Relationship between air pollution, *NFE2L2* gene polymorphisms and childhood asthma in a Hungarian population

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Abstract Air pollution and subsequent increased oxidative stress have long been recognized as contributing factors for asthma phenotypes. Individual susceptibility to oxidative stress is determined by genetic variations of the antioxidant defence system. In this study, we analysed the association between environmental nitrogen dioxide (NO₂) exposure and single nucleotide polymorphisms (SNP) in *NFE2L2* and *KEAP1* genes and their common impact on asthma risk.

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We genotyped 12 SNPs in a case–control study of 307 patients diagnosed with asthma and 344 controls. NO₂ concentration was collected from the period preceding the development of asthma symptoms. Multiple logistic regression was applied to evaluate the effects of the studied genetic variations on asthma outcomes in interaction with NO₂ exposure. Our data showed that genotypes of rs2588882 and rs6721961 in the regulatory regions of the *NFE2L2* gene were inversely associated with infection-induced asthma (odds ratio (OR)=0.290, $p=0.0015$, and OR=0.437, $p=0.007$, respectively). Furthermore, case-only analyses revealed significant differences for these SNPs between asthma patients that lived in modestly or highly polluted environment (OR=0.43 (0.23–0.82), $p=0.01$, and OR=0.51, $p=0.02$, respectively, in a dominant model). In conclusion, our results throw some new light upon the impact of *NFE2L2* polymorphisms on infection-induced asthma risk and their effect in gene–environment interactions.

Keywords Infection-induced asthma · *NFE2L2* · Nitrogen dioxide · Oxidative stress · SNP

Abbreviations

AA	Atopic asthma
HWE	Hardy–Weinberg equilibrium
IIA	Infection-induced asthma
NFE2L2	Nuclear factor erythroid-derived 2-like 2
NO ₂	Nitrogen dioxide
KEAP1	Kelch-like ECH-associated protein 1
OR	Odds ratio
SNP	Single nucleotide polymorphism

Introduction

Asthma is a multifactorial disease influenced by complex interactions between multiple genetic and environmental factors. Over the past decades, an increase in asthma incidence and prevalence rates has been observed in numerous developed countries (Braman 2006). Many lines of evidence suggest that one of the most important causative factors of this critical increase is the raised exposure to environmental pollutants and airborne allergens especially if associated with a susceptible genetic background of the person exposed.

Traffic-related air pollutants such as nitrogen dioxide, particulate matter and ozone have been shown to increase the risk of asthma as well as exacerbate symptoms in patients with an already existing airway disease, leading to the production of free radicals and enhancing the inflammatory response (Braback and Forsberg 2009). There is increasing evidence that the pulmonary inflammatory response triggered by air pollutants is mediated via redox-sensitive signalling pathways; thus, genes involved in the oxidative stress response are candidates for susceptibility to the respiratory effects of air pollutants.

The oxidative stress response is centrally mediated by the redox-sensitive transcription factor, nuclear factor erythroid-derived 2-like 2 (NFE2L2, also known as Nrf2) and its negative regulator protein Kelch-like ECH-associated protein 1 (KEAP1). Low levels of oxidative stress following exposure to a pollution episode leads to the liberation of NFE2L2 from its repressor KEAP1, activating more than 200 genes which encode many antioxidant or phase II detoxifying enzymes and related stress response proteins (Kwak et al. 2003). The protective role of NFE2L2 is widely evident not only in oxidative processes but also in inflammatory disorders (Kim et al. 2010).

It appears likely that oxidative stress is related, in part, to the presence of decreased antioxidant defences. Numerous human studies have already been conducted to reveal the interactions between the level of air pollutants, the onset of asthma or other airway disorders and the genetic/epigenetic variations of the genes whose products are involved in the regulation of antioxidant defences (Minelli et al. 2011). In an ovalbumin-challenged asthmatic mouse model, Nfe2l2 deficiency resulted in increased airway inflammation, airway hyperreactivity and an elevated level of Th2 cytokines, indicating the critical role of Nfe2l2 in asthma pathogenesis (Rangasamy et al. 2005). Furthermore, these mice were proven to be highly susceptible to cigarette smoke-induced emphysema (Iizuka et al. 2005).

The most commonly studied outdoor pollutants in connection with respiratory health outcomes are ozone, nitrogen dioxide and particulate matter. Animal studies have shown that repeated exposures to increased nitrogen dioxide levels can damage the lung and might contribute to the development

of different lung diseases (Poynter et al. 2006). Furthermore, there is evidence that NO₂ increases the risk of asthma exacerbations following upper respiratory infections even at relatively low levels of exposure (Linaker et al. 2000). Conversely, it is also proven that continuous exposure to nitrogen dioxide might increase the risk of respiratory infections (Cienciewicki and Jaspers 2007).

The existence of a genetic background that determines a different susceptibility to asthma following NO₂ exposure is supported by animal experiments (Zhang et al. 1995; Holroyd et al. 1997) and the growing, but still limited, set of association studies applying NO₂ as an indicator of air pollution (Minelli et al. 2011). These latter studies showed that polymorphisms in the genes encoding GSTP1, NQO1, TNF, TLR2 and TLR4 significantly modified the effect of air pollution on lung function, allergic sensitization and asthma prevalence (Lee et al. 2004; Melen et al. 2008; Kerkhof et al. 2010; Castro-Giner et al. 2009).

