

Relations between isoprene and nitric oxide in exhaled breath and the potential influence of outdoor ozone: a pilot study

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Abstract

The role of endogenous isoprene in the human body, if any, is unclear because previous research is inconsistent and mechanistic evidence for the biologic function of isoprene is lacking. Given previous evidence that exhaled isoprene is elevated in systemic inflammatory states, we hypothesized that exhaled isoprene would be positively associated with a breath biomarker of airway inflammation, the fractional concentration of exhaled nitric oxide (FENO). We examined relationships of exhaled breath isoprene with FENO and with outdoor ozone given that ozone chemically reacts with isoprene and has been positively associated with FENO in past studies. Sixteen elderly subjects were followed with ≤ 12 weekly exhaled hydrocarbon and FENO collections at the subjects' retirement community. Outdoor ozone concentrations were measured continuously on site. Mixed-effects regression analyses tested relations of FENO with isoprene, and FENO and isoprene with ozone, adjusted for temperature. We found FENO was inversely associated with isoprene, and this was not confounded by ozone. Isoprene was inversely related to ozone. FENO was positively related to ozone and this relation was not confounded by isoprene. In contrast to hypothesized relations, we conclude that exhaled isoprene is inversely associated with FENO as well as outdoor ozone, which suggests possible protective ozone-scavenging functions of endogenous isoprene. Findings may indicate chemical reactions of isoprene oxidation by ozone and by hydroxyl radicals in the presence of O₂ that is dependent on NO concentration. These preliminary results need to be confirmed in additional studies of human subjects, particularly as they apply to FENO monitoring in asthma.

Introduction

A number of volatile organic compounds (VOCs) exist in exhaled human breath, with exhaled isoprene, acetone, ethanol, methanol and other alcohols being among the most abundant (Fenske and Paulson 1999). Measurement of VOCs in exhaled breath is a non-invasive method of disease or

exposure assessment (Pleil and Lindstrom 1997). Recent studies have found that some of these compounds may prove to be useful as clinical biomarkers of systemic ketosis (Likhodii *et al* 2002), acute myocardial infarction (Mendis *et al* 1994), oxidative and metabolic stress (Pabst 2007), and cancer (Schmutzhard *et al* 2008). Breath sulfides have potential as a biomarker of exacerbations in cystic fibrosis patients and methyl nitrate as an indicator of hyperglycemia in children who have insulin dependent diabetes mellitus (Kamboures *et al*

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2005, Novak *et al* 2007). Exhaled isoprene may function as a useful biomarker of inflammation and has been shown to be produced endogenously (King *et al* 2009 and 2010), but its exact functions within the human body, if any, have not yet been established (Salerno-Kennedy and Cahsman 2005).

While at this stage analysis of exhaled breath for disease biomarkers remains largely investigational, there is great clinical potential for these non-invasive, easily reproducible tests. The fractional concentration of exhaled nitric oxide (FE_{NO}) is an example of a non-invasive test of airway inflammation and now can be used in certain cases to diagnose and monitor subjects with asthma (Dweik *et al* 2011, Jones *et al* 2001, Taylor 2012). FE_{NO} has recently been integrated into clinical practice since it has been found to be associated with asthma control and with established measures of airway inflammation in numerous studies (e.g., Jones *et al* 2001). In 2011, the American Thoracic Society published clinical guidelines on the use of FE_{NO} to assess airway disease (Dweik *et al* 2011). Though FE_{NO} has been used as a clinical biomarker of airway inflammation in patients with asthma, the potential of exhaled volatile hydrocarbons such as isoprene in the same capacity is not clear.

Elevated breath isoprene levels have been reported after smoking cigarettes. Smoking just one cigarette increases isoprene by 70% in exhaled breath (Senthilmohan *et al* 2001). Research on isoprene in humans and mammals still remains limited and no clear mechanism pointing to its function, if any, has been elucidated (Salerno-Kennedy and Cashman 2005). Numerous studies have extrapolated to humans data from mice and rats on isoprene toxicokinetics, however, the differences between these species and humans in terms of metabolism and reactivity of metabolites makes it difficult to predict human toxicokinetics (Watson *et al* 2001).

Previous studies have hypothesized that exhaled isoprene may be a clinical indicator of mevalonate biosynthesis from hydroxymethylglutaryl-coenzyme A (HMG-CoA, an early step leading to cholesterol synthesis). This was first demonstrated in the *in vivo* biosynthesis of isoprene from DL-melavonate in the cytosolic fraction of rat liver (Deneris *et al* 1985). Isoprene is a by-product during the conversion of mevalonate to mevanolate-5-pyrophosphate and isopentenyl pyrophosphate. Interestingly enough, there is some evidence that the use of statins to treat hypercholesterolemia (statins inhibit HMG-CoA reductase that catalyzes production of mevalonate from HMG-CoA) has been associated with reduced levels of isoprene in exhaled breath (Karl *et al* 2001, Stone *et al* 1993). Findings of reduced isoprene levels in exhaled breath in those with end stage liver disease has been found to be linked directly to the reduction of serum cholesterol providing further support for the role of isoprene in cholesterol biosynthesis (Sehnert *et al* 2002).

