ELECTRONIC LETTER

Association of lung function decline with the heme oxygenase-1 gene promoter microsatellite polymorphism in a general population sample. Results from the European Community Respiratory Health Survey (ECRHS), France

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Inducible heme oxygenase (HO-1) acts against oxidants that are thought to play a major role in the pathogenesis of chronic obstructive pulmonary disease (COPD), characterised by impaired lung function. A (GT)_n repeat polymorphism in the HO-1 gene promoter can modulate the gene transcription in response to oxidative stress. We hypothesised that this polymorphism could be associated with the level of lung function and decline in subjects exposed to oxidative aggression (smokers). We genotyped 749 French subjects (20–44 years, 50% men, 40% never smokers) examined in both 1992 and 2000 as part of the ECRHS. Lung function was assessed by forced expiratory volume in 1 second (FEV₁) and FEV₁/forced ventilatory capacity (FVC) ratio. We compared long (L) allele carriers ((GT)_n ≥ 33 repeats for one or two alleles) to non-carriers. Cross sectionally, in 2000, L allele carriers showed lower FEV₁/FVC than non-carriers. During the 8 year period, the mean annual FEV₁ and FEV₁/FVC declines were –30.9 (31.1) ml/year and –1.8 (6.1) U/year, respectively. FEV₁/FVC decline was steeper in L allele carriers than in non-carriers (–2.6 (5.5) v –1.5 (6.4), p=0.07). There was a strong interaction between the L allele and smoking. In 2000, the L allele was associated with lower FEV₁ and FEV₁/FVC in heavy smokers (≥20 cigarettes/day) only (p for interaction=0.07 and 0.002 respectively). Baseline heavy smokers carrying the L allele showed the steepest FEV₁ decline (–62.0 (29.5 ml/year) and the steepest FEV₁/FVC decline (–8.8 (5.4 U/year) (p for interaction=0.009 and 0.0006). These results suggest that a long (L) HO-1 gene promoter in heavy smokers is associated with susceptibility to develop airway obstruction.

Impairment and a rapid decline in lung function, are strong predictors of respiratory morbidity and mortality.¹ Oxidative stress, which occurs when there is an excess of oxidants in cells compared to their antioxidant capacity, is thought to impair lung function and to accelerate its decline. This can lead to airway obstruction and chronic obstructive pulmonary disease (COPD).² The oxidative stress hypothesis may explain why smokers have a higher risk of COPD; they are exposed to cigarette smoke, which contains high concentrations of oxidants such as reactive oxygen species (ROS).³ However, smoking usually results in COPD in only 15–20% of smokers,⁴ suggesting a genetic susceptibility to lung function decline and to the development of COPD in smokers. This is supported by studies showing that early

onset COPD showed familial clustering,⁵ and that lung function decline showed familial correlation.⁶

Heme oxygenase (HO) is the rate limiting enzyme in the catabolism of heme, resulting in the release of carbon monoxide, free iron, and biliverdin.⁷ At low concentration, carbon monoxide exerts an anti-inflammatory action and protects against oxidative injury.⁸ Biliverdin is subsequently reduced to bilirubin. HO-1 is the inducible isoform of HO and is considered to be an antioxidant enzyme because bilirubin is an efficient local scavenger of ROS.⁷

A (GT)_n dinucleotide repeat in the 5' flanking region of the human HO-1 gene promoter shows a length polymorphism.⁹ A low number of (GT)_n repeats has been related to both increased HO-1 basal promoter activity¹⁰ and transcriptional upregulation in response to oxidants.¹⁰ Thus, smokers having a high number of (GT)_n repeats may be more susceptible to a rapid decline in lung function. However, the effect of the HO-1 gene promoter microsatellite polymorphism on lung function decline and its interaction with smoking, in a general population has never been studied.^{8–11} The French data collected as part of the international multicentre longitudinal European Community Respiratory Health Survey (ECRHS)¹² provided the ideal opportunity to explore the relationships between lung function and its rate of decline, and HO-1 gene promoter polymorphism in a general population.