In this study, our aim was to discover associations between the genetic variations in the master regulator elements of oxidative stress response and traffic-related air pollution on asthma prevalence in a Hungarian population, especially regarding the infection-induced asthma phenotype. Because up to the present data have not been published on the genotype distribution of the *NFE2L2* and *KEAP1* genes between asthma cases and controls, we first investigated whether polymorphisms in these genes are associated with the development of asthma or some of its endophenotypes, such as atopic or infection-induced asthma status. Afterwards, in a case-only study, we evaluated the interactive effects of these genetic variations and the concentrations of NO₂ on asthma risk.

Methods

Subjects

The study population comprised 651 unrelated individuals of a Hungarian (Caucasian) population. Approximately 5% of the tested subjects were probably of Gypsy origin (estimate based on state population statistics).

All the asthmatic children had specialist physician-diagnosed asthma with the following characteristics: (1) recurrent breathlessness and expiratory dyspnea requiring treatment; (2) physician-diagnosed wheeze; and (3) reversibility of the wheezing and dyspnea by bronchodilator treatment measured as forced expiratory volume 1 s (FEV₁) by a spirometer. All the asthmatics (or their parents) were instructed to record accurately for 2 weeks their symptoms, treatment and twice daily (in the evening and in the morning) peak expiratory flow (PEF). PEF 100% was determined by calculation from the personal best value and the expected value according to the height of the patient.

If the patient is younger than 5 years, the determination of lung function tests (PEF or FEV₁) is usually not possible. In that case, the diagnosis and classification of the disease were made according to the frequency and severity of other symptoms. Atopy was defined by a positive skin prick test to at least one allergen (wheal diameter 3 mm greater than saline control) and/or positive total or specific IgE levels. If the onset of asthma or the asthma exacerbations have been associated with an infection-related acute respiratory illness, the asthma was classified as infection-induced asthma. Cases were recruited from the Budai Children's Hospital and from the Heim Pal Hospital, Budapest.

The control children were randomly selected from outpatients from the Orthopaedic Department in the Budai Children's Hospital or from the Urological Department of Heim Pal Hospital, Budapest. Children in the control group had mild musculoskeletal alterations, phimosis, or other small urogenital problems and showed no symptoms of asthma and required no medication. The adult controls were healthy blood donors and recruited by the Hungarian National Blood Transfusion Service, Budapest. For information about the study subjects, detailed questionnaires were filled out and subjects were called back to clarify information if inconsistencies were identified. None of the controls has had asthma in their childhood.

Both patients and controls were recruited in the period 2001–2004. Only subjects whose place of residence had available air pollution data for this period were included. The study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Ethics Committee of the Hungarian Medical Research Council. Written informed consent was obtained from all patients or the parents or guardians of the minors involved in the study.

Detailed characteristics of the study participants are presented in Table 1.

Determination of air pollution

As an indicator of local traffic-related air pollution, we used the concentrations of NO₂.

Its level for the years preceding the onset of asthma (1999–2003) was provided by the Reference Center of the Hungarian Air Quality Network (under the governance of the Ministry of Rural Development, Hungary). Concentrations of NO₂ were measured by automatic stations located within 10 km of the residence of each study participant.

When considering NO₂ concentration as a discrete parameter, we regarded the concentration of 32 µg/m³ as a threshold level, and all NO₂ concentrations below this threshold were classified as low and above as high. The threshold was determined at the median of the average NO₂ concentrations of the studied geographical locations.

Table 1 Characteristics of the study subjects

Characteristics	Patients with asthma (n=307)	Control patients (n=344)
Age (years±SD)	10.55±4.74	21.77±13.88
Gender (male/female)	200/107	173/171
Endophenotypes		
Atopy, N (%)	191 (62.21)	–
Exercise-induced asthma, N (%)	63 (20.52)	–
Infection-induced asthma, N (%)	88 (28.66)	–
Intrinsic asthma, N (%)	59 (19.22)	–
Severity of asthma ^a		
Mild asthma	117 (38.11%)	–
Moderate asthma	165 (53.74%)	–
Severe asthma	25 (8.14%)	–
Air pollution of the district		
High (≥32 µg/m ³)	116 (37.78%)	285 (82.84%)
Low (≤32 µg/m ³)	191 (62.21%)	59 (17.15%)

n number of patients involved

^a Severity was classified based on the GINA criteria

Selection of SNPs

Tagging single nucleotide polymorphisms (SNPs) located in the regulatory regions of the *NFE2L2* and *KEAP1* genes were assigned using the International HapMap data for European ancestry (CEU) (<http://www.hapmap.org>). A minor allele frequency of ≥5% and pairwise tagging with a minimum of 0.80 were applied to capture the common variants within the linkage disequilibrium (LD) blocks. A complete list of the selected SNPs is shown in Table 2.