Since cholesterol formation is related to inflammation (Kleeman and Kooistra 2005) and isoprene is a byproduct of cholesterol biosynthesis, there is thus the potential of exhaled isoprene to serve as a marker of inflammation (Salerno-Kennedy and Cahsman 2005). Numerous studies have found elevated exhaled isoprene levels in humans in acute conditions of inflammation such as acute myocardial infarctions

and certain cancers (Salerno-Kennedy and Cahsman 2005, Likhodii *et al* 2002, Mendis *et al* 1994, Pabst *et al* 2004, Schmutzhard *et al* 2008). Though exhaled isoprene levels were found elevated in patients after undergoing hemodialysis for end stage renal disease, the authors of this study hypothesized that though these results may be due to oxidative stress it may instead be due to Henry's law constant (Lirk *et al* 2003). Yet, not all studies have found such positive relations between exhaled isoprene and different inflammatory states (Bajtarevic *et al* 2009, Schubert *et al* 2005, McGrath *et al* 2001) and thus the relationship between exhaled isoprene and inflammation in humans is not well defined.

There is a great need to understand the role of endogenous exhaled isoprene because previous research is inconsistent and mechanistic evidence for the biologic function of isoprene is lacking. In the present pilot study we aimed to investigate the relationship of isoprene in exhaled breath with FE_{NO} to determine if exhaled isoprene may also have potential as a biomarker of airway inflammation. We hypothesized that exhaled isoprene would be positively associated with FE_{NO}. Given our previous findings that the pro-oxidant air pollutant outdoor ozone (O₃) is positively associated with FE_{NO}, (Delfino *et al* 2010), we also aimed to investigate the relation of O₃ to both FE_{NO} and exhaled isoprene in the present study population.

Methods

Population and design

Data were derived from our study to evaluate the pro-inflammatory and cardiovascular effects of air pollution exposure in four senior assisted living communities (Delfino *et al* 2010). The present study collected supplemental data from that larger cohort in one of the retirement communities where we collected exhaled breath to analyze hydrocarbons using high resolution methods (described in methods below). For the present study, 16 elderly subjects were recruited from a senior assisted living community in Riverside, California. In order to be included in the study, the subject had to be 65 years or older, a nonsmoker, without tobacco smoke exposure at home, and with a confirmed history of coronary artery disease (CAD). Those with a diagnosis of CAD were included in the original study as this is a population at potentially high risk from the cardiovascular effects of air pollutants, which was the focus of the parent study. An earlier analysis of FE_{NO} in the larger cohort panel of 60 subjects found positive associations between FE_{NO} and markers of exposure to photochemical oxidant air pollution, including outdoor ozone (Delfino *et al* 2010). FE_{NO} was thus available for evaluation of exhaled isoprene as a potential exhaled breath marker of inflammation. There were 27 volunteers for the retirement community in the present analysis of exhaled breath isoprene, of which five were not eligible according the above stated criteria and six either dropped out or had insufficient or invalid biomarker data, leaving 16 subjects.

The subjects were followed for six weeks in two seasonal periods, for a total of 12 weeks. Measurements were collected

once at the same time of day on Fridays of each of the 12 monitored weeks. The overall average number of weeks per subject was 10 and the range was 6–12. The first phase was in September and October 2006, which are warmer months for the study region. The second phase was in January and February 2007, which are cooler months. These two distinct seasonal periods were chosen to enhance differences in photochemical activity where warmer months have higher photochemically-related air pollutants such as outdoor ozone compared to cooler months.

Exhaled NO measurements

Subjects were instructed to refrain from exercise and food and beverage intake for 1 h prior to exhaled breath sampling. This was done to avoid any possible alterations in FE_{NO} levels that may result from these activities. We collected FE_{NO} using standard offline procedures specified by the American Thoracic Society and European Respiratory Society (2005) and described by us in more detail elsewhere (Delfino *et al* 2010). Subjects performed a slow vital capacity maneuver into an offline apparatus (Sievers Deadspace Discard Bag Collection & Sampling Kit, Ionics Inc., Boulder, CO) that was attached to a non-reactive 1.5 L Mylar reservoir bag after venting approximately 200 ml of dead-space air. The exhalation flow rate was 100 ml s^{-1} , and was controlled by having the subjects maintain a constant breath pressure of 19 cm H_2O by monitoring a needle on a pressure gauge. To control for inspired ambient NO, an NO/ NO_2 chemisorbent filter was placed at the air intake of the apparatus, and subjects breathed through it for 15 s (≥ 2 tidal breaths) before sampling. Furthermore, to control for nasal NO and ambient NO, a nose clip was used to prevent unfiltered air from entering the lungs. Subjects were instructed to keep their mouth tightly around the mouthpiece at all times until the end of test. Samples were collected in triplicate. An indoor air sample was collected using the same equipment and concurrent with the breath sample to assess influence of indoor NO on exhaled NO (there was no influence). Refrigerated (6°C) sealed Mylar bags were analyzed within 20 h for FE_{NO} concentration with a chemiluminescence NO analyzer (NOATM 280i Sievers, GE Analytical Instruments, Boulder, CO). Exhaled NO pairs that were within two SD of the within-individual population average were averaged and retained for analysis.