METHODS

Subjects

ECRHS is a multicentre two stage study. The protocol has been published elsewhere.^{12–13} Briefly, at both time points (ECRHS I in 1992 and ECRHS II in 2000), subjects were given standardised questionnaires and underwent lung function tests with a water sealed bell spirometer (Biomedin srl, Padova, Italy). Written informed consent was obtained from each subject before inclusion and the protocol was approved the French Ethics Committee for Human Research.

Of the 1650 subjects examined in the Paris, Grenoble and Montpellier centres for ECRHS I in 1992, 1066 were followed up for ECRHS II in 2000 (participation rate 64.6%). Of these, 229 could not come to the hospital for examination, there was no blood sample for 37, there was a blood sample but no valid spirometry results from ECRHS I and/or ECRHS II for

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; ECRHS, European Community Respiratory Health Survey; FEV₁, forced expiratory volume in 1 second; FVC, forced ventilatory capacity; HO, heme oxygenase; HO-1, inducible heme oxygenase; L, long; LHS, Lung Health Study; ROS, reactive oxygen species

23, and 28 were not screened for *HO-1* gene promoter microsatellite polymorphisms.

For the analyses, we included the remaining subjects—that is the 749 subjects in the Grenoble, Montpellier, and Paris centres for whom spirometry had been determined in both 1992 and 2000, and who had been screened for *HO-1* gene promoter microsatellite polymorphisms. We found no differences with regard to sex, body mass index (BMI), forced expiratory volume in 1 second/forced ventilatory capacity (FEV₁/FVC) <70%, or FEV₁ between the baseline characteristics of the analysed subjects and those of the subjects not included in the analyses (either because they did not participate in ECRHS II or because data were missing). Analysed subjects were slightly older (2 years on average), had slightly lower FEV₁/FVC (mean (SD) 84.6 (7.5)% v 86.9 (7.0)%, $p < 0.0001$), were more likely to have never been smokers, and less likely to be heavy smokers (≥ 20 cigarettes/day) (43% v 40% and 12% v 18%, respectively, $p = 0.03$). However the number of pack years was not significantly different between included and excluded subjects.

Analysis of the length variability of (GT)_n repeats in the *HO-1* gene promoter

Genomic DNAs were extracted from leucocytes in peripheral venous blood using the QIAamp blood kit (Qiagen) according to the manufacturer's protocol. The *HO-1* gene 5' flanking region was amplified by PCR^{9,14} using a fluorescently labelled sense primer and an unlabelled antisense primer (5'-AGAGCCTGCAGCTTCTCAGA-3' and 5'-ACAAAGTCTGGCC-ATAGGAC-3') covering the (GT)_n repeats.^{10,15} The PCR products were subsequently run on a denaturing polyacrylamide gel and analysed using a laser based automated DNA sequencer (ABI Prism 310 genetic analyser with Genescan Analysis software, version 1.2; Applied Biosystems). Each repeat number was calculated with respect to an internal size standard. The allele sizes observed were confirmed by sequencing DNA of homozygous patients. The investigators of genetic analysis were blinded with respect to the characteristics (sex, smoking habit) of the subjects.

Statistical analysis

All analyses were carried out using SAS software (SAS Institute, Cary NC, USA). A lack of underlying population stratification for the polymorphism (Hardy-Weinberg structure) was tested using the Arlequin software package.¹⁶ Statistical significance was set at $p = 0.05$ (two tailed).

For cross sectional analyses, lung function was expressed as a percentage of predicted FEV₁ (FEV₁%predicted)¹⁷ and as FEV₁/FVC ratio. Confounding factors included centre, sex, age, BMI, and smoking status (divided into four categories: never smokers, ex-smokers (>1 year), moderate smokers (smokers and ex-smokers <1 year, with <20 cigarettes/day), and heavy smokers (≥ 20 cigarettes/day)).¹⁸ Age and BMI were introduced as quantitative variables. For longitudinal analyses, both FEV₁ decline (ml/year) and FEV₁/FVC decline (U/year) were calculated as the value in 2000 minus the value in 1992, divided by the length of follow up. Baseline values of confounders and baseline values of lung function were included in the models.

