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ORIGINAL ARTICLE

Evaluation of the oxidant and antioxidant balance in the pathogenesis of chronic obstructive pulmonary disease☆,☆☆

C. Cristóvão[a,*], L. Cristóvão[b,c,d,e], F. Nogueira[a], M. Bicho[e]

[a] *Serviço de Pneumologia do Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz, Lisboa, Portugal*
[b] *Faculdade de Engenharia e Ciências Naturais, Universidade Lusófona de Humanidades e Tecnologias de Lisboa, Lisboa, Portugal*
[c] *Faculdade de Ciências Biomédicas, Universidade Lusófona de Humanidades e Tecnologias de Lisboa, Lisboa, Portugal*
[d] *Unidade de Biotecnologia Ambiental, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Lisboa, Portugal*
[e] *Laboratório de Genética, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal*

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Abstract Chronic obstructive pulmonary disease (COPD) is one of the most common chronic diseases and a major cause of morbidity and mortality. An imbalance between oxidants and antioxidants (oxidative stress) has been proposed as a critical event in the pathogenesis of COPD. The increased oxidative stress in patients with COPD is the result of exogenous oxidants namely pollutants and cigarette smoke as well as endogenous oxidant production during inflammation. The aim of the present study was to clarify the hypothesis about the presence of an imbalance between oxidants and the antioxidant defences associated to COPD. In this study, we evaluated a biomarker of oxidative stress (malondialdehyde, a lipid peroxidation derived product) and non-enzymatic antioxidants (vitamin C and the sulphydryl groups) in COPD patients and healthy controls. The marker of oxidative stress was found to be significantly ($p < 0.001$) higher in COPD patients when compared with control group. No age dependent changes in the plasma levels of lipid peroxidation products were found. COPD patients had a significant ($p < 0.001$) decrease in antioxidant *status* as compared with the control group. Our results show that oxidative stress is an important pathophysiologic change in COPD.

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* Corresponding author.
E-mail address: cristina.cristovao@iol.pt (C. Cristóvão).

PALAVRAS-CHAVE
Doença pulmonar obstrutiva crónica;
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Peroxidação lipídica;
Antioxidantes

Avaliação do equilíbrio entre oxidantes e antioxidantes na patogénese da doença pulmonar obstrutiva crónica

Resumo A doença pulmonar obstrutiva crónica (DPOC) é uma das doenças crónicas mais comuns e representa uma importante causa de morbilidade e mortalidade. Um desequilíbrio entre oxidantes e antioxidantes (*stress* oxidativo) tem sido proposto como um acontecimento importante na patogénese da DPOC. O aumento do *stress* oxidativo em doentes com DPOC é o resultado da presença de oxidantes exógenos, nomeadamente, poluentes e fumo do tabaco, assim como oxidantes endógenos produzidos durante a inflamação. O objetivo do presente estudo consistiu em clarificar a hipótese sobre a existência de um desequilíbrio entre oxidantes e as defesas antioxidantes associado à DPOC. Neste estudo, avaliou-se um biomarcador do *stress* oxidativo (malonildialdeído, um produto resultante da peroxidação lipídica) e antioxidantes não-enzimáticos (vitamina C e grupos sulfidrilo), em doentes com DPOC e em controlos saudáveis. Observou-se um aumento significativo ($p < 0{,}001$) do marcador do *stress* oxidativo nos doentes com DPOC comparativamente ao grupo controlo. Não foram encontradas alterações dependentes da idade nos níveis dos produtos da peroxidação lipídica. Os doentes DPOC apresentaram uma diminuição significativa ($p < 0{,}001$) do *status* antioxidante, comparativamente ao grupo controlo. Os nossos resultados evidenciam que o *stress* oxidativo representa uma importante alteração fisiopatológica na DPOC.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most common chronic diseases and represents an important cause of morbidity and mortality.[1–3] Experimental studies have provided evidence about an imbalance between oxidants/antioxidants, in favor of reactive oxidizing species (oxidative stress), associated with COPD.[2,4–8] The involvement of oxidative stress in the pathogenesis of COPD appears to be crucial for the manifestation of the inflammatory response of the lung.[9–13] The increase of oxidative stress in patients with COPD results from the action of exogenous oxidants (*e.g.* air pollutants and tobacco components) as well as endogenous oxidants produced during the inflammatory process. However, there are numerous inconsistent results in the studies associated with oxidant and antioxidant imbalance in the pathogenesis of COPD.[14,15] One of the main targets of oxidative stress are the polyunsaturated fatty acids present in cell membranes,[16,17] the oxidizing species leading to a multistep process in which a whole is classified as lipid peroxidation. There are several products resulting from lipid peroxidation, among those to consider is a dialdehyde (malonyldialdehyde [MDA]) which has a recognized effect on the level of the human genome and is considered a clastogenic and genotoxic agent.[18] This means that the evaluation of MDA in biological samples can be considered as an indicator of increased lipid peroxidation and, therefore, an indicator of oxidative damage *in vivo*.[16,19,20]

