 104.5)**	97.88 (93 – 102.75)	100.22 (98.8 – 101.6)
	Maternal ITS (n=23)	**96.7 (92.92 – 100.49) p=0.01**	**97.06 (93.4 – 100.72) p=0.043**	90.36 (83.85 – 96.89) p=0.07	98.88 (97.0 – 100.8) p=0.27
GSTM1 non-null	No ITS (n=41)	102.56 (99.51 -105.61)	102.83 (99.82 – 105.84)	95.38 (90.06 – 100.69)	98.78 (96.87 – 100.69)
	Maternal ITS (n=12)	100.5 (96.63 – 104.37) p=0.41	100.05 (96.68 – 103.43) p=0.229	92.54 (85.18 – 99.89) p=0.54	99.78 (97.48 -102.1) p=0.51
GSTT1 null	No ITS (n=18)	**102.26 (98.64 – 105.88)**	100.44 (95.96 – 104.92)	**100.84 (94.73 – 106.96)**	100.74 (98.19 – 103.29)
	Maternal ITS (n=3)	**91.94 (83.98 – 99.9) p=0.021**	91.74 (80.78 – 102.7) p=0.15	**85.63 (76.2 – 95.08) p=0.008**	99.8 (95.51– 104.09) p=0.71
GSTT1 non-null	No ITS (n=75)	**102.85 (100.43 – 105.26)**	**102.6 (100.34 – 104.86)**	95.96 (91.8 – 100.12)	99.37 (98.08 – 100.65)
	Maternal ITS (n=32)	**98.8 (95.94 – 101.67) p=0.034**	**98.9 (96.32 – 101.4) p=0.031**	91.75 (86.49 - 97.01) p=0.22	99.19 (97.63 – 100.75) p=0.86

Mean difference in % predicted lung function for those exposed to maternal *in utero* tobacco smoke, compared with not exposed to maternal *in utero* tobacco smoke, by GST genotype. Number of subjects=128; number of assessments = 411Bold font indicates $p<0.05$

Figure 2. Mean lung function from 6-24 years for GST null versus non-null genotype, by maternal ITS exposure





Mean lung function longitudinally from 6-24 years, % predicted based on GLI reference range. *p<0.05 ; ** p≤ 0.01

Supplementary Table 1: Lung function from 6-24 years by passive smoke exposure

	FEV1% predicted	FVC % predicted	FEF25-75% % predicted	FEV1/FVC % predicted
No passive smoke exposure	102.37 (99.83 – 104.91)	101.57 (99.16 – 103.97)	96.02 (91.67 – 100.36)	99.93 (98.56 - 101.31)
Maternal ITS	98.55 (95.88 – 101.22)	98.45 (95.97 – 100.93)	91.94 (87.21 – 96.67)	99.36 (97.95 – 100.77)
Paternal ITS	100.95 (98.34 – 103.56)	100.84 (98.29 – 103.39)	95.18 (90.89 – 99.47)	99.29 (98.01 – 100.57)
Postnatal smoke exposure only	102.08 (97.87 – 106.29)	102.44 (98.23 – 106.65)	96.2 (89.46 – 102.9)	98.69 (96.5 – 100.87)

No passive smoke exposure – no in utero or postnatal tobacco smoke exposure from either parent or household member; Maternal ITS – mother smoked at all during the pregnancy; Paternal ITS – father smoked at all during the pregnancy; Postnatal smoke exposure only- no maternal *in utero* tobacco smoke exposure, but postnatal smoke exposure in the home.

Supplementary Table 2: *GST* polymorphisms and lung function from 6 to 24 years

	Mean FEV_1 % predicted	Mean FVC% predicted	Mean FEF25-75% % predicted	Mean FEV1/FVC %predicted
GSTT1 null	100.45 (96.68 – 104.21)	98.91 (94.43 – 103.39)	98.17 (92.47 – 103.87)	100.57 (98.34 – 102.8)
GSTT1 non-null	101.52 (99.61 – 103.43)	101.37 (99.61 – 103.14)	94.59 (91.28 – 97.9)	99.31 (98.3-100.31)
	p=0.62	p=0.32	p=0.29	p=0.31
GSTM1 null	100.94 (98.56 – 103.3)	100.29 (98.04 – 102.54)	95.53 (91.55 – 99.51)	99.79 (98.65 – 100.94)
GSTM1 non-null	101.96 (99.5 – 104.42)	102.02 (99.64 – 104.4)	94.56 (90.2 – 98.9)	99.07 (97.56 -100.59)
	p=0.56	p=0.3	p=0.75	p=0.46

Mean lung function from 6-24 years by GST genotype. % predicted based on GLI reference values. Unadjusted values