Crude means (SD), and p values of the association between lung function and *HO-1* gene promoter polymorphism after adjustment for potential confounders were assessed by analyses of covariance. The results of these analyses are expressed as adjusted means and their 95% confidence interval (CI) of lung function according to the *HO-1* genotype. The p values for linear trend were obtained by including a CONTRAST statement in the PROC GLM used to perform our multiple linear regression models.¹⁹

The interaction between *HO-1* gene promoter polymorphism and smoking status was tested by adding the term genotype $\times$ heavy smoking, both coded as binary variables, to the aforementioned confounders in the regression model.

RESULTS

Characteristics of the study population

The characteristics of the population studied are shown in table 1. Half of the subjects were men. Mean (SD) age in ECRHS I was 36.9 (7.1) years. As expected in this population of young adults, FEV₁ level and FEV₁/FVC level were normal. Only 3% and 4% of subjects in ECRHS I and in ECRHS II, respectively, had airways obstruction (FEV₁/FVC <70%).

The number of (GT)_n repeats in the *HO-1* gene promoter ranged from 11 to 41. In the total population, the distribution of (GT)_n repeats was trimodal with peaks at 23, 30, and 38 repeats (fig 1).

This trimodal distribution was similar in the three studied centres. Therefore, we classified the alleles into three subclasses according to the number of (GT)_n repeats, as previously reported:^{10,20,21} class S (<27 (GT)_n repeats), class M (27–32 (GT)_n repeats), and class L (>32 (GT)_n repeats). The subjects were then classified as having L/L, L/M, L/S, M/M, M/S, or S/S genotypes. The allelic and genotypic distributions are shown in table 1. We observed no deviation from the Hardy-Weinberg structure ($p = 0.77$ in pooled centres, $p = 0.86$ in Grenoble, $p = 0.72$ in Montpellier, and $p = 0.65$ in Paris).

We pooled carriers of the L allele (L/L, L/M, L/S) and compared them with the three other genotypes either separately or pooled. We designated L allele carriers as group L+ (13.0% of the population studied). When genotypes M/M, M/S, and S/S were pooled, they were designated as group L– (without the L allele).

Cross sectional association between lung function level and *HO-1* gene promoter polymorphism

FEV₁/FVC was significantly associated with the *HO-1* genotype in 2000 (ECRHS II) (table 2). There was no significant association for FEV₁%predicted in 2000 and for both outcomes in 1992 although the pattern did not seem to be different.

There was a significant interaction between smoking and *HO-1* polymorphism on FEV₁%predicted and FEV₁/FVC (fig 2). Heavy smokers in group L+ had the lowest FEV₁%predicted (90.1%; 95% CI 80.6 to 99.5) and the lowest FEV₁/FVC (74.7%; 95% CI 70.7 to 78.7) (p for interaction = 0.07 and 0.002, respectively).

In ECRHS I, 8 years before, we observed no association between the *HO-1* genotype and either FEV₁%predicted or FEV₁/FVC levels (table 2) and no interaction between heavy smoking and group L+ for lung function.

Association between lung function decline and *HO-1* gene promoter polymorphism

The 749 subjects studied were followed up at 8.6 (0.8) years (mean (SD)). The mean (SD) annual FEV₁ decline and FEV₁/FVC decline were –30.9 (31.1) ml/year and –1.8 (6.1) U/year, respectively. Between the two studies, 79.1% of the subjects had the same smoking status, 9.4% either took up or increased smoking, and 11.5% stopped or reduced smoking.

FEV₁/FVC decline but not FEV₁ decline was more rapid in the L allele carriers and slower for the S/S genotype carriers than in the M/M or M/S genotype carriers (table 3).

We observed a strong interaction between heavy smoking at baseline and group L+ for FEV₁ decline and for FEV₁/FVC decline (p for interaction = 0.009 and 0.0006, respectively; fig 3). In heavy smokers, there was a more rapid decline in group L+ than in group L– for FEV₁ decline (–62.0 ml/year

Figure 1 Distribution of the number of (GT)_n repeats of the *HO1* gene promoter in all subjects (n=749; 1498 alleles).

(95% CI -79.6 to -44.4) ν -37.7 ml/year (95% CI -44.2 to -31.1), $p = 0.051$) and for FEV₁/FVC decline (-8.8 U/year (95% CI -12.0 to -5.6) ν -2.1 U/year (95% CI -3.3 to -1.0), $p = 0.0007$). For the other smoking status, we found no difference between group L+ and group L- for either decline in FEV₁ (-28.5 ml/year (95% CI -34.8 to -22.3) ν -30.5 ml/year (95% CI -33.1 to -28.0), $p = 0.93$) or for decline in FEV₁/FVC (-1.5 U/year (95% CI -2.7 to -0.4) ν -1.2 U/year (95% CI -1.6 to -0.7), $p = 0.93$).

