

## Breath isoprene during acute respiratory exacerbation in cystic fibrosis

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*Breath isoprene during acute respiratory exacerbation in cystic fibrosis. L.T. McGrath, R. Patrick, P. Mallon, L. Dowey, B. Silke, W. Norwood, S. Elborn. ©ERS Journals Ltd 2000.*  
**ABSTRACT:** Patients with cystic fibrosis (CF) experience a combination of chronic systemic oxidative stress, generation of free radicals in the lungs due to a hyperimmune response and a diminished ability to scavenge free radicals secondary to malabsorption and increased consumption. The authors asked the question, "Does breath isoprene content reflect systemic oxidative stress?"

The study involved 12 CF patients and 12 matched healthy controls. The patients were sampled during acute respiratory exacerbation (increased respiratory symptoms, reduction in forced expiratory volume (FEV<sub>1</sub>) of >10%, and a decision to treat with intravenous antibiotics) and after two weeks of antibiotic treatment. Blood samples were examined for markers of oxidative stress. Breath samples were analysed for isoprene content.

Malondialdehyde (MDA), erythrocyte membrane polyunsaturated fatty acids, protein sulphhydryls and protein carbonyls all showed evidence of increased oxidative stress which was moderated by antibiotic treatment. Breath isoprene production rate was significantly lower in patients during exacerbation than in controls with a mean difference of -39 (95% confidence interval (CI) -11–57) pmol·min·kg<sup>-1</sup> and increased to normal values following treatment (mean change 63 (95% CI 42–84) pmol·min·kg<sup>-1</sup>).

In conclusion, breath isoprene cannot be considered a reliable marker of oxidative stress.

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The contribution of oxygen derived free radicals to the pathogenesis of many diseases is now generally accepted. These include atherosclerosis, cancer and post ischaemic reperfusion-induced injury [1–3]. Free radical species contain an unpaired electron and have the ability to damage biological macromolecules [4]. In biological systems the majority of free radicals are derived from dioxygen, *e.g.* the superoxide anion and the hydroxyl radical.

A major role for oxygen derived free radicals has been proposed in the damage to lung tissue found in cystic fibrosis (CF) [5]. The dominant source of free radicals found in the bronchi/alveoli is the accumulation of polymorphonuclear neutrophils (PMNs) associated with repeated cycles of infection and persistent inflammation. One of the mechanisms of action of the PMNs is the generation of radicals. While these are important in killing the pathogens they may damage the pulmonary epithelium by way of peroxidative destruction of the cell membrane [6].

There have always been problems in assessing free radical damage *in vivo*. This is a function of both the methodologies available and the fact that free radical tissue damage tends to be a highly compartmentalized process. The most widely used methods measure markers of lipid peroxidation (malondialdehyde (MDA)) or protein oxidation (protein carbonyls) in the systemic circulation [7]. A novel method of assessing free radical damage in real

time by analysing exhaled breath, has used the properties of lipid hydroperoxides produced by free radical attack on polyunsaturated fatty acids.

Lipid hydroperoxides fragment to produce numerous degradation products. One of the products is believed to be the volatile isoprene (2-methyl-1,3 butadiene) which can be detected in breath [8]. The source of this compound, in addition to that contributed from the systemic circulation, will be directly from the fragmenting lipid hydroperoxides in the lung epithelia. This should avoid the problems associated with using a systemic marker to assess a compartmentalized process. Some doubts however, have been cast upon the validity of this compound as a marker of free radical activity [9]. It has demonstrated that the degree of oxidative stress in CF patients is related to the presence of acute respiratory exacerbation [10]. As part of the present study the authors have subsequently examined the production of isoprene to address the question, "Does breath isoprene content reflect systemic oxidative stress?"