Breath samples were collected in electropolished 1.9 L stainless steel canisters that were fitted with Swagelok Nupro metal Bellow valves (see figure 1). Simultaneous samples of room air for hydrocarbon measurements were taken because levels of hydrocarbons in air can vary greatly during a short period of time and exhaled sample levels of hydrocarbons need to be corrected for inspired levels. Samples of exhaled breath were taken in the same centrally ventilated rooms in both phases of the study. Subjects were directed to take three deep breaths, each time exhaling slowly through the tube, before filling lungs to total capacity and exhaling into the evacuated canister. In order to avoid upper airway contamination of the sample, the initial 2 s of the exhaled breath was bypassed at the canister valve and not collected.

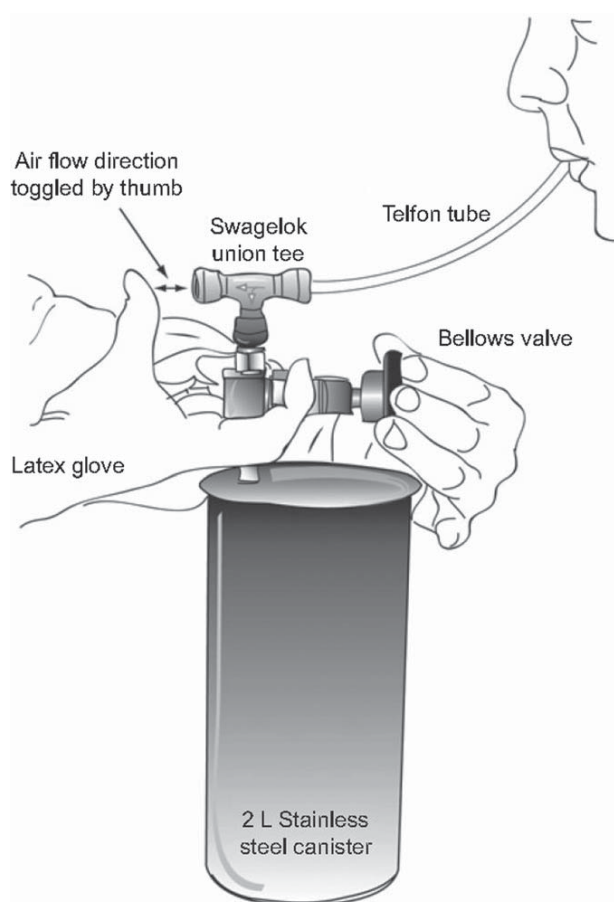


Figure 1. Diagram of a hydrocarbon/ CO_2 breath sampling apparatus (Adapted from Gorham *et al* (2009)).

Exhaled breath and indoor air hydrocarbon measurements

Breath and room samples were analyzed for hydrocarbons by cryogenic preconcentration and injection (264 cm^3 at STP) into a multi-column/detector chromatography system. This system employed the use of two flame ionization detectors (FID), two electron capture detectors (ECD), and a quadrupole mass spectrometer. Trace gases including exhaled isoprene were quantified using five different column/detector combinations: DB-1/FID, PLOT+DB-1/FID, Restek1701/ECD, DB-5+Restek1701/ECD and DB-5 ms/MSD. CO_2 concentrations in breath and room samples were measured using a separate GC system. The samples were analyzed within two weeks of sampling. We have done canister stability studies for a variety of gases, isoprene included, and the loss rate for exhaled isoprene was $<1\%$ /week.

Exhaled CO_2 was used to normalize exhaled isoprene levels, which along with subtracting room isoprene, produced an estimate of endogenously produced exhaled isoprene. For analysis of CO_2 , aliquots of breath samples were injected onto a Carboxphere 80/100 packed column output to a thermal conductivity detector. Alveolar gradient concentrations of exhaled gases were calculated by subtracting the background room concentration from the corresponding breath concentration.

Exhaled isoprene levels were expressed as:

$$(\text{ppt/ppm CO}_2) = (\text{isoprene.breath} - \text{isoprene.room}) / \text{CO}_2 \text{ breath.}$$

To further understand the relation between exhaled isoprene and FE_{NO} , we evaluated the possible role of outdoor ozone. Previous studies in a larger cohort of 60 subjects from which the present study was derived found a positive association between FE_{NO} and outdoor ozone (Delfino *et al* 2010). Although no similar data exists to our knowledge in animal cells, it is of interest that plants release higher concentrations of isoprene when exposed to ozone, thus limiting oxidant damage to their cells (Velikova *et al* 2005, 2008).

Hourly outdoor ozone measurements were made at the retirement community where subjects resided. Federal reference methods were used to measure ozone in a mobile air monitoring station located at the outdoor home environment of the community.

Statistical analysis

Exhaled NO had a skewed distribution and was log transformed to normalize its distribution. Analyses of the relationships between biomarkers of interest (FE_{NO} in relation to exhaled isoprene) were done using linear mixed-effects models allowing a multivariate analysis with individual repeated measures, which are not independent. The focus of the models is on within-individual exposure-response relations. A generalized linear mixed-effects model has fixed effect regression coefficients as well as an additional random-effects intercept to account for the clustered within-subject observations.