When the analyses were conducted without adjustment, or with supplemental adjustment for change either in smoking status or in BMI, similar pattern of results was observed.

DISCUSSION

In this study, we explored the relationships between the length of the *HO-1* gene promoter microsatellite polymorphism,

and the level of lung function and decline in lung function, in a European general population. We found that over 8 years, subjects with a long promoter (L allele carriers) had a significantly more rapid decline in FEV₁/FVC than subjects with a shorter promoter (without the L allele). Moreover, we observed a host-environment interaction: in heavy smokers (≥ 20 cigarettes/day), the decline was steeper in subjects with than in subjects without the L allele for both FEV₁ and FEV₁/FVC. These results suggest that a long promoter in the *HO-1* gene may be associated with susceptibility for developing airways obstruction, particularly in heavy smokers.

Validity

The protocol of the ECRHS was highly standardised and the quality of data was strictly controlled.¹² Adherence to the protocol was assessed by a quality control visit by a member of the coordinating team to the Paris centre, and by subsequent visits by a nominated member of this centre to the other centres in France. Water sealed bell spirometers were regularly checked and all blood samples were genotyped at the same place and at the same time.

Not having all data available for all subjects may have affected the representativeness of the study. Analysed subjects were < 2 years older than excluded subjects. However, such a small difference is unlikely to have biased the association between lung function decline and *HO-1* gene promoter polymorphism, owing to the young age of the study population and the small range of ages (20–44 years in 1992). Analysed subjects were more frequently non-smokers and less frequently heavy smokers. This would only have weakened the overall association between *HO-1* polymorphism and lung function decline, as this association was stronger in heavy smokers.

We found associations between polymorphism and lung function levels in ECRHS II but not in ECRHS I. Subjects recruited in ECRHS I were aged from 20 to 44 years, whereas airway obstruction is rarely observed before the age of 45 years. In ECRHS II, our study population was older and thus more likely to have experienced longer environmental exposure to tobacco smoking, for example. However, spirometry values in 2000 were still within the normal range, and only 4% of the subjects had FEV₁/FVC $< 70\%$. The participants in the study could only be at a very early stage of airway obstruction.

Among the subjects with the lowest levels of lung function at baseline, the association between lung function decline and polymorphism was not stronger than in subjects with

Table 1 Characteristics of the 749 participants

Variable	ECRHS I	ECRHS II
Centre		
Grenoble, %	39.5	–
Montpellier, %	18.7	–
Paris, %	41.8	–
Sex, men %	49.7	–
Age, years	36.7 (7.1)	45.1 (7.2)
Smoking status		
Never smokers, %	43.5	40.0
Ex-smokers, %	24.8	31.7
Moderate smokers, %	19.4	19.1
Heavy smokers, %	12.3	9.2
Pack years	8.4 (12.8)	10.3 (15.4)
BMI		
Underweight, %	17.0	11.9
Normal, %	62.2	56.2
Overweight, %	18.0	24.7
Obese, %	2.8	7.2
FEV ₁ , l/s	3.7 (0.8)	3.4 (0.8)
FEV ₁ %predicted	105.7 (13.5)	105.0 (14.5)
FEV ₁ /FVC	84.6 (7.5)	83.0 (7.4)
Allelic* distribution		
Class L, %	6.8	–
Class M, %	55.1	–
Class S, %	38.1	–
Genotypic distribution		
L/L, %	0.7	–
L/M, %	7.2	–
L/S, %	5.1	–
M/M, %	30.6	–
M/S, %	41.9	–
S/S, %	14.5	–

*For this count, n = 1498 alleles.

