Effect of age on the breath methylated alkane contour, a display of apparent new markers of oxidative stress

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Reactive oxygen species (ROS) are toxic byproducts of mitochondrial energy production that inflict oxidative stress, a constant barrage of damage to DNA, proteins, lipids, and other biologically important molecules. Oxidative stress has been implicated as a pathologic mechanism in aging and in several diseases. We developed a display of apparent new markers of oxidative stress in human beings, the breath methylated alkane contour (BMAC). The BMAC is a three-dimensional display of C4 to C20 alkanes and monomethylated alkanes in breath, with x-axis = carbon chain length, z-axis = methylation site, and y-axis = alveolar gradient (relative abundance in breath minus relative abundance in room air). In 102 normal human subjects of 9 to 89 years of age, alveolar gradients of components of the BMAC increased significantly with age. The mean alveolar gradient of all components of the BMAC varied from negative in the youngest quartile (ages 9 to 31 years) to positive in the oldest quartile (ages 74 to 89 years)($P < 2.10^{-9}$). These findings were consistent with an increase in oxidative stress with advancing age, although an age-related decline in clearance by cytochrome p450 may have contributed. The BMAC provides a display of apparent new markers of oxidative stress with potential applications in aging research, clinical diagnosis, pharmacology, and toxicology. (J Lab Clin Med 2000;136:243-9)

Abbreviations: AUC = area under curve; BMAC = breath methylated alkane contour; PUFA = polyunsaturated fatty acid; RA = relative abundance; ROS = reactive oxygen species; VOC = volatile organic compound

Power plants commonly produce toxic byproducts. In biologic systems, mitochondrial power plants generate the energy that sustains mammalian life, but they also convert oxygen to toxic and potentially lethal byproducts. Oxygen is the final acceptor of electrons in oxidative metabolism, but electron leakage from the mitochondria in the form of ROS inflicts oxidative stress, a constant barrage of oxidative damage to DNA, proteins, lipids, and other biologically important molecules[1,2] (Fig 1). The toxic effects of oxygen were first described in laboratory animals in 1878, and oxygen was recognized as the cause of retrolental fibroplasia in premature neonates in the 1940s.[3] In the 1950s, the oxidative stress theory of aging was proposed, as follows: the molecular basis of aging may be the cumulative oxidative damage inflicted by ROS on virtually all biologic molecules, including PUFAs, proteins, and DNA. This theory attracted little credence at first because free radicals were thought unlikely to occur in biologic systems; however, this changed with the discovery in 1969 of superoxide dismutase, an enzyme that clears superoxide radical.

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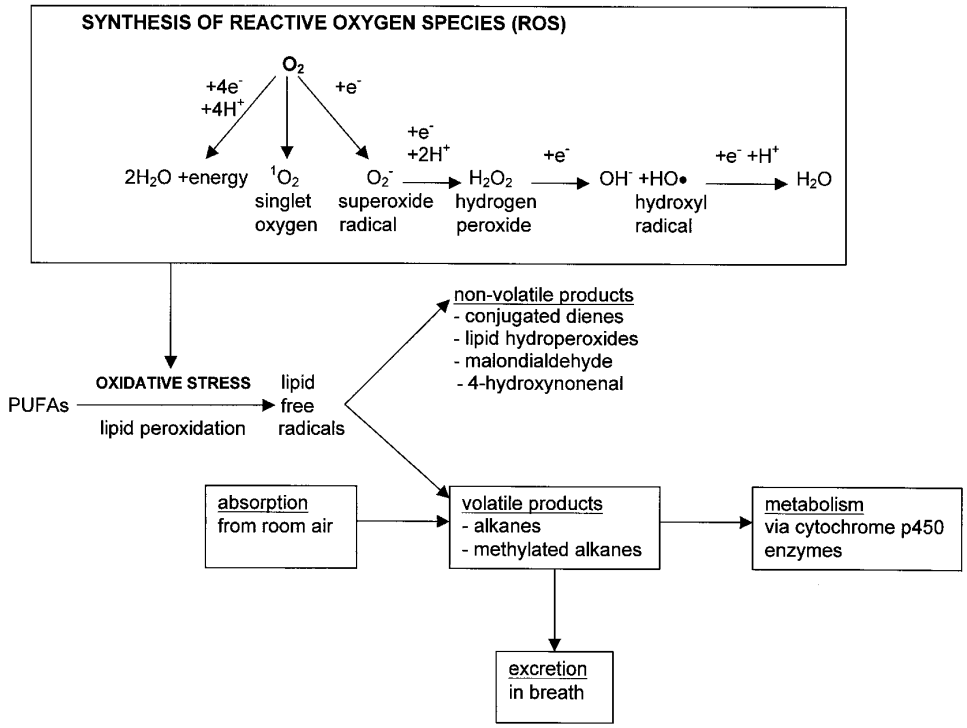