DNA isolation and genotyping

Genomic DNA was isolated from whole blood samples using iPrep PureLink gDNA Blood Kit on iPrep Purification Instrument (Invitrogen). A total of 12 SNPs were genotyped using the Sequenom iPLEX Gold MassARRAY technology at the McGill University and Génome Québec Innovation Centre, Montréal (Québec), Canada.

Statistical analysis

Allele frequencies in groups of cases and normal subjects were estimated by gene counting and tested for deviation from the Hardy–Weinberg equilibrium (HWE) by the software programme DeFinetti (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>). For the significant deviation threshold, we used $p=0.05$. Using SPSS v17 (SPSS Inc., Chicago, IL, USA), logistic regression

Table 2 Description of selected single nucleotide polymorphisms

SNP	SNP rs#	Position according to NCBI Genome Build 37.1	Alleles on the forward strand	Gene location	MAF in controls	MAF in cases	<i>p</i> value ^a
<i>KEAP1</i>		<i>Chr:19</i>					
1	rs11085735	10602180	C/A	Intron	0.07	0.06	0.50
2	rs8113472	10608064	C/A	Intron	0.07	0.08	0.37
3	rs11668429	10616303	T/G	Promoter	0.34	0.33	0.68
4	rs7246953	10621108	G/A	Promoter	0.19	0.18	0.68
<i>NFE2L2</i>		<i>Chr:2</i>					
1	rs2588882	178087165	T/G	3' region	0.08	0.11	0.06
2	rs2706110	178092162	C/T	3' region	0.15	0.18	0.23
3	rs10183914	178097666	C/T	Intron	0.29	0.28	0.79
4	rs1806649	178118152	C/T	Intron	0.22	0.20	0.50
5	rs6721961	178130037	G/T	Promoter (−617)	0.13	0.14	0.70
6	rs6706649	178130071	C/T	Promoter (−651)	0.12	0.11	0.71
7	rs35652124	178130073	T/C	Promoter(−653)	0.34	0.36	0.35
8	rs2364725	178132988	T/G	Promoter	0.45	0.43	0.49

MAF minor allele frequencies in our study population, *NFE2L2* nuclear factor erythroid-derived 2-like 2, *KEAP1* Kelch-like ECH-associated protein 1

^a Unadjusted *p* value was calculated by a one degree of freedom Pearson's chi-square test on allele counts

analyses were completed for asthma, atopic asthma and infection-induced asthma using additive (11 vs. 12 vs. 22), recessive (11/12 vs. 22), or dominant (11 vs. 12/22) models. Since the age and sex distributions were not equal in the asthma and control groups, logistic regression analyses were adjusted for these factors. Confidence intervals were calculated at the 95% level. For single marker analyses, alpha levels of $p < 0.0033$, that is, 0.05 after Bonferroni correction considering multiple testing for 12 SNPs and three genetic models, were considered as significant. To compare the genotype distributions between the asthma endophenotypes and to estimate the gene–environment interactions, a case-only design was applied (Botto and Khoury 2001).

LD and the distribution of haplotype frequencies in cases and controls were analysed using the software programme Haploview 4.1. (<http://www.broad.mit.edu/mpg/haploview/>). Odds ratios for haplotypes were counted using the MedCalc 10.0.2 (MedCalc Software, Mariakerke, Belgium) software.

Results

SNP association analysis

Genotyping mean success rate was 96%. The genotype distributions of all SNPs tested were found to be in HWE ($p > 0.05$). Genotype distributions and single marker analyses of the studied SNPs are shown in Table 3.

Between asthma cases and controls, none of the studied SNPs could withstand the correction for multiple testing; however, rs2588882 located in the 3' region of *NFE2L2* was nominally significant with asthma in the additive model (OR=1.53, 95% CI=1.01–2.33, $p=0.05$).

The strongest evidence for association was found between infection-induced asthma status and rs2588882 as its minor allele was significantly less prevalent in the infection-induced asthmatic group (IIA) compared with the non-infection-induced asthmatic (non-IIA) group (OR=0.28, 95% CI=0.13–0.60, $p=0.0005$). The genotype associations were significant in both dominant and additive models (OR=0.28, 95% CI=0.12–0.62, $p=0.0017$, and OR=0.29, 95% CI=0.13–0.62, $p=0.0015$, respectively). Furthermore, another *NFE2L2* SNP, rs6721961, also showed association with IIA in the recessive model, but it failed to reach statistical significance after Bonferroni correction (OR=0.44, 95% CI=0.28–0.80, $p=0.007$).

Only nominal significance was found between atopic asthma (AA) and rs6721961 ($p=0.035$) and rs7246953 ($p=0.016$) in the recessive models at both cases. No other associations were seen for the markers in *KEAP1* with either risk of asthma, AA or IIA.

