Eur Respir J 2007; 29: 51–55
DOI: 10.1183/09031936.00023606

8-Isoprostane as a marker of oxidative stress in nonsymptomatic cigarette smokers and COPD

V.L. Kinnula*, H. Ilumets*, M. Myllärniemi*, A. Sovijärvi# and P. Rytilä¶

ABSTRACT: 8-Isoprostane is a potential *in vivo* marker for oxidant burden, but its usefulness in induced sputum of smokers and chronic obstructive pulmonary disease (COPD) has not been investigated.

The current study investigated 58 subjects comprising 11 never-smokers, 11 ex-smokers, 13 healthy current smokers and 23 COPD with stage 0–III disease (according to the Global Initiative for Chronic Obstructive Lung Disease criteria). 8-Isoprostane was determined from induced sputum by enzyme immunoassay.

Sputum 8-isoprostane levels were similar in the never-smokers and ex-smokers, but were elevated in the healthy smokers compared with nonsmokers, and in those with stage I–III COPD. Sputum 8-isoprostane levels could not differentiate nonsymptomatic smokers from those with Stage 0 COPD. There was a correlation between sputum 8-isoprostane level and lung function parameters (forced expiratory volume in one second/forced vital capacity and sputum neutrophils.

In conclusion, sputum 8-isoprostane levels correlate with the severity of chronic obstructive pulmonary disease. However, they do not appear to differentiate healthy smokers from those who are at risk of developing chronic obstructive pulmonary disease (Global Initiative for Chronic Obstructive Lung Disease stage 0).

KEYWORDS: Chronic obstructive pulmonary disease, cigarette smoking, oxidant, sputum

Chronic obstructive pulmonary disease (COPD) is generally diagnosed when lung function parameters have become significantly reduced and a major part of the lung has been damaged. The pathogenesis of COPD has been strongly associated with reactive oxygen species [1–4], although it is not known how oxidative/nitrosative stress predicts the disease progression. Several oxidant markers and ''footprints'' of oxidative/nitrosative damage have been detected in COPD lung tissue, sputum, exhaled air and exhaled breath condensate [3–6], but it has not been unequivocally resolved whether these biomarkers can be used in the early assessment of cigarette-smoke-related lung diseases, their progression or whether they relate to smoking alone.

8-Epi-prostaglandin$F_{2\alpha}$ (8-isoprostane) has been suggested to be the most reliable approach to monitor oxidative stress *in vivo* [7, 8]. Isoprostanes are formed by free-radical-catalysed lipid peroxidation of arachidonic acid and cell membrane phospholipids. Isoprostanes can also be released into the circulation, secretions and urine where levels have been found to be stable and reproducible in many experimental approaches [8, 9]. Isoprostanes also have potent biological actions and therefore they may significantly contribute to the progression of oxidant-mediated lung diseases, such as COPD.

Several studies have shown elevated 8-isoprostane in the exhaled breath condensate of COPD patients [10–13]. There are, however, a number of uncertainties with respect to the usefulness and standardisation of exhaled breath condensate [14–17]. One recent study also failed to detect 8-isoprostane in exhaled breath condensate in the majority of cigarette smokers [18]; the reasons suggested were the high dilution of all biological constituents in the breath condensate and the low sensitivity of the 8-isoprostane enzyme immunoassay (EIA) method. Induced sputum is a standardised method that reliably reflects local airway inflammation. 8-Isoprostane has not been previously investigated using the induced sputum of smokers.

AFFILIATIONS
*Dept of Medicine and Divisions of Pulmonary Medicine,
#Clinical Physiology, and
¶Allergology, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland.

CORRESPONDENCE
V.L. Kinnula
Dept of Medicine
University of Helsinki
P.O. Box 22
00014 Helsinki
Finland
Fax: 358 947176107
E-mail: vuokko.kinnula@helsinki.fi

Received:
February 14 2006
Accepted after revision:
September 19 2006

SUPPORT STATEMENT
This work was supported by the Juselius Foundation, The Finnish Antituberculosis Association Foundation, Yrjö Jansson Foundation and by the Helsinki University Hospital (HUCH-EVO). The authors declare that they have no competing interests.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

The present study was undertaken to obtain a more accurate insight into the significance of the local oxidant burden in the airways of healthy smokers and smokers who are evidently at risk of developing COPD (stage 0 COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria) [19] by analysing the concentrations of 8-isoprostane from induced sputum specimens. The study included age-matched subjects, never-smokers, ex-smokers, healthy smokers, smokers with stage 0 COPD (symptoms with normal lung function parameters, GOLD criteria [19]), and those with stable COPD (stage I–III).