There is evidence that oxidative stress reaches the circulation by a fall in the plasma antioxidant capacity (vitamin C, vitamin E, β-carotene and sulphydryls) associated to smoking. In addition, a similar fall in plasma antioxidant occurs in exacerbations of COPD.[5,13] Epidemiological studies have shown that high dietary intake of antioxidants vitamins C and E related to a lower prevalence of chronic bronchitis in smokers.[21]

The main objective of this study was to clarify the hypothesis of the existence of an imbalance between oxidant species production and antioxidant defence associated with the pathogenesis of COPD.

Materials and methods

Study population

Twenty patients (mean age 71.30 ± 7.68 years) with stable COPD (at least more than 6 months without exacerbation history) were studied. Fifteen of them had a past history of smoking and five were non-smokers, with passive smoking exposure or occupational exposure. Clinical and physiological characteristics of COPD group are shown in Table 1. The ex-smokers had stopped smoking at least 1 year before their participation in the study. Fifty healthy subjects (mean age 41.60 ± 12.31 years; 12 female, 38 male and body mass index, BMI $27.322 \pm 4.310\,kg/m^2$) with no history of lung

Table 1 Clinical and physiological characteristics of COPD group.

Clinical and physiological parameters	Values
Age, years	$71{,}30 \pm 7{,}68$
Male/female, *n*	11/9
Body mass index, kg/m^2	$27{,}918 \pm 1{,}724$
Smoking status: ex-smokers/non-smokers, *n*	15/5
Packs-years in ex-smokers	$68{,}8 \pm 10{,}754$
GOLD stage: I/II/IV, *n*	2/4/14
FVC, % predicted	$77{,}100 \pm 5{,}190$
FEV1, % predicted	$58{,}130 \pm 5{,}332$
FEV1/FVC, %	$59{,}453 \pm 3{,}805$
paO2, mmHg	$66{,}700 \pm 2{,}71$

disease were used as control group. Twelve of them were current smokers and thirty-eight had never smoked.

Oxidative stress was assessed in plasma through the determination of the levels of a biomarker such as MDA in COPD patients and in control group. Antioxidant *status* was evaluated by the quantification of vitamin C and total sulphydryl (—SH) groups, using spectrophotometric methods.

The study was conducted according to the rules of the declaration of Helsinki. Informed consent was obtained from all subjects participating in the study.

Methods

Determination of plasmatic malonyldialdehyde

A biomarker of systemic oxidative stress has been assessed in plasma through the quantification of the levels of a lipid peroxidation derived product, MDA. MDA concentrations were measured spectrophotometrically in terms of thiobarbituric acid reactive substances (TBARS) using a spectrophotometric method modified from Ohkawa et al.[22] The absorbances were read at 532 nm corresponding to the colored complex formed between the MDA and thiobarbituric acid (TBA). The concentration of TBARS was calculated using the MDA concentration and using a calibration curve previously prepared. The concentration of MDA was expressed in nmol/mL of plasma.