Exhaled isoprene and FE_{NO} levels were analyzed in relation to each other and both in relation to outdoor community levels of 24 h average ozone over the five-days leading up to the breath measurements. We adjusted for splines of the 24 h average of temperature before each breath measurement. This type of smoothing spline was implemented to fit a curve to nonlinear data as can happen with hourly to daily variations in temperature. To account for the possibility of subject outliers influencing the data, analysis of FE_{NO} predicted by exhaled isoprene was repeated after excluding two influential subject clusters that were identified in residual analyses (discussed below). All regression coefficients were standardized to the interquartile (25th–75th) percentile range (IQR) of each predictor variable.

Results

Table 1 shows descriptive statistics for study subjects consisting of 13 males and three females, all of Caucasian descent. All subjects had a confirmed history of CAD and were non-smokers. None of the participants had a confirmed history of diabetes mellitus or asthma; however, one patient did have a diagnosis of chronic obstructive pulmonary disease. Half of the subjects were on statins.

Table 1. Demographics of study population.

Variable	Value ^a
Age (years); mean $\pm$ SD	83 $\pm$ 4
Sex	
Males	13 (81)
Females	3 (19)
BMI; mean $\pm$ SD	26.6 $\pm$ 3.23
Past smokers ^b	7 (44)
Cardiovascular history	
Myocardial infarction	6 (38)
Congestive heart failure	0
Hypertension	8 (50)
Hypercholesterolemia	12 (75)
Medications	
Angiotensin-converting enzyme inhibitor	6 (38)
Statins	8 (50)
Platelet inhibitors	6 (38)

^a Values are number (%), unless otherwise indicated.

^b All subjects are nonsmokers.

Descriptive data of our variables of interest (exhaled isoprene, FE_{NO} , and 24 h outdoor ozone) are provided in table 2. Exhaled isoprene was normally distributed. As stated in the methods section, FE_{NO} was skewed in an approximately log normal distribution. The intra-subject coefficient of variation for breath CO_2 (not shown) was as little as 5% in one subject (subject average of $4.42\% \pm 0.24$) and as large as 22% in another subject ($3.64\% \pm 0.79$). This suggests that our breath sampling method was fairly repeatable within each subject based on the stability of exhaled CO_2 , although some week-to-week variation is expected, thus justifying the CO_2 correction.

In mixed model analyses we found an inverse relation between FE_{NO} and exhaled isoprene, adjusted for 24 h average temperature splines up to the time of breath measurements. The per cent decrease in FE_{NO} predicted by an interquartile range increase in exhaled isoprene was 9.9% with a 95% confidence interval of (−16.9, −2.9), p -value < 0.006, where the per cent change in FE_{NO} is calculated from $[e^{(\beta \times \text{IQR})} - 1] \times 100\%$. In analysis of influential clusters for $\ln(\text{FE}_{\text{NO}})$ predicted by exhaled isoprene, we identified two influential subject clusters. When one subject cluster was removed it decreased the magnitude of association from −9.9% to −7.4%, but was still significant ($p < 0.04$, 95% CI: −14.1%, −0.7%). On the other hand, removal of the other subject cluster increased the magnitude to −14.2% as well as the significance of association ($p < 0.0001$, 95% CI: −20.6%, −7.9%). Therefore, the inverse relationship between FE_{NO} and exhaled isoprene remained even after removal of these opposing subject clusters.

A limitation of the breath sampling method is the possibility that residual isoprene in the airways from ambient air is not completely accounted for by subtracting background room concentrations. Therefore, as discussed in the Methods section exhaled isoprene was normalized to exhaled CO_2 (we prefer to express results directly as exhaled isoprene ppt/ppm CO_2 using the ratio ppt/ppm) to obtain an estimate of endogenously produced exhaled isoprene. This resulted in the reduction of isoprene variability by ~10–20% in most cases. Therefore, we performed a sensitivity analysis using uncorrected exhaled isoprene (but still subtracting background

Table 2. Distribution of variables.

Variables	N	Mean (SD)	25th% tile	Median	75th% tile
Indoor Isoprene (ppb)	163	1.58 (0.81)	1.01	1.23	2.06
Exhaled Raw Isoprene (ppb)	163	97.5 (47.4)	62.9	87.7	122.3
Exhaled Corrected ^a Isoprene (ppt/ppm CO ₂)	163	2.17 (0.89)	1.53	2.09	2.70
Exhaled NO (ppb)	159	19.8 (9.75)	14.6	16.4	20.9
24 h ozone (ppb)	163	31.0 (7.92)	23.1	33.9	35.8

^a Corrected for indoor isoprene and exhaled CO₂ (see text).**Table 3.** Linear mixed-effects model for change in isoprene (ppt/ppm CO₂) predicted by an interquartile range (25th–75th percentile)^a increase in 24 h average outdoor home ozone.^b

Ozone multi-day averaging times	Effect estimate (95% confidence limit)	p-value
One-day	−0.284 (−0.499, −0.068)	0.0102
Two-day	−0.455 (−0.751, −0.160)	0.0028
Three-day	−0.395 (−0.688, −0.103)	0.0085
Five-day	−0.352 (−0.621, −0.083)	0.0107

^a 12.7 ppb ozone.^b Adjusted for splines of 24 h average temperature of the same multi-day averaging time.**Table 4.** Linear mixed effects for per cent change in exhaled NO predicted by interquartile range (25th–75th percentile)^a increase in 24 h average outdoor home ozone.^b