To obtain more statistical evidence for the associations, we performed haplotype analyses. Analysing our genotype data, two distinctive LD blocks were formed in *NFE2L2* consisting of the tag SNPs 1–2 and 4–8, respectively. Significant associations were found only between the IIA and non-IIA groups; however, these

Table 3 Associations between the studied *NFE2L2/KEAP1* gene polymorphisms and different asthma phenotypes

SNP	Gene	Model	Asthma		Atopic asthma		Infection-induced asthma	
			<i>p</i> value	OR (95% CI)	<i>p</i> value	OR (95% CI)	<i>p</i> value	OR (95% CI)
rs2588882	NFE2L2	Dominant	0.11	1.45 (0.91–2.31)	0.71	0.89 (0.49–1.61)	0.002 ^a	0.28 (0.12–0.62)
		Recessive	0.06	7.43 (0.85–64.57)	0.58	0.65 (0.14–3.03)	0.99	NA
		Additive	0.05	1.53 (1.00–2.33)	0.62	0.88 (0.53–1.46)	0.002 ^a	0.29 (0.13–0.62)
rs2706110	NFE2L2	Dominant	0.27	1.24 (0.83–1.85)	0.63	1.13 (0.67–1.92)	0.16	0.66 (0.37–1.18)
		Recessive	0.06	2.72 (0.94–7.86)	0.34	1.78 (0.53–5.89)	0.07	0.15 (0.02–1.21)
		Additive	0.11	1.31 (0.93–1.83)	0.45	1.17 (0.76–1.80)	0.06	0.63 (0.38–1.02)
rs10183914	NFE2L2	Dominant	0.73	1.06 (0.73–1.53)	0.46	1.19 (0.73–1.94)	0.14	1.47 (0.87–2.47)
		Recessive	0.94	1.02 (0.49–2.10)	0.18	2.17 (0.68–6.94)	0.01	3.49 (1.32–9.12)
		Additive	0.76	1.04 (0.77–1.40)	0.25	1.26 (0.84–1.89)	0.02	1.61 (1.06–2.45)
rs1806649	NFE2L2	Dominant	0.98	0.99 (0.68–1.44)	0.64	1.12 (0.68–1.85)	0.48	1.20 (0.71–2.02)
		Recessive	0.68	0.83 (0.35–1.98)	0.95	1.04 (0.29–3.67)	0.22	2.10 (0.63–6.97)
		Additive	0.86	0.97 (0.71–1.33)	0.67	1.09 (0.71–1.68)	0.30	1.25 (0.81–1.95)
rs6721961	NFE2L2	Dominant	0.68	1.09 (0.72–1.64)	0.17	0.68 (0.40–1.18)	0.01	0.43 (0.23–0.83)
		Recessive	0.92	1.06 (0.31–3.62)	0.04	0.09 (0.01–0.84)	0.99	NA
		Additive	0.69	1.07 (0.74–1.54)	0.05	0.62 (0.38–1.00)	0.007	0.44 (0.28–0.80)
rs6706649	NFE2L2	Dominant	0.58	0.88 (0.57–1.36)	0.93	1.02 (0.57–1.81)	0.63	1.15 (0.63–2.07)
		Recessive	0.20	0.28 (0.04–1.98)	0.74	1.63 (0.08–31.14)	0.99	NA
		Additive	0.42	0.84 (0.56–1.27)	0.88	1.04 (0.60–1.79)	0.83	1.06 (0.60–1.86)
rs35652124	NFE2L2	Dominant	0.55	1.11 (0.77–1.61)	0.89	0.96 (0.59–1.58)	0.38	1.26 (0.74–2.15)
		Recessive	0.73	0.91 (0.52–1.56)	0.25	1.54 (0.73–3.25)	0.25	0.61 (0.26–1.42)
		Additive	0.79	1.03 (0.79–1.35)	0.62	1.09 (0.76–1.56)	0.94	1.01 (0.69–1.48)
rs2364725	NFE2L2	Dominant	0.78	1.05 (0.71–1.56)	0.48	0.82 (0.49–1.39)	0.72	0.90 (0.52–1.55)
		Recessive	0.71	0.91 (0.58–1.44)	0.58	0.84 (0.44–1.57)	0.19	0.61 (0.29–1.27)
		Additive	0.97	0.99 (0.77–1.28)	0.43	0.87 (0.61–1.23)	0.33	0.83 (0.57–1.21)
rs11085735	KEAP1	Dominant	0.28	0.75 (0.44–1.27)	0.80	1.09 (0.52–2.31)	0.29	1.48 (0.70–3.10)
		Recessive	0.54	0.47 (0.04–5.33)	0.99	NA	0.99	NA
		Additive	0.26	0.75 (0.45–1.23)	0.75	1.12 (0.54–2.32)	0.18	1.60 (0.79–3.25)
rs8113472	KEAP1	Dominant	0.91	1.02 (0.61–1.72)	0.44	1.30 (0.66–2.56)	0.49	0.77 (0.37–1.60)
		Recessive	0.28	5.70 (0.23–137.4)	0.39	0.33 (0.02–4.20)	0.93	1.10 (0.09–13.48)
		Additive	0.75	1.08 (0.66–1.77)	0.62	1.16 (0.63–2.14)	0.54	0.81 (0.42–1.58)
rs11668429	KEAP1	Dominant	0.98	0.96 (0.68–1.43)	0.99	1.00 (0.61–1.63)	0.22	1.38 (0.81–2.33)
		Recessive	0.82	0.93 (0.51–1.69)	0.73	0.87 (0.40–1.90)	0.72	0.85 (0.36–2.01)
		Additive	0.90	0.98 (0.74–1.29)	0.87	0.97 (0.67–1.40)	0.45	1.15 (0.78–1.706)
rs7246953	KEAP1	Dominant	0.89	0.97 (0.67–1.42)	0.80	1.06 (0.64–1.77)	0.05	1.68 (0.99–2.85)
		Recessive	0.97	1.02 (0.28–3.64)	0.02	0.07 (0.01–0.61)	0.50	1.65 (0.37–7.27)
		Additive	0.91	0.98 (0.69–1.38)	0.51	0.86 (0.54–1.34)	0.05	1.56 (0.99–2.48)