Fig 1. Origin of oxidative stress and the generation of metabolic markers. ROS (superoxide radical, hydrogen peroxide, and hydroxyl radical) are synthesized in the mitochondria; electron leak into the cytoplasm generates oxidative stress, a constant barrage of damage to DNA, proteins, and PUFAs. This diagram illustrates the effect of oxidative stress on PUFAs, generating alkanes and methylated alkanes that are either metabolized or excreted in the breath. These pathways are well documented for alkanes but hypothetical for methylated alkanes.

The in vivo level of oxidative stress tends to increase with age in insects and mammals, as indicated by increasing exhalation of alkanes; this has been ascribed mainly to an increased rate of production of ROS rather than a decline in antioxidant defenses. There is additional supportive evidence for a link between aging and oxidative stress. The life span of *Drosophila* is extended by overexpression of human superoxide dismutase in motorneurons[4] and is shortened by exposure to high levels of oxygen.[5] Similarly, the life span of human diploid fibroblasts in vitro also is shortened by exposure to high levels of oxygen[6] and is extended by hypoxia.[7] Food restriction extends the life span of rats and is accompanied by a reduction in breath pentane excretion.[8]

Oxidative stress has been implicated as a pathologic mechanism in aging and several diseases, but measuring its intensity in vivo has been difficult.[9] Various markers of oxidative stress have been proposed, including malonaldehyde and conjugated dienes in the blood and hydrocarbons and hydrogen peroxide in the breath.[10,11] Volatile markers of oxidative stress have attracted attention because breath tests are intrinsically noninvasive and painless.[12] Increased breath alkanes, particularly ethane and pentane, have demonstrated increased oxidative stress in breast cancer,[13] rheumatoid arthritis,[14] heart transplant rejection,[15] acute myocardial infarction,[16] schizophrenia[17] and bronchial asthma.[18] Breath ethane and pentane are of limited value in screening for these disorders, because their sensitivity and specificity are poor.

However, oxidative stress appears to generate other degradation products that are excreted as VOCs in the breath. We have previously reported that the breath of normal human beings[19] and patients with lung cancer[20] contains longer-chain alkanes and that the concentrations of several of the C4 to C20 alkanes increases with age.[21] We report here the results of examining another molecular dimension—the alkane methylation site—to produce a three-dimensional graph, the BMAC, and its variation with age.

METHODS

Collection of breath and air samples. VOCs in breath and room air were collected with a breath collection apparatus, a portable microprocessor-controlled device. Each subject wore a nose clip while inspiring and expiring through a low-resistance disposable mouthpiece into a wide-bore breath reservoir (1.0-inch diameter) open to the atmosphere at its distal end. The breath reservoir was heated to prevent condensation. Alveolar breath was pumped from the breath reser-