### Materials and methods

#### *Study subjects and study design*

Twelve patients with CF (eight males) and 12 healthy controls (nine males) were studied. Patients with CF had a

prior diagnosis of CF by genotype and or sweat testing (sweat sodium concentration  $>70 \text{ mmol}\cdot\text{L}^{-1}$ ) and *in vitro* cultures of sputum showing chronic colonization by  $\geq 1$  organisms associated with CF. All patients were receiving vitamin E supplementation ( $200 \text{ mg}\cdot\text{day}^{-1}$ ). Patients were recruited at the time of symptomatic exacerbation of their lung disease defined as a reduction in forced expiratory volume in one second (FEV<sub>1</sub>) of  $>10\%$  compared to the best in the previous year, an increase in respiratory symptoms and a decision to treat with intravenous antibiotics. Samples were collected at the time of hospitalization before commencement of treatment and again following two weeks of antibiotic treatment. Control subjects had no personal or immediate family history of CF and no evidence of illness at the start of or during the trial. Controls were excluded if they were on any antimicrobial medication at the start of or at any time during the trial. The study was approved by the local ethics committee and informed consent was obtained from all participants prior to initiation of the protocol.

#### Sample collection and treatment

The sampling protocol was designed to minimize oxidation after collection of blood [11]. Twenty millilitres of venous blood was collected from resting, fasting subjects and prepared for the analysis of red cell membrane fatty acids, plasma MDA, carbonyls and protein thiols as previously described. All of these samples were stored at  $-80^\circ\text{C}$  and analysed within 4 weeks. Breath was collected *via* a two-way nonrebreathing valve into a 60 L gas collection bag. The subjects nose was clamped and the collection tubes equilibrated for 60 s, the breath being vented to air *via* a three-way tap. The breath was then transferred into the collection bag and collected for 3 min and the volume recorded. A sample of ambient air was collected at the same time. A measured aliquot ( $\sim 1.5 \text{ L}$ ) of sample was drawn through a gas absorption tube packed with poropak-Q<sup>TM</sup>. All samples of breath were collected at 10:00 h.

#### Pulmonary function tests

Measurements taken included, FEV<sub>1</sub> and forced expiratory volume (FVC) using a vitalograph spirometer. These procedures were repeated in the patients at the end of the two-week period.

#### Biochemical analyses

MDA was determined by high performance liquid chromatography (HPLC) and total cholesterol enzymatically as previously described [12]. Total plasma sulphhydryls and protein carbonyls were assayed spectrophotometrically [13, 14]. Protein carbonyl results were expressed per gram of protein. The results of the MDA analyses were lipid standardized and expressed per mole of total cholesterol. Lipid was extracted from red blood cell membranes, saponified, derivatized with pentafluorobenzene and analysed by capillary gas chromatography (GC) with electron capture detection as previously described [15]. The percentage of the total fatty acids comprised of

polyunsaturated fatty acids (PUFAs) was calculated from the individual fatty acids. This was expressed as moles per cent.

#### Isoprene

The absorbed organic breath contents were analysed for isoprene by thermal desorption GC mass spectrometry using the following procedure. The analytical system comprised a Perkin Elmer ATD 400 automated thermal desorption unit (Perkin Elmer, Norwalk, CT, USA), coupled to a Hewlett Packard 5890 GC (Hewlett Packard, Palo Alto, CA, USA) with a Restek RT  $\times$  % 30 m fused silica capillary column. The column was directly interfaced to a VG Trio bench-top mass spectrometer (VG Trio, Thermo VG, West Sussex, UK). The sample tubes were purged at  $200^\circ\text{C}$  for 10 min and the desorbed components collected on a cold trap packed with Tenax and maintained at  $-30^\circ\text{C}$ . The cold trap was ballistically heated to  $250^\circ\text{C}$ , which initiated the GC. The column was held at  $40^\circ\text{C}$  for 5 min and then heated at  $10^\circ\text{C}\cdot\text{min}^{-1}$  to a final temperature of  $280^\circ\text{C}$ . Isoprene, which eluted after  $\sim 3$  min, was detected by monitoring the ion at  $m/z$  68. Levels were quantified relative to toluene, which was introduced on to a tube by direct injection in methanol. The concentration of isoprene in breath was used to calculate the rate of production  $\cdot\text{min}^{-1}$ . This was then related to body mass and expressed as  $\text{pmol}\cdot\text{min}\cdot\text{kg}^{-1}$ .