SUBJECTS AND METHODS

Subjects

A total of 58 subjects were included in the present study, comprising 11 never-smokers, 11 ex-smokers (who had stopped smoking $\geqslant$20 yrs before the study with a smoking history of <15 pack-yrs), 13 healthy nonsymptomatic smokers, nine smokers with symptoms (St George's questionnaire for symptoms such as cough and sputum production) whose lung function parameters were normal (stage 0 COPD) [19], and 14 COPD patients with stage I–III disease. The diagnosis of COPD was based on the GOLD criteria with post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) <70% with the post-bronchodilator effect <12%. Atopy and allergies were excluded. Healthy controls and stage 0 COPD subjects had not been treated with any anti-inflammatory medication for 2 months, but two individuals had been prescribed a short-acting bronchodilator. The medications for stage I–III included inhaled short-acting bronchodilators in 100% of cases, long-acting bronchodilators in 79% and inhaled steroids in 64%. None of the subjects were allowed to smoke during the 12 h prior to specimen collection and none had suffered from any viral infection for 2 months. Each subject underwent spirometry with the bronchodilator test and an assessment of total lung capacity and diffusion capacity [20].

This study was approved by the Ethics Committee of Helsinki University Hospital with written consent obtained from every subject. The study is registered by the hospital (www.hus.fi/clinicaltrials).

Sputum processing

Sputum was induced, as described by the European Respiratory Society Task Force, with 4.5% physiological saline solution [21], and samples processed as previously described [22]. Briefly, expectorated samples were processed with four volumes of dithioerythritol (DTE; Sigma, Munich, Germany). Suspensions were filtered through 70-μm nylon gauze and centrifuged at $400 \times g$ at 4°C for 10 min. In preliminary studies, the sputum was also collected by the same protocol in PBS without DTE to test the possible effect of DTE on the 8-isoprostane EIA analysis. After centrifugation, the pellet was resuspended, and the viabilities and absolute numbers of cells were calculated by the trypan blue exclusion test. All samples in DTE or PBS were immediately frozen at -80°C. Cytospins were prepared and stained using the May–Grunwald–Giemsa method for cell differential counts. The cytospins were frozen at -20°C.

Analyses

Free 8-isoprostane was analysed by EIA according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA). The values were expressed as $pg \cdot mL^{-1}$. The EIA method is highly specific for 8-isoprostane and has been used previously for the assessment of 8-isoprostane from exhaled breath condensate [10, 23], bronchoalveolar lavage [24], plasma [25] and induced sputum [26]. The method has been found to show strong association with the results obtained with gas chromatography-mass spectroscopy (GC-MS) [26]. To test the analysis further, sputum samples were analysed at three different dilutions, giving good reproducibility (% coefficient of variation 10.5, intra-class correlation coefficient 0.87). When one individual sputum specimen (divided originally in small aliquots) was run in 10 separate assays the reproducibility was good; the values of this specimen ranged 29.7–37.1 $pg \cdot mL^{-1}$ (SD 3.8). The effect of DTE had been tested in the preliminary studies by adding the corresponding DTE concentration as in the sputum specimens to the standards; these determinations showed very consistent results ($r=0.99$). In addition, eight inductions had been processed with PBS without DTE. These specimens were further divided into two parts and treated with DTE or PBS, and analysed for 8-isoprostane. The results with or without DTE were also very similar ($r=0.75$). The dilutions were made in the buffer provided by the manufacturer.

Statistics

Data are presented as mean±SEM or median (range) for not normally distributed data. Data for all groups were analysed by the Kruskall–Wallis test and differences between individual variables from two groups were analysed by the Mann–Whitney U-test. Correlations between variables were sought using the Spearman rank correlation test. A p-value <0.05 was considered significant. For values below the detection limit in 8-isoprostane analysis, random number interpolation was used; this was only needed for five samples in the healthy control group.