Ozone multi-day averaging times	Per cent change in eNO (95% confidence limit)	p-value
One-day	10.6 (1.2, 20.1)	<0.05
Two-day	22.9 (5.6, 40.2)	<0.05
Three-day	25.6 (10.5, 40.8)	0.001
Five-day	24.1 (10.3, 37.8)	0.001

^a 12.7 ppb ozone.^b Adjusted for splines of 24 h average temperature of the same multi-day averaging time.

room isoprene) to determine if correcting for CO₂ affected the regression results presented above. The regression results for uncorrected exhaled isoprene showed a major decrease in the per cent change of FE_{NO} predicted by an interquartile range increase in isoprene that was only borderline significant (−1.3%, 95% CI: −2.8, 0.13) indicating that our approach may have reduced a major source of error in sample collection.

Table 3 describes the results for exhaled isoprene predicted by outdoor community ozone measurements. There was an inverse relation between exhaled isoprene and outdoor ozone levels averaged across different numbers of days, all of which were statistically significant. Table 4 describes the results for changes in FE_{NO} predicted by 24 h average outdoor community ozone measurements adjusted for splines of 24 h average temperature. Unlike the relation of FE_{NO} with exhaled isoprene, FE_{NO} and outdoor ozone were positively associated. There also appears to be a multi-day cumulative increase in the magnitude of the relation between FE_{NO} and ozone up to the three-day average of ozone.

Given that ozone is associated with both exhaled isoprene and FE_{NO}, it is possible that ozone could confound the inverse relationship that was found between exhaled isoprene

and FE_{NO}. Therefore we compared models unadjusted and adjusted for ozone (figure 2). The inverse relationship between FE_{NO} and exhaled isoprene changed only nominally (<10%) and remained statistically significant. The positive relationship of FE_{NO} with ozone remained as well with exhaled isoprene in the model (<10% change).

As discussed previously, it has been hypothesized that use of statins is associated with a decrease in isoprene levels in exhaled breath (Karl *et al* 2001, Stone *et al* 1993). In order to test this hypothesis with our data we performed a linear mixed effect model for change in exhaled isoprene predicted by statins. We unexpectedly found that compared with subjects not taking statins, subjects taking statins had an overall higher exhaled isoprene level of 0.32 ppt ppm^{−1} CO₂ (*p*-value < 0.05, 95% CI: 0.0003, 0.62).

Discussion

The objective of our pilot study was to determine if isoprene in exhaled breath may be a potential clinical biomarker of airway inflammation by assessing its relationship with FE_{NO}, which has already been established as a biomarker of airway inflammation. Interestingly, we observed that instead of a positive relation between exhaled isoprene and FE_{NO}, an inverse relation was found in this group of retirement community subjects with CAD. These results remained significant when outdoor ozone was included in the regression model. Exhaled isoprene was also found to have an inverse relationship with 24 h multi-day averages of outdoor ozone. FE_{NO}, as predicted, had a positive relationship with one-day through five-day average 24 h outdoor ozone measurements. This finding is consistent with previous studies that have observed a positive relation of FE_{NO} and ambient ozone in children (Berhane *et al* 2011, Delfino *et al* 2006) and in elderly people (Adamkiewicz *et al* 2004, Delfino *et al* 2010). One of these studies (the larger cohort for the present study) additionally found that FE_{NO} was positively associated with both outdoor ozone levels and markers of secondary organic chemicals in PM suggesting oxidized air pollutants in general were important to airway inflammatory responses (Delfino *et al* 2010). Using the same breath hydrocarbon measurements as the present study, Gorham *et al* (2009) reported that repeated weekly measurements of ethane and n-pentane were not associated with FE_{NO} but were associated with concentrations of air pollutant gases (NO, NO₂ and CO) in the indoor and outdoor community environment. We concluded that ethane and n-pentane were more likely to be biomarkers of air

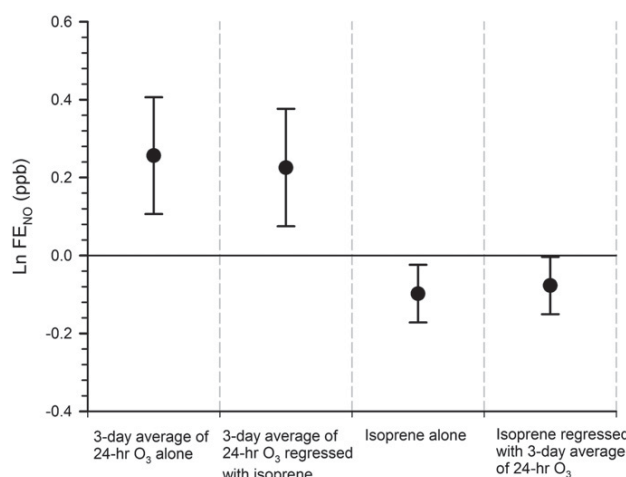


Figure 2. Per cent changes of natural log transformed FE_{NO} in relation to concurrent isoprene and to the three-day average of 24 h average outdoor O₃: single predictor and two-predictor models. All models are adjusted for splines of three-day average temperature.

pollutant exposures not biomarkers of airway inflammation as previously believed.