Table 2 Adjusted* mean FEV₁%predicted, and FEV₁/FVC in genotypes of the *HO1* gene promoter

	HO1 genotype				p for association	p for trend
	L/L+L/M+L/S	M/M	M/S	S/S		
ECRHS I (1992)						
FEV ₁ %predicted, %	104.4 (101.7 to 107.2)	105.1 (103.2 to 106.9)	105.4 (103.8 to 107.0)	106.2 (103.6 to 108.7)	0.81	0.33
FEV ₁ /FVC, %	85.3 (84.0 to 86.7)	85.8 (84.9 to 86.6)	85.3 (84.6 to 86.1)	86.2 (85.0 to 87.5)	0.60	0.43
ECRHS I (2000)						
FEV ₁ %predicted, %	102.7 (99.8 to 105.8)	104.1 (102.1 to 106.2)	103.6 (101.8 to 105.4)	104.9 (102.1 to 107.7)	0.73	0.34
FEV ₁ /FVC, %	82.5 (81.2 to 83.7)	83.8 (82.9 to 84.6)	83.2 (82.5 to 84.0)	84.9 (83.8 to 86.1)	0.02	0.008

Data are means (95% CI) (cross-sectional analyses). *Multiple linear regression including centre, sex, age, smoking status, and BMI.

higher levels (data not shown). Thus it can reasonably be hypothesised that the cross sectional association observed in 2000 (ECRHS II) may actually be the consequence of the longitudinal relationship.

HO-1 gene promoter polymorphism and respiratory outcomes

The number of (GT)_n repeats in the *HO-1* gene promoter had a trimodal distribution in our population, as was also seen in three previous studies with respiratory outcomes.^{21–23} As described in these studies, we classified the subjects into three allelic subclasses according to the number of (GT)_n repeats. Our allelic frequencies were the same as those seen in a north American study²⁰ and slightly different from those in two Japanese studies.^{21–22} The allelic frequencies in these two Japanese studies were very similar. In one study, on male smokers only, a long promoter was associated with higher

risks of emphysema,²² whereas in the other study on men and women with different smoking status, a long promoter was associated with lung adenocarcinoma.²¹ In this second study, the authors found an association with lung adenocarcinoma in male smokers but not in female non-smokers. Male non-smokers and female smokers could not be analysed because of small sample sizes.

The North American study by He *et al* used data from the Lung Health Study (LHS). Initially, the LHS was a clinical trial studying the effects of smoking intervention associated with the use of a bronchodilator on the progression of COPD in smokers with mild to moderate COPD (FEV₁/FVC ≤ 70% and FEV₁%predicted between 55% and 90%).²³ This study concentrated on continuing smokers and compared, in this subsample, 281 smokers with a rapid decline in lung function (mean (SD) annual FEV₁ decline was –152 (2.5) ml/year) to 304 smokers with no decline in lung function (mean (SD) annual FEV₁ decline was 15 (1.5) ml/year).²⁰ No association between a rapid lung function decline and *HO-1* gene promoter polymorphism was observed.

Several reasons may explain the lack of agreement between our study and the study of He *et al*. Firstly, this lack may reflect the differences in the subject selection criteria and methods of analysis. Subjects in the ECRHS were recruited from the general population, were 20–44 years old at baseline and were followed up for 8 years on average. Subjects in the LHS were recruited from smokers with mild to moderate COPD, were aged 35–60, and were followed up for 5 years. Baseline FEV₁%predicted was 105.7 (13.5)% in ECRHS I, whereas it was already low in the LHS, being 72.6 (8.8)% in subjects having a rapid decline in lung function and 75.7 (8.1)% in subjects having no decline in lung function. In our study, the outcomes were continuous variables and centre, sex, BMI, age, and smoking status (plus baseline spirometry for longitudinal analyses) were taken into account, as these are known to be related to lung function levels.^{24–25} In the LHS, the outcome was a binary variable (either a rapid decline or not) and adjustments only included age, smoking history, and methacholine responsiveness. The effect of treatment (smoking intervention with or without bronchodilator) on lung function decline was not evaluated. Thus, the two studies may not be directly comparable. Secondly, as explained by the same team of researchers about another polymorphism in the LHS,²⁶ the genetics of COPD susceptibility and COPD severity may be different.²⁶ In ECRHS, we studied the effect of the *HO-1* polymorphism as a genetic determinant for the onset of airways obstruction in heavy smokers, whereas in the study of He *et al*, the effect of this microsatellite polymorphism can be seen as a genetic determinant for the progression and severity of airway obstruction in heavy smokers. Finally, we cannot rule out the hypothesis of linkage disequilibrium with a nearby gene present in some populations and missing in others.