In the case of sufficient patient number, logistic regression was performed using additive (11 vs. 12 vs. 22), recessive (11/12 vs. 22) or dominant (11 vs. 12/22) models, the common homozygote signed as 11

NA not applicable (due to the absence of 22 genotypes), OR odds ratio, CI confidence interval, *NFE2L2* nuclear factor erythroid-derived 2-like 2, *KEAP1* Kelch-like ECH-associated protein 1

^a Significant *p* values after Bonferroni correction

results were driven mainly by rs2588882 and thus simply reflected the single effect of this variation (data not shown). With regard to *KEAP1*, three haplotypes were formed by rs8113472 and rs11668429, but none of them proved to be significant in any of the comparisons.

Analyses of gene–environment interactions

Because of the marked population bias in the air pollution data between the control and case groups (shown in Table 1), the relation of the genotypes and NO₂ level was assessed

within the asthma group only (Botto and Khoury 2001). Because the case-only approaches have been shown to be efficient only under the assumption of independence between the environmental and genetic factors in the control population (Albert et al. 2001), to assess the independency of the genotypes and NO₂ levels, we analysed our data in the control population separately and found no relationships ($p > 0.54$). Significant results of our case-only analyses are presented in Table 4. The results show that the rare alleles of rs2588882 and rs6721961 in the *NFE2L2* gene were significantly more prevalent in those children who lived in a modestly polluted environment (in the dominant model: for rs2588882—OR (95% CI)=0.43 (0.23–0.82), $p=0.01$; for rs6721961—OR (95% CI)=0.51 (0.29–0.90), $p=0.02$, respectively). No similar differences were found studying the atopic or infection-induced asthma groups.

In line with the results of the individual SNP analyses, haplotypes carrying the minor allele of both rs2588882 and rs6721961 were more prevalent within the low air pollution group (5.6% vs. 12.3%, OR=2.31, 95% CI=1.22–4.37, $p=0.0073$).

Discussion

To our knowledge, this is the first study investigating the possible links between *NFE2L2/KEAP1* polymorphisms, asthma and level of air pollution. Promoter polymorphisms of the *NFE2L2* and *KEAP1* genes have already been implicated in some diseases such as COPD, acute lung injury and Parkinson (Marzec et al. 2007; von Otter et al. 2010; Siedlinski et al. 2009). In our study, none of the investigated polymorphisms in the *NFE2L2* and *KEAP1* genes were found to be more prevalent in the asthma group compared with controls, questioning the elemental effect of these SNPs on the development of an asthma phenotype. However, when analysing the presence of infection-induced exacerbations (i.e. infection-induced asthma group) within the asthma group, genotypes carrying the variant alleles of rs2588882 and

rs6721961 appeared to have a significant protective effect. Additionally, these SNPs showed significant differences between asthma cases that lived in a highly or modestly polluted environment. These data suggest a very complex interaction between *NFE2L2* genotype and air pollution-dependent development of asthma and infectious exacerbations.

Whilst a growing number of studies exist investigating the impact of the promoter SNP rs6721961 (at position –617) on *NFE2L2* transcription, the exact in vivo function of this variant is not yet clear. But according to the scientific literature, some plausible theories can be suggested to explain our observations. In transient transfection assays, promoter constructs carrying the wild-type allele of rs6721961 showed a significantly higher luciferase activity than constructs bearing the minor alleles of rs6721961 or rs6706649 (–651) polymorphisms, suggesting that these variations affect the basal level expression of *NFE2L2* (Marzec et al. 2007).

It is widely recognized that *NFE2L2* regulates the transcription of antioxidant and phase II enzyme genes through binding the antioxidant response elements (AREs) located in their upstream regulatory regions. Interestingly, the proximal region of the *NFE2L2* promoter also bears two distinct ARE-like elements, which suggest an autoregulatory mechanism on its own expression (Kwak et al. 2002). Though multiple ARE sites are necessary for the maximal transcriptional activation of the target genes (Favreau and Pickett 1995), the binding of *NFE2L2* protein to its own ARE-like elements can contribute to the persistent nuclear accumulation of *NFE2L2*. Because rs6721961 is located in one of these ARE-like sites, Marzec et al. (2007) investigated the effects of this polymorphism on *NFE2L2*–DNA complex formation. As was speculated, they found that *NFE2L2* binds less efficiently to ARE-like sequences that contain the –617 A allele, reducing the boosting effect on its own transcriptional activity.