Speculations regarding these preliminary findings

As previously cited, studies have observed that isoprene may protect plants against short term temperature changes and exposure to ozone. The antioxidant effect of exhaled isoprene may be due to a direct reaction with ozone and other reactive oxygen species in the gas phase (Logan *et al* 2000, Loreto and Velikova 2001). Isoprene has also been reported to quench NO either by direct scavenging of NO or ONOO⁻ or by lowering the oxidative burst resulting in less NO (Velikova *et al* 2005, Velikova *et al* 2008, Sharkey and Yeh 2001). This may have led to the reduced damage observed in ozonated leaves by modifying NO-triggered hypersensitivity and cell death responses after ozone exposure (Sharkey and Yeh 2001).

In contrast to our finding of a negative relation between outdoor ozone and exhaled isoprene, an experimental study found that in six human subjects exposed experimentally to high ozone (to 350 ppb) during exercise, isoprene increases significantly 19 ± 1 h after ozone exposure as compared with filtered air (Foster *et al* 1996). These ozone levels are a magnitude higher than the present study. Authors hypothesized that damage to cell membranes by ozone could be followed by increased sterol synthesis and compensatory increases in isoprene.

We speculate that in humans, isoprene may also play a role as an antioxidant. Like in plants, isoprene may scavenge for reactive oxygen species. In particular it may react with H₂O₂ and peroxynitrite (ONOO⁻) in the lungs, which are the products of the oxidation of NO by superoxide anion (O₂⁻). This may explain why research on isoprene in humans has found that exhaled isoprene levels correlate with acute inflammation. Subjects who had acute myocardial infarctions, subjects immediately after sternotomy from cardiac surgery, and subjects with a higher cardiac index have been found to

have elevated exhaled isoprene levels (Mendis *et al* 1994, Pabst *et al* 2007). Patients who were diagnosed with head and neck tumors were also found to have elevated levels of isoprene in exhaled breath compared to subjects in a control group, a high risk group, as well as a post therapy group (Schmutzhard *et al* 2008).

The function of isoprene may also be explained in terms of a chemical reaction in humans rather than a biological mechanism. Isoprene reacts with the OH radical, NO₃ radical, Cl radical, and O₃. The OH-radical initiated oxidation of isoprene is responsible for 50–100% of ambient air ozone production attributed to VOC oxidation in the United States. In addition to ozone, this oxidation of isoprene in the atmosphere is responsible for a significant portion of secondary organic aerosol formation. These reactions can cause NO to form alkoxy radicals in the presence of molecular oxygen. Through a series of experiments, it has been demonstrated that there is OH cycling in isoprene oxidation. There is a time-dependent formation of OH as a function of O₂ and NO. Thus, oxidation of isoprene in the presence of O₂ is dependent on NO concentration (Ghosh *et al* 2010).

Origination of exhaled isoprene

As stated earlier in the introduction, via experimental modeling, exhaled isoprene has been shown to originate endogenously in humans. This was demonstrated in a studies performed by King *et al* (2009 and 2010) in which real time measurements of exhaled isoprene were collected under ergometer challenges. Although isoprene is involved in cholesterol biosynthesis, their results support that there is a peripheral tissue source of isoprene in humans which is linked to local gas exchanges. Both studies performed ergometer challenges using leg exercises (pedaling). The latter of the two studies found a rise and peak of exhaled isoprene that correlated with cardiac output, alveolar ventilation, and ventilation perfusion ratio. When both legs were exercised, exhaled isoprene levels decreased and leveled off to baseline levels during the exercise. This indicates that there may possibly be a reserve of isoprene that became depleted. However, when only one leg was used at a time, another peak of exhaled isoprene was noted when subjects switched their legs. The authors note that the exact tissue groups involved in the origin of exhaled isoprene remains unknown but hypothesize that the skeletal locomotor muscles and/or the vascular tree are involved due to high blood flow during exercise (King *et al* 2010). Since our subjects were at rest at each and every weekly repeated measurement, this source of exhaled isoprene should not have affected our results.

Limitations

Although our study had a limited number of subjects we were able to collect repeated measures for each subject for our statistical analysis in which essentially each subject acts as their own control over time. Nevertheless, the sample size of 16 subjects limits the generalizability of the results. As stated previously, numerous studies have proposed that isoprene is a by-product of mevalonate biosynthesis. The

small number of subjects using statins (eight subjects) did limit our ability in analyzing between-subject differences in exhaled isoprene levels predicted by statin use and may explain our non-significant and unexpected results showing generally higher levels of exhaled isoprene among subjects taking statins. Another limitation of this study is that even though we had outdoor air samples to measure ozone levels we did not have personal exposure assessments. Personal exposure assessments allow a more accurate measurement of exposure of an individual. Given the potential influence of physical activity on isoprene levels, another limitation of the present study is the lack of activity monitoring data. This in turn, could have also biased our results although all subjects were tested at rest during the exhaled breath testing after sitting in the clinic intake area. Also, although subjects may have hyperventilated while providing a breath sample, it is unlikely since we had the subjects breath in and out deeply three times and then blow out slowly. Furthermore, intra-individual CO₂ values as described in the methods section were found to be fairly consistent and if a subject was hyperventilating, they would have done that with each weekly measurement. As mentioned above, normalizing isoprene by CO₂ reduced the overall coefficient of variation by 15.7% (10–20% in most cases). Furthermore, removing the normalization of isoprene by CO₂ led to a much weaker association with FE_{NO} that was only borderline significant. This is likely to have occurred because of the increased variance of raw exhaled isoprene compared with the CO₂-normalized data. The implications are that other studies not performing this adjustment may have been biased. Limitations in the FE_{NO} data are as follows. We discarded 200 mL of dead-space air before collection of breath samples for the collection of exhaled NO, but the appropriate volume of gas to be discarded is not known and likely varies from subject to subject (ATS/ERS 2005). Also, it is possible that some contamination from sinus NO occurred despite the venting of deadspace air and use of a nose clip.