A fourth recent study²⁷ showed, firstly, that in a family based study (the Boston Early Onset COPD families), the

Figure 2 Adjusted* mean (95% CI) FEV₁%predicted (A) and FEV₁/FVC (B) according to the *HO-1* genotype (group L+ (one or two L alleles) v group L- (no L alleles)) and heavy smoking status (heavy smokers versus the others), in 2000 (ECRHS II). *Multiple linear regression including centre, sex, age, BMI, *HO-1* genotype, heavy smoking, and interaction of *HO-1* genotype with heavy smoking.

Table 3 Adjusted* mean annual decline in FEV₁ (ml/year) and FEV₁/FVC (U/year) in genotypes of the *HO1* gene promoter

	<i>HO1</i> genotype				p for association	p for trend
	L/L+L/M+L/S	M/M	M/S	S/S		
Decline in FEV ₁ , ml/year	-33.3 (-39.6 to -27.3)	-31.5 (-35.6 to -27.5)	-33.4 (-37.0 to -29.9)	-30.8 (-36.5 to -25.1)	0.80	0.66
Decline in FEV ₁ /FVC, U/year	-2.56 (-3.67 to -1.46)	-1.51 (-2.26 to -0.77)	-1.80 (-2.45 to -1.15)	-0.56 (-1.61 to -0.46)	0.058	0.014

Data are means (95% CI). *Multiple linear regression including centre, sex, and ECRHS I data for age, smoking status, BMI, and spirometry (that is, baseline FEV₁ for decline in FEV₁ and baseline FEV₁/FVC for decline in FEV₁/FVC)

(GT)₃₁ allele was associated with several respiratory outcomes (FEV₁, FEV₁/FVC, mild to severe airways obstruction (that is, FEV₁ <80% predicted, and FEV₁/FVC <90% predicted) and moderate to severe airways obstruction (that is, FEV₁ <60% predicted and FEV₁/FVC <90% predicted)), and secondly that in a case-control study the (GT)₃₀ allele was underrepresented in COPD subjects (FEV₁ ≤65% predicted) compared with controls, although no significant association was reported when alleles were coded into the same three subclasses as mentioned above.²⁸

Possible mechanisms

Several cellular and molecular mechanisms could explain the association between relatively low expression of HO-1 and susceptibility for accelerated lung function decline in heavy smokers. Firstly, because of low expression, HO-1 may release less carbon monoxide and bilirubin, which have anti-inflammatory and antioxidant properties, respectively, when breaking down heme.⁸ Secondly, low expression of

HO-1 has been shown to potentiate oxidant induced proliferation of human airway smooth muscle cells *in vitro* and bronchial wall thickness *in vivo* in guinea pigs.²⁸ Increased bronchial wall thickness and consequently reduced airway lumen could be involved in airways obstruction observed in L+ subjects. Similarly, low levels of HO-1 have been shown to increase oxidant driven airway smooth muscle contractility,²⁹ another factor that may participate in airway obstruction.

CONCLUSION

For the first time in a general population, this study explored the relationships between the *HO-1* gene promoter length and lung function level and decline. Our results suggest that a long *HO-1* gene promoter, which may lead to low protein expression and activity, is associated with low lung function and accelerated lung function decline, especially in heavy smokers. Heavy smokers with a long gene promoter had an FEV₁ decline twice as rapid as that observed in the other subjects of this young adult general population. Thus, the *HO-1* gene promoter may be a genetic determinant explaining why tobacco smoking results in airways obstruction only in 15–20% of smokers⁴ and may also affect people who have never smoked but who are exposed to environmental risk factors. These findings need to be confirmed in a larger population.

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Figure 3 Adjusted* mean (95% CI) decline in FEV₁ (A) and FEV₁/FVC (B), according to the *HO1* genotype (group L+ (one or two L alleles) v group L- (no L alleles)) and baseline heavy smoking status (heavy smokers v the others). *Multiple linear regression including centre, sex, *HO1* genotype, and ECRHS I data for FEV₁/FVC, age, BMI, heavy smoking, and interaction of *HO1* genotype with heavy smoking.

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