Quantification of vitamin C

Vitamin C levels were monitored by the method described by Omaye et al.[23] In brief, the dinitrophenylhydrazine reacts with oxidized vitamin C (oxidized ascorbic acid) to give a colored product. The absorbance was measured at 520 nm and is directly proportional to the vitamin C concentration. The levels of vitamin C were expressed in μg/mL of plasma.

Determination of non-protein sulphydryl groups

The non-protein sulphydryl groups are mainly in the form of reduced glutathione (GSH). We evaluated the sulphydryl group using a spectrophotometric method involving the use of Ellman's reagent.[24] The 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) undergoes disulfide exchange with sulphydryl groups and 5-thio-2-nitrobenzoate anion (TNB) is formed. The absorbance of the reduced chromogen was measured at 412 nm and is directly proportional to the GSH concentration. The levels of sulphydryl groups were expressed in μmol/mL of plasma.

Statistical analyses

The results are expressed as mean ± SEM of the concentrations of plasma parameters evaluated. The appropriate nonparametric test was chosen for data not normally distributed. Comparisons between two groups were tested using unpaired *t*-test or Mann–Whitney *U*-test. A difference with $p < 0.05$ was considered statistically significant.

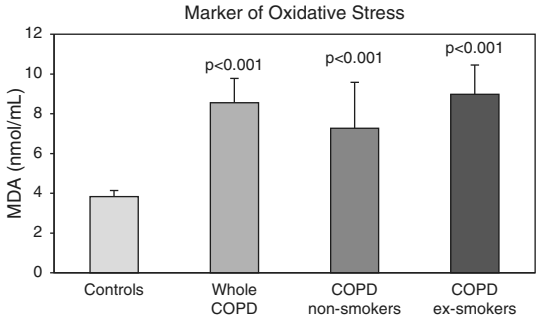

Figure 1 Levels of malondialdehyde (MDA) in plasma from control subjects and COPD patients. Values are expressed as mean ± SEM. $p < 0.001$ significantly different between controls and whole COPD group. $p < 0.01$ significantly different between controls and non-smoker patients. $p < 0.001$ significantly different between controls and ex-smoker patients. There was no significant differences between non-smokers and ex-smokers COPD patients.

Results

Oxidative stress and antioxidant *status*

The plasma concentrations of MDA, vitamin C and sulphydryl groups of COPD patients (whole group, non-smoker and ex-smoker patients) and control subjects are shown in Table 2. The marker of oxidative stress (TBARS evaluated as the amount of MDA produced) was found to be significantly ($p < 0.001$) higher in COPD patients when compared with control group. Fig. 1 shows the MDA plasma levels in controls and COPD patients. We did not find significant differences in MDA levels associated to age but we did observe a significant difference between controls and COPD patients non-smokers ($p < 0.01$) and ex-smokers ($p < 0.001$). Our results have shown no significant difference in TBARS in ex-smokers COPD patients as compared with non-smokers COPD patients. However, we observed an increase in the marker of oxidative stress associated to the ex-smoker patients.

COPD patients had a significant ($p < 0.001$) decrease in antioxidant *status* (vitamin C and sulphydryl groups) when compared with the control group (Fig. 2). No significant difference was found between non-smokers and ex-smokers in COPD patients. But we observed a decrease in vitamin C associated to the ex-smoker patients.

Discussion and conclusions

Oxidants promote lipid peroxidation in cell membrane. The detection of a product of lipid peroxidation in plasma, namely MDA, is a useful tool to show the occurrence of an oxidative stress *in vivo*. The aim of the present study was to compare the production of MDA and the levels of antioxidants vitamin C and the thiol groups in COPD and healthy controls. Our results show that oxidative stress is an important pathophysiologic change in COPD. The increase in the lipid peroxidation products in plasma of patients with COPD, supports the hypothesis of oxidative stress associated with the disease. According to our results, an increased

Table 2 Plasma MDA levels and antioxidant *status* of the study groups.