Future research

Our results have shown interesting associations of exhaled isoprene with FE_{NO} in this cohort panel, but it is difficult to extrapolate this information to a broader population because our cohort is a narrow subset of the general population. As stated in the methods section, this is a cohort of elderly subjects in an assisted living area who all have a diagnosis of CAD and are non-smokers. A similar study among a broader population, those consisting of a mixture of subjects in different age groups and with different co-morbidities (especially asthma) as well as those who are healthy, would be valuable to see if these results could be reproduced. This would help to support or contradict (as with the presented results) our original hypothesis, that exhaled isoprene would be positively associated with FE_{NO} and may thus be a potential biomarker of inflammation.

Conclusions

Our results confirm a previous study in the larger 60-subject cohort that found FE_{NO} to rise with increasing outdoor ozone air pollution (Delfino *et al* 2010). From these pilot

data we found no supportive data that isoprene could be considered as a positive biomarker of airway inflammation due to the inverse relationship between exhaled isoprene and FE_{NO}. However, a significant inverse association between exhaled isoprene and FE_{NO} as well as an inverse association between exhaled isoprene and outdoor ozone suggests some potentially important chemical reactions may be occurring in the lungs. These findings also suggest interesting mechanisms as discussed regarding the potential antioxidant function of isoprene in humans although this is purely speculative. Finally, we speculate that since FE_{NO} may be used to help diagnose asthma and to monitor asthma status in certain cases as discussed, it is conceivable that isoprene may confound that relation in some individuals with high or variable exhaled isoprene levels. The potential biochemical effects of isoprene could also have implications in the individual variation in acute asthma severity. Further research to confirm or reject the present preliminary findings is suggested, particularly in populations of subjects with and without asthma.

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References

- Adamkiewicz G, Ebelt S, Syring M, Slater J, Speizer F E, Schwartz J, Suh H and Gold D R 2004 Association between air pollution exposure and exhaled nitric oxide in an elderly population *Thorax* **59** 204–9
- American Thoracic Society (ATS) and European Respiratory Society (ERS) 2005 ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide *Am. J. Respir. Crit. Care Med.* **171** 912–30
- Bajtarevic A *et al* 2009 Noninvasive detection of lung cancer by analysis of exhaled breath *BMC Cancer* **9** 348
- Berhane K, Zhang Y, Linn W S, Rappaport E B, Bastain T M, Salam M T, Islam T, Lurmann F and Gilliland F D 2011 The effect of ambient air pollution on exhaled nitric oxide in the children's health study *Eur. Respir. J.* **37** 1029–36
- Delfino R J, Staimer N, Gillen D, Tjoa T, Sioutas C, Fung K, George S C and Kleinman M T 2006 Personal and ambient air pollution is associated with increased exhaled NO in children with asthma *Environ. Health Perspect.* **114** 1736–43