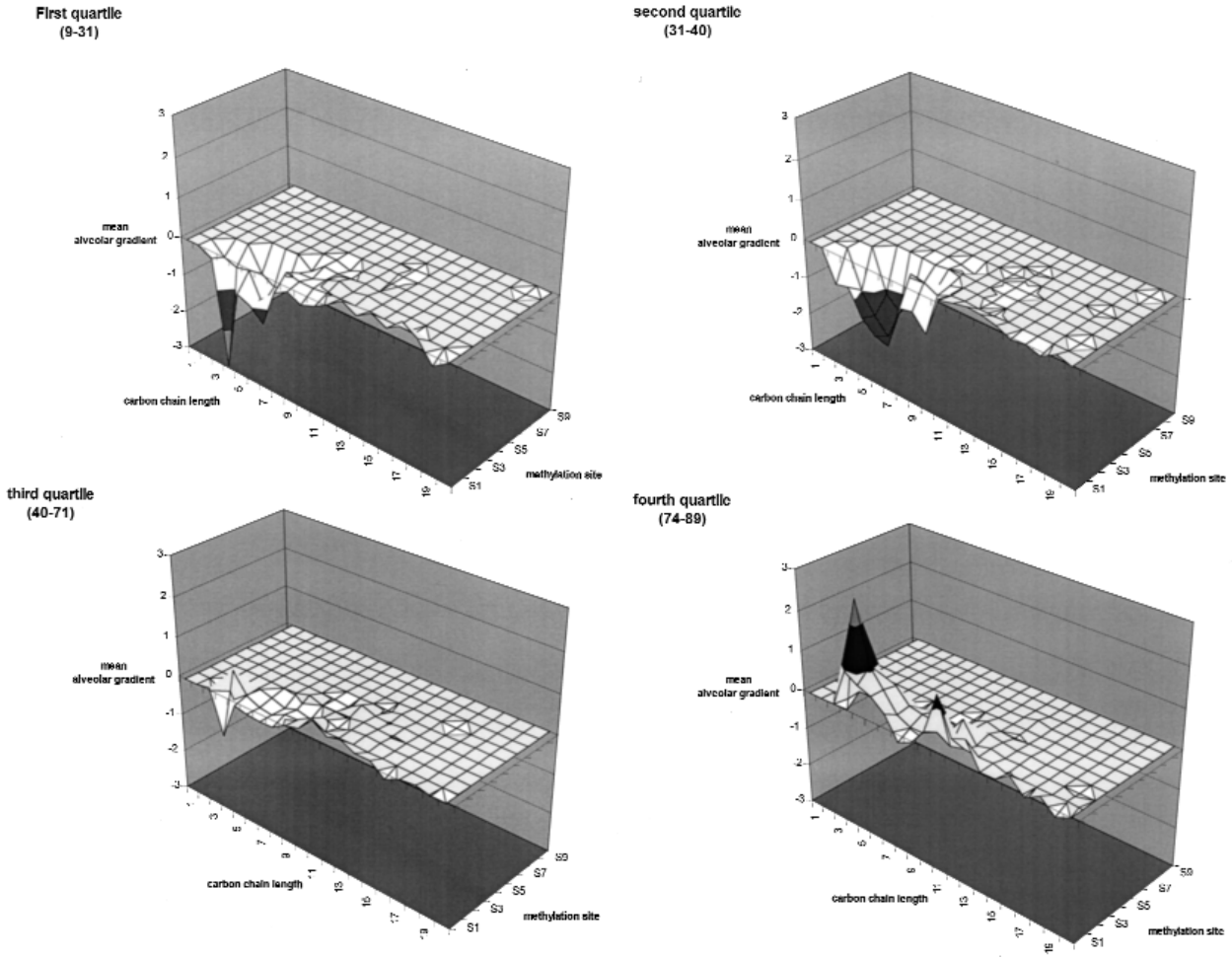

Fig 2. BMACs in normal subjects. The mean BMACs are shown for 102 normal human subjects, separated into quartiles by age. The compounds shown are straight chain n-alkanes methylated at one site. This figure includes n-alkanes, showing them as methylated at C1. For example, an alkane with carbon chain length = 4 (butane) becomes the C5 alkane pentane when methylated at C1. The alveolar gradient is the RA in breath minus the RA in room air and varies with the rate of synthesis minus the rate of clearance. There was a progressive change with age from predominantly negative to predominantly positive alveolar gradients of several methylated alkanes, consistent with increasing oxidative stress with age. Statistically significant differences between younger and older subjects are shown in Table I and Fig 3.

voir through a sorbent trap where the breath VOCs were captured on activated carbon (200 mg Carbotrap C [20/40 mesh] and 200 mg Carbopack B [60/80 mesh]; Supelco Inc, Bellefonte, PA). The geometry of the system ensured that the sample comprised alveolar breath virtually uncontaminated by dead-space air. Two separate samples were collected: alveolar breath and background room air, each containing VOCs in 1.0 L collected over 2.0 minutes at 0.5 L/min. The collection method has been described in greater detail elsewhere.[19,22]