#### Statistical analyses an expression of results

Data were analysed using the SPSS package (SPSS Inc., Chicago, IL, USA). Comparisons between the two groups (patients during exacerbation and controls) were made using the Mann-Whitney rank sum test and comparisons between patients before and after treatment were made using Wilcoxon's matched pairs signed ranks test. Absolute values are summarized in tables 1–2 and expressed as mean  $\pm$  SE. The mean differences between patients and controls and mean changes within the patient group following treatment are given with 95% confidence intervals (CI) for the difference/change. A p-value of  $\leq 0.05$  was considered significant.

## Results

#### Subjects

The average age of patients was 25 yrs (range 22.4–27.7) and of the controls was 24.9 yrs (range 23.8–6.0). Of the patients, 7 (58%) were chronically colonized by *Burkholderia cepacia* while 5 (42%) were chronically colonized with *Pseudomonas aeruginosa*.

#### Clinical markers

Absolute mean  $\pm$  SEM levels of the clinical markers in patients and the controls are summarized in table 1. During acute respiratory exacerbation the patients had a significantly lower FEV<sub>1</sub> ( $1.60 \pm 0.30$  L) than the controls ( $4.43 \pm 0.30$ ), mean difference  $-2.82 \text{ L}$  (95% CI 2.12–3.52)

Table 1. – Values for pulmonary function tests and inflammatory markers in patients during exacerbation and after antibiotic treatment and controls

	CF patients		Controls
	Exacerbation	Post-treatment	
FEV1 L	1.6±0.3*	1.89±0.3* <sup>+,#</sup>	4.43±0.3
FVC L	2.37±0.35* <sup>+</sup>	2.70±0.40* <sup>+,#</sup>	5.16±0.30

Values are expressed as mean±SEM FEV1: forced expiratory volume in one second; FVC: forced vital capacity; CF: cystic fibrosis. \*: p<0.05; \*\*: p<0.01; <sup>+</sup>: versus control; <sup>#</sup>: versus value during exacerbation. n=12 for both patients and controls.

L (p<0.05). Similarly, FVC was lower in the patients than in the controls (2.37±0.35 versus 5.16±0.30), mean difference -3.79 L (95% CI 3.03–4.55) L (p<0.05). Both FEV1 and FVC increased significantly in the patients following treatment (FEV1 1.89±0.30) L, mean change 0.29 (95% CI 0.18–0.40) L, FVC 2.70±0.40 L, mean change 0.33 L (0.23–0.43) L; both p<0.05). The values remained lower than in controls (p<0.05).

#### Markers of free radical damage and total cholesterol

These are summarized in table 2. Total protein sulphhydryls were significantly lower in patients during infection than in controls (299±59 versus 421±75), mean difference -122 (95% CI -77–167) µmol·L<sup>-1</sup>, p<0.005) and increased significantly after treatment to 349±55 µmol·L<sup>-1</sup> (mean change 50 (32–68) µmol·L<sup>-1</sup>, p<0.01). They continued to remain lower than the levels in control subjects, although they were not statistically significant. Lipid standardized MDA was higher during respiratory exacerbation than in control subjects (362±136 versus 169±24, mean difference 193 (107–279) nmol·mmol<sup>-1</sup> cholesterol, p<0.05) and remained high following treatment (306±73 nmol·mmol<sup>-1</sup> cholesterol) compared with controls (p<0.05). MDA levels decreased following treatment (mean difference -56 (-28–84) but this change did not reach significance. There was no change between the two observations in patient subjects. Protein carbonyl concentrations during respiratory exacerbation were not significantly different from control subjects (0.58±0.14 versus 0.53±0.14 µmol·g protein<sup>-1</sup>) but increased after treatment (0.97±0.37 µmol·g protein<sup>-1</sup>) to become significantly higher than during exacerbation (mean change 0.39 (0.11–0.67) µmol·g protein<sup>-1</sup>, p<0.05). They were also higher than the levels in control subjects (mean difference 0.44 (0.14–0.74) µmol·g<sup>-1</sup>, p<0.005). The percentage of PUFAs in red cell membranes of the patients was lower during respiratory exacerbation than in

control subjects (35.0±8.2) versus 39.4±4.7, mean difference -4.4 (-2.6–6.2) moles per cent, p<0.01). PUFAs did not increase with treatment. PUFAs remained lower after treatment (26.7–46.3 moles per cent, mean difference 2.9 moles per cent, p<0.05) compared to controls and were not different from baseline.