- Delfino R J, Staimer N, Tjoa T, Arhami M, Polidori A, Gillen D L, George S C, Shafer M M, Schauer J J and Sioutas C 2010 Associations of primary and secondary organic aerosols with airway and systemic inflammation in an elderly panel cohort *Epidemiology* **21** 892–902
- Deneris E S, Stein R A and Mead J F 1985 Acid-catalyzed formation of isoprene from a mevalonate-derived product using a rat liver cytosolic fraction *J. Biol. Chem.* **260** 1382–5
- Dweik R, Boggs P B, Erzurum S C, Irvin C G, Leigh M W, Lundberg J O, Olin A C, Plummer A L and Taylor D R 2011 An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FeNO) for clinical applications *Am. J. Respir. Crit. Care Med.* **184** 602–15
- Fenske J D and Paulson S E 1999 Human breath emissions of VOCs *J. Air Waste Manage. Assoc.* **49** 594–8
- Foster W M, Jiang L, Stetkiewicz P T and Risby T H 1996 Breath isoprene: temporal changes in respiratory output after exposure to ozone *J. Appl. Physiol.* **80** 706–10
- Ghosh B, Bugarin A, Connell B T and North S W 2010 Isomer-selective study of the OH-initiated oxidation of isoprene in the presence of O₂ and NO: the major OH addition channel *J. Phys. Chem.* **114** 2553–60
- Gorham K, Sulbaek Andersen M P, Meinardi S, Delfino R J, Staimer N, Tjoa T, Rowland F S and Blake D R 2009 Ethane and n-pentane in exhaled breath are biomarkers of exposure not effect *Biomarkers* **14** 17–25
- Jones S L, Kittelson J, Cowan J O, Flannery E M, Hancox R J, McLachlan C R and Taylor D R 2001 The predictive value of exhaled nitric oxide measurements in assessing changes in asthma control *Am. J. Respir. Crit. Care Med.* **164** 738
- Karl T, Prazeller P, Mayr D, Jordan A, Rieder J, Fall R and Lindinger W 2001 Human breath isoprene and its relation to blood cholesterol levels: new measurements and modeling *J. Appl. Physiol.* **91** 762–70
- Kamboures M A, Blake D R, Cooper D M, Newcomb R L, Barker M, Larson J K, Meinardi S, Nussbaum E and Rowland F S 2005 Breath sulfides and pulmonary function in cystic fibrosis *Proc. Natl Acad. Sci. USA* **102** 15762–7
- King J, Koc H, Unterkofler K, Mochalski P, Kupferthaler A, Teschl G, Teschl S, Hinterhuber H and Amann A 2010 Physiological modeling of isoprene dynamics in exhaled breath *J. Theor. Biol.* **267** 626–37
- King J, Kupferthaler A, Unterkofler K, Koc H, Teschl S, Teschl G, Miekisch W, Schubert J, Hinterhuber H and Amann A 2009 Isoprene and acetone concentration profiles during exercise on an ergometer *J. Breath Res.* **3** 027006
- Kleeman R and Kooistra T 2005 HMG-CoA reductase inhibitors: effects on chronic subacute inflammation and onset of atherosclerosis induced by dietary cholesterol *Curr. Drug Targets – Cardiovas. Haematol. Disord.* **5** 441–53
- Likhodii S, Musa K and Cunnane S C 2002 Breath acetone as a measure of systemic ketosis assessed in a rat model of the ketogenic diet *Clin. Chem.* **48** 115–20
- Lirk P, Bodrogi F, Raifer H, Greiner K, Ulmer H and Rieder J 2003 Elective haemodialysis increases exhaled isoprene *Nephrol. Dial. Transplant.* **18** 937–41
- Logan B A, Monson R K and Potosnak M J 2000 Biochemistry and physiology of foliar isoprene production *Trends Plant Sci.* **5** 477–81
- Loreto F and Velikova V 2001 Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes *Plant Physiol.* **127** 1781–7
- McGrath L T, Patrick R and Silke B 2001 Breath isoprene in patients with heart failure *Eur. J. Heart Fail.* **3** 423–7
- Mendis S, Sobotka P and Euler D 1994 Expired hydrocarbons in patients with acute myocardial infarction *Free Radic. Res.* **23** 117–22
- Novak B J, Blake D R, Meinardi S, Rowland F S, Pontello A, Cooper D M and Galassetti P R 2007 Exhaled methyl nitrate as a noninvasive marker of hyperglycemia in type 1 diabetes *Proc. Natl Acad. Sci.* **104** 15613–1
- Pabst F, Miekisch W, Fuchs P, Kischkel S and Schubert J K 2007 Monitoring of oxidative and metabolic stress during cardiac surgery by means of breath biomarkers: an observational study *J. Cardiothorac. Surg.* **2** 37
- Pleil J D and Lindstrom A B 1997 Exhaled human breath measurement method for assessing exposure to halogenated volatile organic compounds *Clin. Chem.* **43** 723–30
- Salerno-Kennedy R and Cashman K D 2005 Potential applications of breath isoprene as a biomarker in modern medicine: a concise overview *Wien Klin. Wochenschr.* **117** 180–6
- Schmutzhard J, Rieder J, Deibl M, Schwentner I M, Schmid S, Lirk P, Abraham I and Gunkel A R 2008 Pilot study: volatile organic compounds as a diagnostic marker for head and neck tumors *Head Neck* **30** 743–9
- Schubert J K, Miekisch W, Birken T, Geiger K and Nöldge-Schomburg G F 2005 Impact of inspired substance concentrations on the results of breath analysis in mechanically ventilated patients *Biomarkers* **10** 138–52
- Sehnert S S, Jiang L, Burdick J F and Risby T H 2002 Breath biomarkers for detection of human liver diseases: a preliminary study *Biomarkers* **7** 174–87
- Senthilmohan S T, McEwan M J, Wilson P F, Milligan D B and Freeman C G 2001 Real time analysis of breath volatiles using SIFT-MS in cigarette smoking *Redox Rep.* **6** 185–7
- Sharkey T and Yeh S 2001 Isoprene emission from plants *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52** 407–36
- Stone B G, Besse T J, Duane W C, Evans C D and DeMaster E G 1993 Effect of regulating cholesterol biosynthesis on breath isoprene excretion in men *Lipids* **28** 705–8
- Taylor D R 2012 Advances in the clinical applications of exhaled nitric oxide measurements *J. Breath Res.* **6** 047102
- Velikova V, Fares S and Loreto F 2008 Isoprene and nitric oxide reduce damages in leaves exposed to oxidative stress *Plant Cell Environ.* **31** 1882–94
- Velikova V, Pinelli P, Pasqualini S, Reale L, Ferranti F and Loreto F 2005 Isoprene decreases the concentration of nitric oxide in leaves exposed to elevated ozone *New Phytologist* **166** 419–25
- Watson W, Cottrell L, Zhang D and Golding B 2001 Metabolism and molecular toxicology of isoprene *Chem. Biol. Interact.* **135–6** 223–38