RESULTS

Patient characteristics are shown in table 1. As expected, current smokers had higher numbers of neutrophils in the induced sputum than nonsmokers.

The percentage and number of sputum neutrophils were very similar in never-smokers and ex-smokers (who had stopped smoking $\geqslant$20 yrs ago). The percentage of sputum neutrophils tended to be higher in those smokers who had symptoms but normal lung function parameters (stage 0 COPD) compared with nonsymptomatic smokers ($p=0.06$; table 1). However, there was no difference in the total number of neutrophils between these two groups ($p=0.23$).

The levels of 8-isoprostane in the induced sputum were higher in healthy smokers (median 108.4 $pg \cdot mL^{-1}$) than in non-smokers (median 15.3 $pg \cdot mL^{-1}$; $p=0.005$), but did not differ between healthy smokers and stage 0 COPD (median 66.6 $pg \cdot mL^{-1}$; fig. 1a). The levels of 8-isoprostane did not differ between never-smokers and ex-smokers. The levels significantly increased in COPD (median 202.2 $pg \cdot mL^{-1}$, stage I–III; $p<0.0001$ and $p=0.02$ compared with nonsmokers and healthy smokers, respectively). There was a significant correlation between the 8-isoprostane levels and lung function parameters

TABLE 1 Patient characteristics

	Healthy nonsmokers	Healthy smokers	COPD stage 0	COPD stage ⩾1
Subjects n	22	13	9	14
Male/female	18/4	9/4	8/1	7/7
Age yrs	58±1.9	53±1.6	63±1.6	58±1.9
BMI	26±0.8	27±1.3	28±0.8	27±0.8
Pack-yrs	7±3.0	30±3.8	54±5.9	44±3.0
Post-bronchodilator				
FVC L#	4.6±0.24	4.7±0.42	3.4±0.14	2.9±0.10
FVC % pred*	100±3.8	95±5.0	82±4.5	80±2.8
FEV_1 L#	3.7±0.15	3.8±0.33	2.6±0.12	1.7±0.10
FEV_1 % pred#	100±3.9	96±5.0	77±4.1	57±2.8
FEV_1/FVC#	80±1.4	80±1.3	76±1.1	57±1.4
$D_{L,CO}$ %#	96±2.8	86±3.7	80±4.1	58±2.8
Sputum neutrophils				
%*	27 (0–75)	37 (0–93)	72 (32–84)	74 (46–82)
$\times 10^6 \cdot g^{-1}$**	0.10 (0–0.76)	0.31 (0–1.65)	0.50 (0.11–1.32)	0.50 (0.07–1.86)
Sputum macrophages				
%	41 (20–97)	51 (6.8–70)	26 (11–67)	26 (13–99)
$\times 10^6 \cdot g^{-1}$	0.19 (0.04–0.83)	0.21 (0.08–0.90)	0.34 (0.04–1.18)	0.14 (0.06–0.67)

Data are presented as n, mean±SEM or median (range). COPD: chronic obstructive pulmonary disease; BMI: body mass index; FVC: forced vital capacity, % pred: % predicted; FEV_1: forced expiratory volume in one second; $D_{L,CO}$: diffusion capacity of the lung for carbon monoxide. *: $p<0.05$; **: $p<0.01$; #: $p<0.0001$ (between all groups, Kruskall–Wallis test).

(8-isoprostane *versus* FEV_1/FVC $r=-0.66$, $p<0.0001$, *versus* FEV_1 $r=-0.48$, $p=0.006$; fig. 1b) and between the 8-isoprostane level and smoking history evaluated from smoking pack-yrs ($r=0.56$, $p=0.001$; fig. 1c). The 8-isoprostane level in the sputum significantly correlated with sputum neutrophils (total neutrophils $r=0.37$, $p=0.02$; fig. 1d). Three subjects (33%) with stage 0 COPD had stopped smoking ⩾1 yr ago (mean time from quitting 2 yrs). However, there was no difference between these ex-smokers and current smokers in stage 0 COPD group in any of the parameters measured. In patients with stage I COPD or worse, six patients (43%) had stopped smoking (mean time from quitting 3.6 yrs). In this group, current smokers tended to have higher sputum 8-isoprotane levels than ex-smokers but the difference was not significant ($p=0.09$). No significant differences could be found between current smokers and ex-smokers in these COPD patients. When all the subjects were divided into two groups, current smokers and nonsmokers, then the level of 8-isoprostane in sputum was significantly higher in current smokers ($p=0.001$). In the COPD group, 64% of the patients used inhaled steroids. However, there were no significant differences between 8-isoprostane levels in patients with inhaled steroids or without them.