#### Isoprene

This is summarized in table 2. Breath isoprene was significantly lower in patients during acute respiratory exacerbation than in controls (125±23 versus 164±20, mean difference -39 (-11–57) pmol·min·kg<sup>-1</sup>, p<0.05) and increased significantly after treatment to 188±23 pmol·min·kg<sup>-1</sup> (mean change 63 (42–84) pmol·min·kg<sup>-1</sup>, p<0.05).

### Discussion

Free radicals are produced constantly by normal metabolic pathways. A major group of free radicals, the reactive oxygen species (ROS), are derived from molecular oxygen and include the superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the hydroxyl radical (OH<sup>-</sup>) [4]. The OH<sup>-</sup> radical in particular has the potential to cause structural damage to lipids, proteins, carbohydrates and nucleic acids. Whether or not they produce damage is determined by the balance between their generation and the bodies antioxidant capacity [16]. A major source of free radicals are the mitochondria and activated phagocytes [17]. This is particularly relevant to CF patients who show evidence of depleted antioxidant capacity, increased systemic radical generation secondary to leakage of electrons from increased electron transport activity and extensive production from activated neutrophils in the lung in response to infection [5, 18, 19]. It is obvious that an ability to follow the course and extent of free radical

Table 2. – Aqueous phase antioxidant, markers of free radical damage in the plasma and isoprene in the breath of patients during exacerbation and after antibiotic treatment and controls

	CF patients		Controls
	Exacerbation	Post-treatment	
Sulphydryls µmol·L <sup>-1</sup>	299±59*** <sup>+,#</sup>	349±55*** <sup>#</sup>	421±75
MDA nmol·mmol cholesterol <sup>-1</sup>	362±136* <sup>+,#</sup>	306±73* <sup>+,#</sup>	169±24
Protein carbonyls µmol·g protein <sup>-1</sup>	0.58±0.14	0.97±0.37* <sup>+,***,#</sup>	0.53±0.14
PUFAs Moles per cent	35.0±8.2*** <sup>+,#</sup>	36.5±4.9* <sup>+,#</sup>	39.4±4.7
Isoprene pmol·min·kg <sup>-1</sup>	125±23* <sup>+,#</sup>	188±23* <sup>+,#</sup>	164±20

Values are expressed as mean±standard error. MDA: malondialdehyde; PUFAs: polyunsaturated fatty acids; CF: cystic fibrosis. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.005; <sup>+</sup>: versus control; <sup>#</sup>: versus first patient observation.

mediated damage in a disorder such as CF would be a useful clinical tool. The utility of using a volatile marker of free radical damage (isoprene) was examined in the breath of CF patients by comparing it with conventional measures of free radical damage/activity. Patients were studied at a time of maximum oxidative stress during a period of acute respiratory exacerbation and again following two weeks treatment when oxidative stress would be expected to have abated. During acute exacerbation markers including plasma MDA, plasma protein thiols and red cell membrane PUFAs provided evidence of increased oxidative stress. This degree of oxidative stress decreased following antibiotic treatment although it did not reach normal levels. Levels of protein carbonyls increased after treatment. This may reflect different kinetics for damage to proteins and their appearance in the systemic circulation. In contrast to other workers who found increased breath isoprene in conditions associated with increased oxidative stress to lung epithelia the authors found decreased levels during acute respiratory exacerbation which increased towards normal, following treatment [20].

The ability to assess free radical damage/activity has always been compromised by limitations in the assays available. One approach has been to assess peroxidative damage to lipids. This has been assessed, most commonly, by measuring MDA, a break down product of lipid hydroperoxides. Original methods depended upon the reaction with thiobarbituric acid. The original nonspecific method measuring thiobarbituric acid reacting substances (TBARS) has been replaced by methods specific for MDA [21]. Analysing the fatty acid composition of red blood cell membranes may assess peroxidation of membrane phospholipid PUFAs. Consumption of PUFAs is evidence of peroxidative damage.

An alternative approach has been to examine the effect on proteins by measuring protein carbonyls [14]. In addition to these direct measurement methods, free radical activity has been assessed indirectly by measuring the consumption of scavengers. Proteins can scavenge free radicals by donating a hydrogen atom from sulphhydryl side chains. Evidence of increased scavenging is provided by a reduction in reduced protein thiols [13].