Considering the previous results, one would expect the rs6721961 A allele to have a predisposing effect on the development of asthma. Furthermore, as *Nfe2l2*-deficient

Table 4 Interactive effects of genotypes and NO₂ level in asthma patients

	rs2588882	NO ₂ high (n)	NO ₂ low (n)	Case-only ORi (95% CI)	p
NO ₂ high: NO ₂ concentration ≥32 µg/m ³ , NO ₂ low: NO ₂ concentration ≤32 µg/m ³	TT	99 (86.1%)	141 (74.2%)	1	
	TG	13 (11.3%)	45 (23.7%)	0.38 (0.19–0.76)	0.006
	GG	3 (2.6%)	4 (2.1%)	1.20 (0.26–5.58)	0.82
	GG+TG	16 (13.9%)	49 (25.8%)	0.43 (0.23–0.82)	0.01
	rs6721961				
ORi odds ratios for interactive effects between genotype and pollution data (adjusted for age and gender), CI confidence interval, n number of cases	GG	92 (80%)	132 (69.5%)	1	
	GT	21 (18.3%)	54 (28.4%)	0.51 (0.29–0.91)	0.02
	TT	2 (1.7%)	4 (2.1%)	0.75 (0.13–4.23)	0.74
	TT+GT	23 (20%)	58 (30.5%)	0.51 (0.29–0.90)	0.02

mice were proven to be more susceptible to bacterial infection after hyperoxia exposure (Reddy et al. 2009), it could also be speculated that the lower abundance of *NFE2L2* due to the presence of the rs6721961 A allele might lead to infection-induced exacerbations and, thus, to an infection-induced asthma phenotype.

Contrary to these expectations, our results show that asthma exacerbations provoked by infectious agents are inversely associated with some of the *NFE2L2* regulatory polymorphisms. It might be speculated that the observed relative protective effect of these variants and the subsequent decreased *NFE2L2* gene expression level are compensated by other *NFE2L2* polymorphisms or by the upregulation of other genes or mechanisms that participate in the antioxidant defence network. In addition, because *NFE2L2* activity is repressed by *KEAP1*, it is also conceivable that compensation occurs between these two central molecules via, e.g. epigenetic silencing of *KEAP1* (Wang et al. 2008).

Furthermore, the abundance of the variant alleles of these SNPs in asthmatic children who live in a less polluted environment may reflect that *NFE2L2* has a central role in the oxidative stress response only if the oxidative stress level does not exceed a critical level. As is described in the hierarchical oxidative stress model of Li et al. (2003), high levels of pro-oxidative stimuli may overwhelm the defence capacity of the *NFE2L2* pathway, activating additional intracellular cascades which induce the expression of pro-inflammatory molecules. If it can be supposed that long-term high average NO_2 concentration reflects a relatively bad global air quality at a given geographical location (usually at bigger cities), it can be speculated that permanent exposure to high-level pollution may activate inflammatory mechanisms and therefore that the diverse susceptibility is rather dependent on the genetic variations of the genes participating in these pathways than the ones in the *NFE2L2* pathway. However, to clarify this hypothesis, future studies are warranted.

It is noteworthy that facing such discrepancies is not unique in the field of environmental genetics research. In the past few years, a highly similar phenomenon was observed in connection with the interaction of *CD14* polymorphisms and levels of endotoxin exposure on allergy and asthma risk. The preceding inconsistent results were explained by the endotoxin switch theory (Vercelli 2003) which postulates that polymorphisms in genes at the host/environment interface influence the environmental endotoxin load required for the Th1/Th2 switch and thus for the development of a Th2-dominant allergic phenotype. According to the theory, the same genotypes may result in entirely opposite effects depending on the quantity and quality of endotoxin exposure. This assumption has already been confirmed several times (Eder et al. 2005; Simpson et al. 2006).

There is also conflicting evidence from studies examining the modifying effect of cigarette smoke on the associations between *ADRB2* (beta-2 adrenergic receptor) polymorphisms and asthma (Zhang et al. 2007). One explanation for the apparent contradictory results on the role of gene polymorphisms in the development of asthma between never and ever smokers was suggested by Litonjua et al. (2004) who proposed that smoking has such a strong effect on airway hyperresponsiveness that may overwhelm any effects of *ADRB2* variants.

Earlier, Fitzpatrick et al. (2011) showed that children with severe asthma have elevated *NFE2L2* mRNA and protein levels as a function of increased thiol oxidation, but this increase has no effect on the downstream components of the antioxidant pathway, suggesting a posttranslational modification. One of the possible explanations for the *NFE2L2* dysfunction and redox disturbances in severe asthma is the presence of single nucleotide polymorphisms in the *NFE2L2* promoter. In our study, we were unable to confirm this hypothesis as we did not find any differences in the genotype distributions of these variations between mild-to-moderate vs. severe asthma patients (data not shown). Nevertheless, this result may reflect the small sample size of our severe asthma cohort; further work is needed to elucidate this problem.