Breath VOC assay. VOCs were desorbed from the sorbent trap by heating it to 300°C in an automated thermal desorber (ATD 400; Perkin Elmer, Norwalk, CT). A stream of helium flushed the VOCs onto a concentrator, a refrigerated sorbent trap maintained at 0°C. The concentrated sample of VOCs was then heated to 300°C, and the volatilized VOCs were separated by gas chromatography and identified and quantified by mass spectroscopy. The assay method has been described in greater detail elsewhere.[19,22]

Human subjects. Breath samples were collected between 7.00 AM and noon from 102 normal volunteers between 9 and 89 years of age who had fasted from the previous midnight. Subjects sat for approximately 20 minutes before the collections of breath and air to allow time for equilibration between VOCs in room air and in the blood. The institutional review board of the Sisters of Charity Medical Center, St Vincent's Campus, Staten Island, NY, approved the human research.

Analysis of data. The RA of each alkane (C4 to C20) and its monomethylated derivatives were determined from the chromatographic AUC and the AUC of an internal standard (0.25 mL of 2 ppm 1-bromo-4-fluorobenzene; Supelco) ($RA_{VOC} = AUC_{VOC}/AUC_{internal\ standard}$). The alveolar gradient of each VOC was determined as $RA_{breath} - RA_{room\ air}$.[19]

Table I. Alkanes and monomethylalkanes: Significant differences between quartiles

Quartile	1	1	1	2	2	3
vs quartile	2	3	4	3	4	4
Pentane, 2-methyl	—	—	—	—	*	—
Hexane	—	—	*	—	†	—
Hexane, 2-methyl	—	—	*	*	†	—
Hexane, 3-methyl	—	—	*	*	‡	—
Heptane	—	—	†	*	‡	—
Heptane, 2-methyl	—	—	*	†	‡	—
Heptane, 3-methyl	—	—	†	†	‡	—
Octane	—	*	†	†	‡	—
Octane, 3-methyl	—	—	†	†	‡	*
Octane, 4-methyl	*	*	*	—	—	—
Nonane	—	—	—	†	†	—
Nonane, 2-methyl	—	*	†	*	*	—
Decane, 2-methyl	—	—	‡	—	‡	*
Decane, 3-methyl	—	†	—	‡	‡	—
Decane, 5-methyl	—	*	*	*	*	—
Undecane	—	—	—	*	†	—
Undecane, 5-methyl	—	*	—	*	—	—
Dodecane, 5-methyl	—	†	†	†	†	—
Tridecane, 2-methyl	—	—	*	—	*	*
Tridecane, 3-methyl	—	—	—	—	—	—
Tetradecane, 5-methyl	—	†	—	†	—	—
Heptadecane	—	†	—	—	—	*

*$P < .05$.
†$P < .01$.
‡$P < .001$.

In each subject, a BMAC was constructed by plotting alkane carbon skeleton length (x-axis) versus methylation site (z-axis) versus alveolar gradient (y-axis).

RESULTS

Seventy-three different C4 to C20 alkanes and monomethylated alkanes were observed at least once in the breath samples. Subjects, in groups of 25 or 26, were assigned to four quartiles according to their age, ranging from the first quartile (the youngest quarter of the population) to the fourth quartile (the oldest quarter of the population). Breath data were pooled from subjects in each quartile to determine their mean alveolar gradients of alkanes and monomethylated alkanes. These values were displayed as the BMAC for each quartile (Fig 2); significant differences between quartiles were determined with one-way analysis of variance and a Newman-Keuls post hoc test (Table I). The combined mean alveolar gradient of all alkanes and monomethylated alkanes in each quartile are shown in Fig 3; these exhibited highly significant increases in the third and fourth quartiles as compared with the first. When subjects were matched for age, the only difference with sex was 4-methyloctane (greater in females than in males, $P < .05$, two-tailed t test); there was an insufficient number of tobacco smokers in the study group for a meaningful statistical comparison with nonsmokers.

DISCUSSION

Our study demonstrated three main findings: (1) normal human breath contains a greater number of alkanes and monomethylated alkanes than previously reported; (2) the alveolar gradients of several of these VOCs were significantly more positive in older than in younger normal human beings; (3) the combined mean alveolar gradient of all VOCs in the BMAC significantly increased with age, from negative in the youngest to positive in the oldest.