DISCUSSION

The main aim of the present study was to assess 8-isoprostane in induced sputum specimens, since these are probably the most sensitive noninvasive way of assessing oxidative stress in the airways. As far as the current authors are aware, there are no studies on the levels of 8-isoprostane in the induced sputum of COPD. The levels of 8-isoprostane were already significantly elevated in healthy smokers and consistently increased in COPD, and a significant correlation with the lung function parameters was noted.

The EIA method used appeared to be reproducible. Several earlier studies have also assessed 8-isoprostane in the exhaled breath condensate and bronchoalveolar lavage [10, 23, 24]. The current results are also in line with a recent study where this same method was used to examine the induced sputum of asthma and found to have highly significant correlation ($r>0.9$) with GC-MS and 100% spiking recovery [26].

Given that 8-isoprostane was also related to smoking without COPD, the possibility that sputum 8-isoprostane levels may differentiate healthy smokers from those who are at risk of developng COPD was investigated. Therefore, healthy smokers and those who were exhibiting symptoms, *i.e.* stage 0 COPD, were included in the study. The levels of 8-isoprostane were significantly elevated in smokers but there was no difference in the sputum 8-isoprostane level between nonsymptomatic smokers and stage 0 COPD. None of the smokers had smoked for 12 h. 8-Isoprostane was, however, significantly higher in the sputum of COPD (stage⩾1) when compared with stage 0. These results clearly suggest that 8-isoprostane is not a reliable marker in differentiating healthy smokers from those who are probably at risk of developing COPD. It also needs to be emphasised that stage 0 COPD does not necessarily lead to the development of COPD [27–30]. Overall, 8-isoprostane is already elevated in the sputum of smokers but it still remains unclear whether this or other markers of oxidative stress are sensitive enough in finding those smokers who really are at risk for COPD development.

In conclusion, 8-isoprostane levels are clearly increased in the induced sputum of smokers and especially in moderate-to-severe chronic obstructive pulmonary disease, but do not appear to be very sensitive in differentiating healthy smokers