A major draw back of these assays is that they reflect total activity and cannot address the question of the site of origin. This could be addressed by examining bronchiolar lavage for evidence of free radical activity. The membrane of small airway epithelial cells is a major target for free radical damage in these patients, another possible alternative would be to examine a volatile derivative of peroxidized membrane lipids. Measurement of a volatile compound in breath partially meets these conditions in that it is produced directly from the lung surface. This is confounded by the fact that an unquantifiable fraction comes from the systemic circulation.

Measurement of a volatile compound in breath to assess oxidative stress has been used in a number of conditions. Originally pentane was measured as the volatile compound. LEMOYNE *et al.* [22] analysed breath pentane to demonstrate the ability of supplemental vitamin E to reduce oxidative stress. Levels of breath pentane were inversely proportional to vitamin E levels. VAN GOSSUM *et al.* [23] infused lipid rich in linoleic acid into individuals and demonstrated increased breath pentane. HOTZ *et al.*

[24] demonstrated small increases in breath pentane in cirrhotic patients. Many of the methodologies employed GC to analyse breath. KOHLMULLER and KOCHEN [25] carried out an analysis of these methods and found them to have unacceptable limitations, either relating to sensitivity or specificity. They developed a gas chromatographic/mass spectroscopic (GC/MS) method. This showed that using the common GC methods, the "n-pentane" peak was actually composed predominantly of isoprene and these previous studies had misidentified the compound. The method employed in this study was a specific GC/MS method with confirmation of the peak as isoprene. Analysis of breath isoprene has inherent difficulties apart from the methodology, these include a wide distribution within and between individuals, a distinct circadian rhythm and dependence on the state of nutrition and exercise [26, 27]. In an effort to minimize variations all of the subjects in the present study were closely matched, sampled fasting at the same time of day, and were resting. To minimize variations in concentration due to rate and depth of breathing, breath isoprene was calculated as a production rate in mass eliminated per kg of body mass per unit of time rather than as an absolute concentration. This does not resolve the problem encountered in the present study that breath isoprene decreased during the period of maximum oxidative stress and increased towards normal following treatment which would be expected to diminish oxidative stress [28]. This raises the possibility that isoprene either does not track, or is indirectly related to, oxidative stress. The observed results may also be partly explained by altered transmission/diffusion properties of the lung membranes. During periods of acute respiratory exacerbation transmission of isoprene from systemic sources may be reduced. This could lower breath concentration even in the presence of increased systemic production. Improvement due to treatment transmission could increase. This process should have a smaller impact on isoprene generated from lung epithelial membranes. Isoprene from this source would be released directly into the lung space, although this could also be inhibited by mucus accumulation. The work of SCHUBERT *et al.* [29] is interesting in this respect. While they demonstrated a decrease in breath isoprene in critically ill patients who developed pulmonary infection, breath pentane actually increased. These conflicts can only be resolved by measuring isoprene in blood. The exact origin of isoprene in human breath is not known. KOHLMULLER and KOCHEN [25] argue that polyisoprenes, *e.g.* squalene, are possible sources of isoprene *via* a radically mediated *in vivo* peroxidation. Mechanisms which induce an increased rate of reactive oxygen intermediates and are thought to attack membrane PUFAs might also attack polyisoprenes with the consequence that isoprene and n-pentane are produced.

Alternatively STONE *et al.* [9] have suggested that isoprene might also be produced from mevalonate as a by-product of cholesterol synthesis. They provided evidence to support this hypothesis by manipulating cholesterol synthesis in human volunteers and comparing breath isoprene with rate of cholesterol synthesis. In the present study lowest levels of breath isoprene were found during acute exacerbation, the period of highest oxidative stress. When this had been modified by antibiotic treatment levels of cholesterol increased, although not

significantly, markers of oxidative stress decreased and breath isoprene increased towards normal. Animal studies have shown that endotoxin, tumour necrosis factor and interleukin decrease hepatic squalene synthase messenger ribonucleic acid, activity and protein mass [30]. This would reduce the production of isoprene. The present results do not support the hypothesis that breath isoprene can be used as a reliable real time marker of oxidative stress.

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