To interpret these findings, two separate issues need to be considered: (1) the qualitative significance of alkanes and monomethylated alkanes in the breath, and (2) the quantitative significance of their alveolar gradients.

Alkanes in the breath are qualitatively significant as markers of oxidative stress. This has been extensively documented in other reports, most of which have focused on ethane and pentane. However, Kneepkens

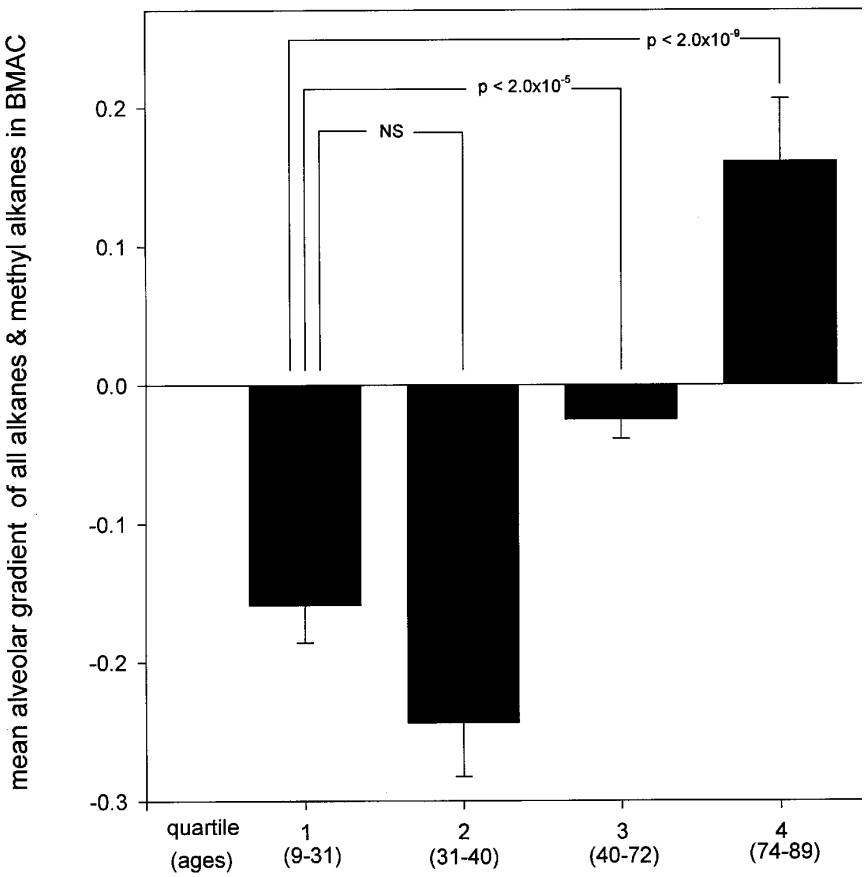

Fig 3. Effect of age on the mean alveolar gradient of all alkanes and methylated alkanes in the BMAC. This figure demonstrates, for each age quartile, the mean alveolar gradient of all the VOCs in the BMACs shown in Fig 2 (*bar* = SEM). This value varied from negative in the first, second, and third quartiles to positive in the fourth quartile, and the changes were highly significant with advancing age in subjects over 40 years of age. This demonstrates that the overall elevation in components of the BMAC with age, which is visually apparent in Fig 2, is statistically significant as well. The probable pathophysiologic basis of these changes was an age-related increase in oxidative stress causing increased synthesis of alkanes and methylated alkanes, although an age-related decline in cytochrome p450 clearance of these compounds may have contributed to the effect.

et al have shown that oxidative stress elicits the production of other alkanes besides ethane and pentane. In summary, ROS peroxidate a PUFA with n carbons to lipid alkoxyl radical, which is then converted by beta scission to an alkane with n-1 carbons. PUFAs composed of three and six carbons are converted to ethane and pentane, respectively, but the same mechanism generates other alkanes including propane, butane, hexane, heptane, and octane.[10,11] Consequently, all of the C4 to C20 alkanes observed in this study appear to be markers of oxidative stress. Methylated alkanes have not been studied in comparable detail, and further research is required to document their biosynthetic pathways. However, our findings suggest that methylated alkanes are also markers of oxidative stress, and this hypothesis could be tested experimentally because it predicts that these VOCs would be manufactured in cells, tissues, or intact animals subjected to pharmacologic oxidative stress.