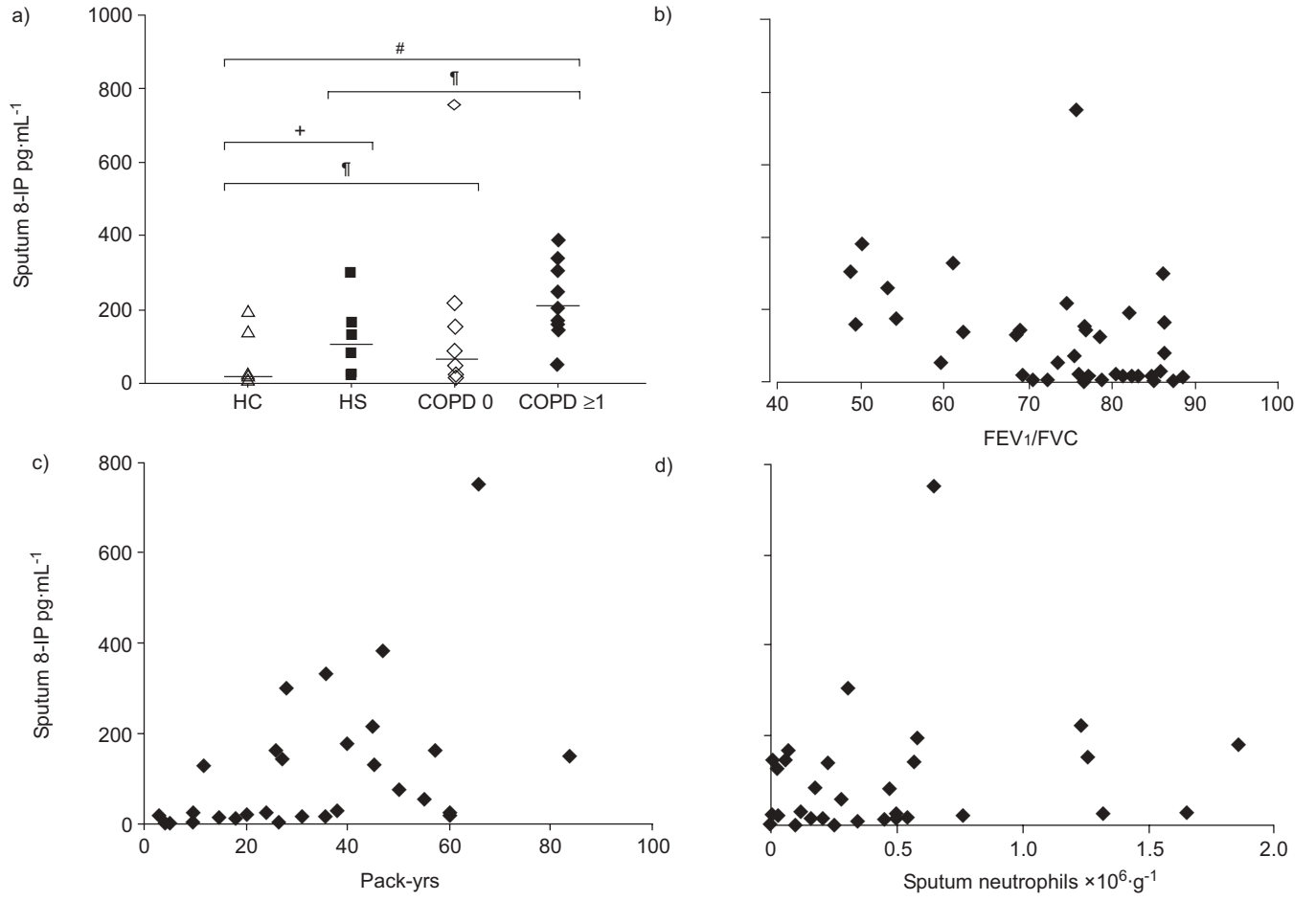

FIGURE 1. a) 8-isoprostane (8-IP) levels (pg·mL^{-1}) in sputum samples. HC: healthy controls; HS: healthy smokers; COPD: chronic obstructive pulmonary disease. $^{\#}$: $p<0.0001$; ¶: $p=0.02$; $^{+}$: $p=0.005$. Horizontal bars indicate median values. p-Values between all groups were calculated with Kruskall–Wallis test and between two groups with Mann–Whitney U-test. Spearman rank correlations between sputum 8-IP and b) forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC; =-0.66, $p=0.0001$), c) pack-yrs ($r=0.56$, $p=0.001$), and d) total number of sputum neutrophils ($r=0.37$, $p=0.02$).

from those who probably have a risk of developing chronic obstructive pulmonary disease.

ACKNOWLEDGEMENTS

The authors would like to thank M. Luukkonen and T. Marjomaa for their excellent technical assistance and T. Rehn for assistance with subject recruitment.

REFERENCES

1 Rahman I, MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 1996; 21: 669–681.
2 Repine JE, Bast A, Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Respir Crit Care Med* 1997; 156: 341–357.
3 Langen RC, Korn SH, Wouters EF. ROS in the local and systemic pathogenesis of COPD. *Free Radic Biol Med* 2003; 35: 226–235.
4 Boots AW, Haenen GR, Bast A. Oxidant metabolism in chronic obstructive pulmonary disease. *Eur Respir J* 2003; 46: Suppl. 22, 14s–27s.
5 Kharitonov SA, Robbins RA, Yates D, Keatings V, Barnes PJ. Acute and chronic effects of cigarette smoking on exhaled nitric oxide. *Am J Respir Crit Care Med* 1995; 152: 609–612.
6 Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2001; 163: 1693–1722.
7 Delanty N, Reilly M, Pratico D, FitzGerald DJ, Lawson JA, FitzGerald GA. 8-Epi PGF2 alpha: specific analysis of an isoeicosanoid as an index of oxidant stress *in vivo*. *Br J Clin Pharmacol* 1996; 42: 15–19.
8 Montuschi P, Barnes PJ, Roberts LJ. Isoprostanes: markers and mediators of oxidative stress. *FASEB J* 2004; 18: 1791–1800.
9 Reilly M, Delanty N, Lawson JA, FitzGerald GA. Modulation of oxidant stress *in vivo* in chronic cigarette smokers. *Circulation* 1996; 94: 19–25.
10 Montuschi P, Collins JV, Ciabattoni G, *et al.* Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med* 2000; 162: 1175–1177.
11 Biernacki WA, Kharitonov SA, Barnes PJ. Increased leukotriene B4 and 8-isoprostane in exhaled breath