The mechanism of rs258882 with which it influences the asthmatic processes is yet unknown. The 3' localization of the polymorphism raises the possibility of disrupting or creating a miRNA binding site, but with the available miRNA predicting tools, we were not able to confirm this assumption.

This study has some limitations that should be taken into consideration when evaluating its results. First, it is well known that smoking and passive smoking has an influence on the development of asthma. Although the asthma cases are children, and we do not assume that they have smoking history, the smoking habit of the people living in the same household could influence the results of this study. Also, as we already mentioned, there is a population bias between the asthma status and the level of air pollution as we have more controls from highly polluted regions. This bias raises the possibility that population stratification could have influenced our findings in the case-control tests; the study must be replicated using an appropriately matched control group. However, it does not question the main results of the study derived from the case-only analyses.

Conclusions

In summary, our results have important implications for the study of gene-environment interactions in the asthma pathomechanism. We showed that polymorphisms in the

regulatory regions of *NFE2L2* are associated with susceptibility to infection-induced asthma, and that differs from what we could expect on the basis of preceding data. Also, we found remarkable differences in the genotype distributions of these polymorphisms between distinctly polluted regions, which indicate an environment-dependent regulation of the antioxidant defence mechanisms. These results not only strengthen the importance of *NFE2L2* in the effects of air pollution on asthma but also accentuate the existence of a complex network between genes and environment and, most importantly, set directions for future research.

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Conflict of interest The authors declare that they have no conflict of interest.

References

- Albert PS, Ratnasinghe D, Tangrea J, Wacholder S (2001) Limitations of the case-only design for identifying gene–environment interactions. *Am J Epidemiol* 154:687
- Botto LD, Khoury MJ (2001) Commentary: facing the challenge of gene–environment interaction: the two-by-four table and beyond. *Am J Epidemiol* 153:1016
- Braback L, Forsberg B (2009) Does traffic exhaust contribute to the development of asthma and allergic sensitization in children: findings from recent cohort studies. *Environ Health* 8:17. doi:10.1186/1476-069X-8-17
- Braman SS (2006) The global burden of asthma. *Chest* 130(1 Suppl):4S–12S. doi:10.1378/chest.130.1_suppl.4S
- Castro-Giner F, Kunzli N, Jacquemin B, Forsberg B, de Cid R, Sunyer J, Jarvis D, Briggs D, Vienneau D, Norback D, Gonzalez JR, Guerra S, Janson C, Anto JM, Wjst M, Heinrich J, Estivill X, Kogevinas M (2009) Traffic-related air pollution, oxidative stress genes, and asthma (ECHRS). *Environ Health Perspect* 117(12):1919–1924. doi:10.1289/ehp.0900589
- Ciencewicz J, Jaspers I (2007) Air pollution and respiratory viral infection. *Inhal Toxicol* 19(14):1135–1146. doi:10.1080/08958370701665434
- Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, Nowak D, Martinez FD (2005) Opposite effects of CD 14/-260 on serum IgE levels in children raised in different environments. *J Allergy Clin Immunol* 116(3):601–607. doi:10.1016/j.jaci.2005.05.003
- Favreau LV, Pickett CB (1995) The rat quinone reductase antioxidant response element. Identification of the nucleotide sequence required for basal and inducible activity and detection of antioxidant response element-binding proteins in hepatoma and non-hepatoma cell lines. *J Biol Chem* 270(41):24468–24474
- Fitzpatrick AM, Stephenson ST, Hadley GR, Burwell L, Penugonda M, Simon DM, et al. (2011) Thiol redox disturbances in children with severe asthma are associated with posttranslational modification of the transcription factor nuclear factor (erythroid-derived 2)-like 2. *J Allergy Clin Immunol* 127:1604–1611
- Holroyd KJ, Eleff SM, Zhang LY, Jakab GJ, Kleeberger SR (1997) Genetic modeling of susceptibility to nitrogen dioxide-induced lung injury in mice. *Am J Physiol* 273(3 Pt 1):L595–L602
- Iizuka T, Ishii Y, Itoh K, Kiwamoto T, Kimura T, Matsuno Y, Morishima Y, Hegab AE, Homma S, Nomura A, Sakamoto T, Shimura M, Yoshida A, Yamamoto M, Sekizawa K (2005) Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema. *Genes Cells* 10(12):1113–1125. doi:10.1111/j.1365-2443.2005.00905.x
- Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, Gehring U, Koppelman GH (2010) Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. *Thorax* 65(8):690–697. doi:10.1136/thx.2009.119636
- Kim J, Cha YN, Surh YJ (2010) A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat Res* 690(1–2):12–23. doi:10.1016/j.mrfmmm.2009.09.007
- Kwak MK, Itoh K, Yamamoto M, Kensler TW (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. *Mol Cell Biol* 22(9):2883–2892
- Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW (2003) Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1–Nrf2 pathway. Identification of novel gene clusters for cell survival. *J Biol Chem* 278(10):8135–8145. doi:10.1074/jbc.M211898200
- Lee YL, Lin YC, Lee YC, Wang JY, Hsiue TR, Guo YL (2004) Glutathione S-transferase P1 gene polymorphism and air pollution as interactive risk factors for childhood asthma. *Clin Exp Allergy* 34(11):1707–1713. doi:10.1111/j.1365-2222.2004.02099.x
- Li N, Hao M, Phalen RF, Hinds WC, Nel AE (2003) Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clin Immunol* 109(3):250–265
- Linaker CH, Coggon D, Holgate ST, Clough J, Josephs L, Chauhan AJ, Inskip HM (2000) Personal exposure to nitrogen dioxide and risk of airflow obstruction in asthmatic children with upper respiratory infection. *Thorax* 55(11):930–933
- Litonjua AA, Silverman EK, Tantisira KG, Sparrow D, Sylvia JS, Weiss ST (2004) Beta 2 adrenergic receptor polymorphisms and haplotypes are associated with airways hyperresponsiveness among nonsmoking men. *Chest* 126:66–74
- Marzec JM, Christie JD, Reddy SP, Jedlicka AE, Vuong H, Lanken PN, Aplenc R, Yamamoto T, Yamamoto M, Cho HY, Kleeberger SR (2007) Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *FASEB J* 21(9):2237–2246. doi:10.1096/fj.06-7759com
- Melen E, Nyberg F, Lindgren CM, Berglund N, Zucchelli M, Nordling E, Hallberg J, Svartengren M, Morgenstern R, Kere J, Bellander T, Wickman M, Pershagen G (2008) Interactions between glutathione S-transferase P1, tumor necrosis factor, and traffic-related air pollution for development of childhood allergic disease. *Environ Health Perspect* 116(8):1077–1084. doi:10.1289/ehp.11117
- Minelli C, Wei I, Sagoo G, Jarvis D, Shaheen S, Burney P (2011) Interactive effects of antioxidant genes and air pollution on respiratory function and airway disease: a HuGE review. *Am J Epidemiol* 173(6):603–620. doi:10.1093/aje/kwq403
- Poynter ME, Persinger RL, Irvin CG, Butnor KJ, van Hirtum H, Blay W, Heintz NH, Robbins J, Hemenway D, Taatjes DJ, Janssen-Heininger Y (2006) Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse. *Am J Physiol Lung Cell Mol Physiol* 290(1):L144–L152. doi:10.1152/ajplung.00131.2005
- Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN, Biswal S (2005) Disruption of Nrf2 enhances susceptibility to severe