	Controls ($n = 50$)	Whole COPD group ($n = 20$)	COPD non-smokers ($n = 5$)	COPD ex-smokers ($n = 15$)
MDA (nmol/mL)	3.830 ± 0.310	8.549 ± 1.228	7.268 ± 2.309	8.976 ± 1.472
VitaminC (μg/mL)	6.230 ± 0.220	1.866 ± 0.353	2.119 ± 0.770	1.782 ± 0.408
Sulphydryl groups (μmol/mL)	0.275 ± 0.011	0.135 ± 0.022	0.130 ± 0.021	0.137 ± 0.029

All data are expressed as mean ± SEM.

oxidative stress is most evident in patients with *smoking status*, but we did not find significant differences in lipid peroxidation and antioxidant *status* in non-smokers and ex-smokers patients. In addition, we did not observe any significant difference in the marker of oxidative stress and antioxidant *status* between healthy smokers and non-smokers. Rahman et al.[25] observed that in smokers with and without COPD, an end product of lipid peroxidation is formed in airway epithelial cells, endothelial cells, neutrophils and macrophages. They concluded that oxidative stress resulting from cigarette smoking appears to be more pronounced in those who developed COPD. Previous studies by other authors showed no correlation between TBARs and hydrogen peroxide levels in the COPD associated to cigarette smoking.[26] Although our results should be regarded as preliminary, we observed a tendency for an increase in MDA levels associated to COPD patients with smoking habits.

Antioxidants are also markers of oxidative stress. We can define an antioxidant as any substance that, when present at low concentrations, compared with those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate.[16] Antioxidants can act at several different stages in an oxidative sequence. Plasma contains a variety of antioxidants, namely vitamin C and thiol groups. The loss of plasma antioxidants may indicate the ongoing biological oxidative stress. When oxidants increase and antioxidant fail, a situation of oxidative stress ensues that leads to excessive molecular damage and tissue injury.[16] Despite some limitations, plasma vitamin C has served as the index for the biochemical evaluation of vitamin C *status*.[23] Plasmatic vitamin C is particularly important because the gas phase of cigarette smoke induces lipid peroxidation in plasma *in vitro* that is decreased by vitamin C.[27] In addition, as a consequence of the role of oxidative stress in the pathogenesis of emphysema, it has been suggested that dietary antioxidants such as vitamins A, C and E could have a protective effect in smokers.[3] In addition, other studies showed decreased total antioxidant capacity in patients with COPD as compared to control subjects.[28–30] However discrepant results were found in other studies regarding the relationship between antioxidant *status* and pulmonary functions in COPD patients.[14] Other studies failed to find a protective effect of antioxidants on the lung function.[30] The molecular mechanisms relating to antioxidant defence in COPD is still lacking.[31,32] In this study we found that COPD patients had a significant ($p < 0.001$) decrease in antioxidant *status* (vitamin C and sulphydryl groups) when compared with control group. The significant decrease in vitamin C and sulphydryl groups associated to COPD patients agrees with the hypothesis that glutathione, the most abundant cellular non-protein sulphydryls, and the glutathione redox cycle may be regarded as an antioxidant mechanism involved in the protection against oxidative stress.[33] We did not find a significant difference in the antioxidant *status* between non-smokers and smokers healthy subjects and between non-smokers and ex-smokers patients but we did observe a tendency toward a decrease in vitamin C associated to COPD patients with smoking habits.

The difference of age between controls and COPD patients could be a limitation in our study. In order to observe the effect of age, we compared the results in healthy subjects younger than 50 years and those over 50 years. We found no significant difference in plasma MDA levels and antioxidant *status* associated to age. Our