The quantitative significance of the BMAC is determined by the physiologic basis of the alveolar gradient. We have previously analyzed the kinetics of breath VOCs and demonstrated the following relationship[19]:

$$\text{Alveolar gradient} = \frac{(R_{synthesis} - R_{clearance})}{RMV}$$

where R = rate of movement of VOC (mol/min) and RMV = respiratory minute volume (L/min). This equation demonstrates that the age-related increase in the alveolar gradients of alkanes and methylated alkanes may have been caused by an increase in their rate of

synthesis, a decrease in their rate of clearance, or both. There is evidence for both processes, and it is not apparent which is quantitatively the most important. Previous studies have shown that breath pentane increases with age in healthy normal human beings.[23,24] Sagai and Ichinose[25] demonstrated age-related increases in lipid peroxidation in rats, as shown by increased excretion of ethane, butane, and pentane in breath, and Sohal et al[26] observed increased exhalation of pentane with age in the male housefly, *Musca domestica*. In all of these studies, the findings were ascribed to an age-related increase in oxidative stress resulting in a commensurate increase in the rate of synthesis of alkanes. However, these changes might also have been influenced by a reduced rate of clearance resulting from the age-related decline in hepatic cytochrome p450 activity that is known to cause a commensurate decline in the clearance of some drugs.[27,28] Whether this process significantly affects alkanes and methylated alkanes in the breath is unclear, because not all hydroxylation enzymes are similarly affected; for example, there is no age-related decline in the activity of cutaneous aryl hydrocarbon hydroxylase[29] nor of the hepatic microsomal hydroxylation of alprazolam.[30]

It will be noted in Figs 2 and 3 that we used the relative abundance of VOCs rather than their absolute concentrations in mass or molar units. The combination of gas chromatography with mass spectroscopy and an internal standard yields a value for the RA of a VOC:

$$RA_{voc} = AUC_{voc} / AUC_{internal\ standard}$$

where *AUC* = area under the curve of chromatographic peak.

The RA value can then be converted into the absolute concentration of the VOC in mass or molar units by reference to a standard curve for that VOC. However, this requires that an individual standard curve must be constructed for every VOC observed. We have previously reported these standard curves for C4 to C20 alkanes but not for methylalkanes. Construction of standard curves for approximately 80 different methylalkanes was not undertaken in this study for two main reasons: (1) the task would be extremely laborious, time-consuming, and technically challenging; (2) it would add very little to the results or the interpretation of the data. Because the relationship between the RA and the molar concentration of a VOC is linear, the appearance of the BMAC would be little changed and the results of statistical analysis of data would not be changed at all.

The main limitation of this study is that it did not demonstrate a direct causal linkage between oxidative stress and the components of the BMAC. This linkage was inferred from two previously documented observations: (1) aging causes increased oxidative stress, and (2) increased oxidative stress causes increased excretion of alkanes in the breath. Because this was a pilot study of normal human beings ranging from children to the elderly, it was not feasible to test this hypothesis by introducing any additional stressor that might elicit oxidative stress. However, we are actively pursuing studies in human subjects, in animals, and in cell cultures to evaluate the effects of disease and induced oxidative stress on the production of alkanes and methylated alkanes.

We conclude that the alveolar gradients of several C4 to C20 alkanes and monomethylated alkanes in the breath increased significantly with age in normal human beings, and the BMAC may provide a clinically useful display of these apparent new markers of oxidative stress. However, further research is needed to confirm this proposed causal linkage between oxidative stress and the BMAC. With this caveat in mind, the breath test may have several potential applications, particularly in diagnostic screening for diseases that are associated with increased oxidative stress. We are currently evaluating the BMAC in multi-center clinical studies of a number of conditions including lung cancer, breast cancer, and heart transplant rejection. The BMAC also has potential applications in other areas of research including the study of aging, in toxicology for estimating the effects of pro-oxidant toxins, and in pharmacology for evaluating the effects of antioxidants.

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