- airway inflammation and asthma in mice. *J Exp Med* 202(1):47–59. doi:[10.1084/jem.20050538](https://doi.org/10.1084/jem.20050538)
- Reddy NM, Suryanarayana V, Kalvakolanu DV, Yamamoto M, Kensler TW, Hassoun PM, Kleeberger SR, Reddy SP (2009) Innate immunity against bacterial infection following hyperoxia exposure is impaired in NRF2-deficient mice. *J Immunol* 183(7):4601–4608. doi:[10.4049/jimmunol.0901754](https://doi.org/10.4049/jimmunol.0901754)
- Siedlinski M, Postma DS, Boer JM, van der Steege G, Schouten JP, Smit HA, Boezen HM (2009) Level and course of FEV1 in relation to polymorphisms in NFE2L2 and KEAP1 in the general population. *Respir Res* 10:73. doi:[10.1186/1465-9921-10-73](https://doi.org/10.1186/1465-9921-10-73)
- Simpson A, John SL, Jury F, Niven R, Woodcock A, Ollier WE, Custovic A (2006) Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. *Am J Respir Crit Care Med* 174(4):386–392. doi:[10.1164/rccm.200509-1380OC](https://doi.org/10.1164/rccm.200509-1380OC)
- Vercelli D (2003) Learning from discrepancies: CD14 polymorphisms, atopy and the endotoxin switch. *Clin Exp Allergy* 33(2):153–155
- von Otter M, Landgren S, Nilsson S, Celojovic D, Bergstrom P, Hakansson A, Nissbrandt H, Drozdik M, Bialecka M, Kurzawski M, Blennow K, Nilsson M, Hammarsten O, Zetterberg H (2010) Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease. *BMC Med Genet* 11:36. doi:[10.1186/1471-2350-11-36](https://doi.org/10.1186/1471-2350-11-36)
- Wang R, An J, Ji F, Jiao H, Sun H, Zhou D (2008) Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem Biophys Res Commun* 373(1):151–154. doi:[10.1016/j.bbrc.2008.06.004](https://doi.org/10.1016/j.bbrc.2008.06.004)
- Zhang LY, Levitt RC, Kleeberger SR (1995) Differential susceptibility to ozone-induced airways hyperreactivity in inbred strains of mice. *Exp Lung Res* 21(4):503–518
- Zhang G, Hayden CM, Khoo SK, Candelaria P, Laing IA, Turner S, Franklin P, Stick S, Landau L, Goldblatt J, Le Souef PN (2007) Beta2-Adrenoceptor polymorphisms and asthma phenotypes: interactions with passive smoking. *Eur Respir J* 30(1):48–55. doi:[10.1183/09031936.00123206](https://doi.org/10.1183/09031936.00123206)