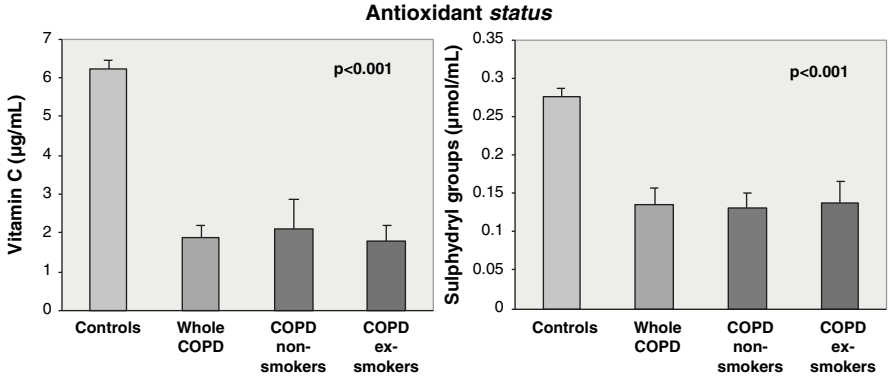

Figure 2 Concentration of plasma vitamin C and sulphydryl groups in control subjects and COPD patients. Values are expressed as mean ± SEM. COPD patients had a significant ($p < 0.001$) decrease in antioxidant *status* (vitamin C and —SH groups) when compared with control group.

observation is supported by the studies of other authors who found no relationship between plasma antioxidant capacity and protein sulphydryls with age in healthy subjects.[21,28] Other studies reported no age dependent change in the plasma MDA levels in the general population.[34,35] In addition, other authors have found a marked reduction in MDA associated to chronic airway disorders, particularly COPD. It has been suggested that the increase in MDA levels related to the pathophysiology of COPD may be a promising marker of oxidative stress in chronic pulmonary diseases.[17]

In our study oxygen therapy was not analyzed. However the effect of this therapy in oxidative stress in COPD patients is still controversial. Our results show a significant difference in oxidative stress and antioxidant *status* between controls and COPD patients (with or without oxygen therapy). No significant difference was found to be associated with oxygen therapy. Further studies are required to clarify the effects of oxygen therapy in COPD patients.

In conclusion, this study showed that elevated levels of TBARS, namely the end product of oxidant stress appears to be more pronounced in COPD patients. Concomitantly, a significant decrease in the protective antioxidant responses was found to be associated to COPD in both non-smokers and ex-smokers patients. The imbalance between oxidants and antioxidants seems to be an important event associated to the development of COPD. This could provide therapeutic implications in the future.

Conflicts of interest

The authors have no conflicts of interest to declare.