condensate of patients with exacerbations of COPD. *Thorax* 2003; 58: 294–298.
12 Kostikas K, Papatheodorou G, Psathakis K, Panagou P, Loukides S. Oxidative stress in expired breath condensate of patients with COPD. *Chest* 2003; 124: 1373–1380.
13 Carpagnano GE, Resta O, Foschino-Barbaro MP, *et al.* Exhaled interleukin-6 and 8-isoprostane in chronic obstructive pulmonary disease: effect of carbocysteine lysine salt monohydrate (SCMC-Lys). *Eur J Pharmacol* 2004; 505: 169–175.
14 Rahman I. Reproducibility of oxidative stress biomarkers in breath condensate: are they reliable? *Eur Respir J* 2004; 23: 183–184.
15 Corradi M, Pignatti P, Manini P, *et al.* Comparison between exhaled and sputum oxidative stress biomarkers in chronic airway inflammation. *Eur Respir J* 2004; 24: 1011–1017.
16 Horvath I, Hunt J, Barnes PJ. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 2005; 26: 523–548.
17 Effros RM, Dunning MB III, Biller J, Shaker R. The promise and perils of exhaled breath condensates. *Am J Physiol Lung Cell Mol Physiol* 2004; 287: L1073–L1080.
18 Van Hoydonck PG, Wuyts WA, Vanaudenaerde BM, Schouten EG, Dupont LJ, Temme EH. Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. *Eur Respir J* 2004; 23: 189–192.
19 Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001; 163: 1256–1276.
20 Viljanen AA. Reference values for spirometric, pulmonary diffusing capacity and body plethysmographic studies. *Scan J Clin Invest* 1982; 42: Suppl. 159, 1–50.
21 Djukanovic R, Sterk PJ, Fahy JV, Hargreave FE. Standardised methodology of sputum induction and processing. *Eur Respir J* 2002; 20: Suppl. 37, 1s–2s.
22 Rytila P, Rehn T, Ilumets H, *et al.* Increased oxidative stress in asymptomatic current chronic smokers and GOLD stage 0 COPD. *Respir Res* 2006; 7: 69.
23 Montuschi P, Corradi M, Ciabattoni G, Nightingale J, Kharitonov SA, Barnes PJ. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med* 1999; 160: 216–220.
24 Montuschi P, Ciabattoni G, Paredi P, *et al.* 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am J Respir Crit Care Med* 1998; 158: 1524–1527.
25 Wood LG, Fitzgerald DA, Gibson PG, Cooper DM, Garg ML. Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. *Lipids* 2000; 35: 967–974.
26 Wood LG, Garg ML, Simpson JL, *et al.* Induced sputum 8-isoprostane concentrations in inflammatory airway diseases. *Am J Respir Crit Care Med* 2005; 171: 426–430.
27 Vestbo J, Lange P. Can GOLD Stage 0 provide information of prognostic value in chronic obstructive pulmonary disease? *Am J Respir Crit Care Med* 2002; 166: 329–332.
28 Willemse BW, ten Hacken NH, Rutgers B, Postma DS, Timens W. Association of current smoking with airway inflammation in chronic obstructive pulmonary disease and asymptomatic smokers. *Respir Res* 2005; 6: 38.
29 Ekberg-Aronsson M, Pehrsson K, Nilsson JA, Nilsson PM, Lofdahl CG. Mortality in GOLD stages of COPD and its dependence on symptoms of chronic bronchitis. *Respir Res* 2005; 6: 98.
30 Stanescu D, Sanna A, Veriter C, Robert A. Identification of smokers susceptible to development of chronic airflow limitation: a 13-year follow-up. *Chest* 1998; 114: 416–425.