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References

1. Repine JE, Bast A, Lankhorst I, Oxidative Stress Study Group. Oxidative stress in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1997;156:341–57.
2. Tuder RM, Voelkel NF. The pathobiology of chronic bronchitis and emphysema. In: Voelkel NF, MacNee W, editors. Chronic obstructive lung diseases. BC Decker Inc.; 2002. p. 90–113.
3. Vestbo J. Epidemiology. In: Voelkel NF, MacNee W, editors. Chronic obstructive lung diseases. BC Decker Inc.; 2002. p. 41–55.
4. Rahman I, MacNee W. Oxidant/antioxidant imbalance in smokers and chronic obstructive pulmonary disease. Thorax. 1996;51:348–50.
5. MacNee W, Rahman I. Oxidants and antioxidants as therapeutic targets in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1999;160:S58–65.
6. MacNee W. Oxidants/antioxidants and COPD. Chest. 2000;117:303S–17S.
7. Hanta I, Kocabas A, Canacankatan N, Kuleci S, Seydaoglu G. Oxidant–antioxidant balance in patients with COPD. Lung. 2006;184:51–5.
8. Park HS, Kim SR, Lee YC. Impact of oxidative stress on lung diseases. Respirology. 2009;14:27–38.
9. Langen RCJ, Korn SH, Wouters EFM. ROS in the local and systemic pathogenesis of COPD. Free Radic Biol Med. 2003;35:226–35.
10. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2005;2:258–66.
11. Dourado VZ, Tanni SE, Vale SA, Faganello MM, Sanchez FF, Godoy I. Systemic manifestations in chronic obstructive pulmonary disease. J Bras Pneumol. 2006;32:161–71.
12. Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease–antiprotease imbalance, and inflammation. Int J Chron Obstruct Pulmon Dis. 2011;6:413–21.
13. MacNee W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2005;2:50–60.
14. Kluchová Z, Petrásova D, Joppa P, Dorková Z, Tkácová R. The association between oxidative stress and obstructive lung impairment in patients with COPD. Phys Res. 2007;56:51–6.
15. Mak JCW. Pathogenesis of COPD. Part II. Oxidative–antioxidative imbalance. Int J Tuberc Lung Dis. 2008;12:368–74.
16. Gutteridge JMC. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem. 1995;41:1819–28.
17. Bartoli ML, Novelli F, Costa F, Malagrinò L, Melosini L, Bacci E, et al. Malondialdehyde in exhaled breath condensate as a marker of oxidative stress in different pulmonary diseases. Mediators Inflamm. 2011:1–6.
18. Rueff J, Laires A, Brás A, Borba H, Chaveca T, Gaspar J, et al. DNA damage and oxygen species. In: DNA repair mechanisms and their biological implications in mammalian cells. New York: Plenum Press; 1989. p. 171–81.
19. Yanbaeva DG, Dentener MA, Creutzberg EC, Wesselin G, Wouters EFM. Systemic effects of smoking. Chest. 2007;131:1557–66.
20. Veskoukis AS, Nikolaidis MG, Kyparos A, Kouretas D. Blood reflects tissue oxidative stress depending on biomarker and tissue studied. Free Radic Biol Med. 2009;47:1371–4.
21. Rahman I, Swarska E, Henry M, Stolk J, MacNee W. Is there any relationship between plasma antioxidant capacity and lung function in smokers and in patients with chronic obstructive pulmonary disease? Thorax. 2000;55:189–93.
22. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–8.
23. Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for determination of ascorbic acid in animal cells, tissues and fluids. In: McCormick DB, Wrigth LD, editors. Methods enzymol, vitamins and coenzymes. New York: Academic Press; 1979. p. 3–11.
24. Rice-Evans C, Diplock A, Symons C. Techniques in free radical research. In: Burdon RH, Knippenberg PH, editors. Laboratory techniques in biochemistry and molecular biology. Elsevier; 1991. p. 227–9.
25. Rahman I, van Schadewijk AAM, Crowther AJL, Hiemstra PS, Stolk J, MacNee W, et al. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2002;166:490–5.
26. Nowak D, Kasielski M, Antczak A, Pietras T, Bialasiewicz P. Increased content of thiobarbituric acid-reactive substances and hydrogen peroxide in the expired breath condensate of patients with stable chronic obstructive pulmonary disease: no significant effect of cigarette smoking. Respir Med. 1999;93:389–96.
27. Cross CE, O'Neill CA, Reznick AZ, Hu ML, Marcocci L, Packer L, et al. Cigarette smoke oxidation of human plasma constituents. Ann N Y Acad Sci. 1993;686:72–90.
28. Rahman I, Morrison D, Donaldson K, MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. Am J Respir Crit Med. 1996;154:1055–60.

29. Agacdiken A, Basyigit I, Özden M, Yildiz F, Ural D, Maral H, et al. The effects of antioxidants on exercise-induced lipid peroxidation in patients with COPD. Respirology. 2004;9:38–42.
30. Nadeem A, Raj HG, Chhabra SK. Increased oxidative stress and altered levels of antioxidants in chronic obstructive pulmonary disease. Inflammation. 2005;29:23–32.
31. Foronjy R, Wallace A, d'Armiento J. The pharmokinetic limitations of antioxidant treatment for COPD. Pulm Pharmacol Ther. 2008;21:370–9.
32. Rahman I. Antioxidant therapeutic advances in COPD. Ther Adv Respir Dis. 2008;2:351–74.
33. Gould NS, Min E, Gauthier S, Chu HW, Martin R, Day BJ. Aging adversely affects the cigarette smoke-induced glutathione adaptive response in the lung. Am J Respir Crit Care Med. 2010;182:1114–22.
34. Bridges AB, Scott NA, Parry GJ, Belch JJ. Age, sex, cigarette smoking and indices of free radical activity in healthy humans. Eur J Med. 1993;2:205–8.
35. Block G, Dietrich M, Norkus EP, Morrow JD, Hudes M, Caan B, et al. Factors associated with oxidative stress in human populations. Am J Epidemiol. 2002;156: 274–85.