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Breath isoprene – aspects of normal physiology related to age, gender and cholesterol profile as determined in a proton transfer reaction mass spectrometry study

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Abstract

Background: This study was performed to clarify variations in breath isoprene concentrations with age, gender, body mass index (BMI) and total serum cholesterol. Our cohort consisted of 205 adult volunteers of different smoking background without health complaints. Total cholesterol in blood serum was measured in 79 of these volunteers.
Methods: Mixed expiratory exhaled breath was sampled using Tedlar bags. Concentrations of isoprene were then determined using proton transfer reaction-mass spectrometry.
Results: Isoprene concentrations ranged from 5.8 to 274.9 ppb, with an overall geometric mean (GM) of 99.3 ppb. There was no statistically significant difference in mean isoprene in breath between males and females (GM 105.4 and 95.5 ppb, respectively). Ageing led to a decrease in concentration in men, with an estimated slope of the regression line for log-transformed isoprene concentrations of –0.0049, but did not influence isoprene levels in women. We did not observe any significant correlation between isoprene breath content and cholesterol level in blood, even after adjusting for the possible influence of age. Similarly, no correlation was found between isoprene levels and BMI.
Conclusions: Isoprene concentrations in exhaled breath showed gender-specific correlations with respect to age. Further investigations are necessary to clarify the relation between isoprene concentrations in exhaled breath and cholesterol levels and synthesis rates in blood.
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Keywords: age; body mass index; breath analysis; gender; isoprene; proton transfer reaction-mass spectrometry (PTR-MS); serum total cholesterol.

Introduction

In recent years, rapid and precise determination of volatile organic compounds (VOCs) in human breath has become possible with accuracy and reproducibility sufficient to provide new non-invasive diagnostic tests (1–7). The main techniques for exhaled breath analysis are gas chromatography with mass spectrometric detection (GC-MS) (3, 8), proton transfer reaction-mass spectrometry (PTR-MS) (7, 9–14), selected ion flow tube mass spectrometry (SIFT-MS) (15, 16), laser spectrometry (17–19), ion mobility spectrometry (20, 21), photoacoustic spectrometry and sensor techniques (22).

Nitric oxide (NO) is a recent example of a volatile compound now used in clinical practice, e.g., for the screening of asthma (23–25). It can be measured using a relatively cheap chemical sensor (sensor cost ~€100) in short measurement times of ~2 min. Typical research devices for exhaled breath analysis are much more expensive, but are much more versatile. A modern proton transfer reaction time-of-flight mass spectrometer (PTR-TOF-MS), for example, may measure hundreds of mass-to-charge ratios (corresponding to hundreds of compounds) within ~1 s. PTR-MS and SIFT-MS also allow on-line measurement of exhaled breath during continuous sampling (4, 5, 13, 26, 27). Apart from determining endogenous compounds produced by the human or animal body, exhaled breath analysis can also be used to measure metabolites of ^{13}C-labelled compounds administered to patients or volunteers (28, 29). This is particularly applied to clinical diagnosis of infection with *Helicobacter pylori*, whereby a small dose of ^{13}C-labelled urea (metabolised in the stomach by this bacterial species) is

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administered and the $^{13}CO_2/^{12}CO_2$-ratio in exhaled breath is then measured.

When introducing these technical achievements into the clinical setting, it is important to know age- and gender-related peculiarities as well as the biochemical origins of breath VOCs as biomarkers for the diagnosis and monitoring of human diseases.

Isoprene (2-methyl-1,3-butadiene) is the major hydrocarbon contained in exhaled human breath (30) and is normally detected in exhaled air at levels ranging from a few parts per billion (ppb) to a few hundred ppb (31, 32). Although breath isoprene is of endogenous origin (30, 33–39) and is not produced within the airways (40), we do not know all its different biochemical sources. Different evidence suggests that isoprene is a by-product of cholesterol biosynthesis (35–37, 41–49). Small amounts of this VOC may arise from peroxidation of squalene, a cholesterol precursor (41).

Administration of lipid-lowering (statin) therapy results in a proportional decrease in breath isoprene and plasma total cholesterol concentrations in healthy volunteers (43, 49) and intensive care patients (48). On the other hand, a cholesterol-rich diet also decreases isoprene levels in exhaled breath (43) due to feedback inhibition of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) (50, 51). Impaired synthesis of cholesterol and intermediary metabolites of its biosynthesis (isoprene, squalene, lanosterol and lathosterol), together with malnutrition and energy deficiency, causes symptomatic hypocholesterolaemia in many acute conditions such as injury, severe infection associated with sepsis and septic shock, post-operative syndrome, myocardial infarction and neoplastic diseases (52).

In view of the above-mentioned findings, breath isoprene might be considered as a biological marker for monitoring lipid and cholesterol status and the efficiency of lipid-lowering therapy. The development of a new non-invasive diagnostic test complementary to invasive blood tests for control of cholesterol levels is highly attractive. However, it has not yet been demonstrated whether levels of breath isoprene correlate with the degree of cholesterolaemia, although it would be logical to suppose such relationship since breath isoprene is considered to reflect the rate of cholesterol production.

The relationships between concentrations of exhaled isoprene, age, gender and body mass index (BMI) are not yet clear and differ significantly in different studies (30, 31, 38, 39, 53–56). Therefore, the present study was performed to clarify variations in breath isoprene concentrations with age, gender, BMI and total serum cholesterol in adults.

Materials and methods

Subjects

The study cohort consisted of 205 adults of different smoking background (Table 1) who had no health complaints and attended a physician for a prophylactic health check. All subjects had fasted for 12 h prior to breath sampling. Heart and respiratory rates and blood pressure were not determined in the study subjects.

Table 1 Smoking status of the study cohort.

	Men	Women	Total
Smokers	15	30	45
Non-smokers	49	76	125
Ex-smokers	17	18	35

PTR-MS instrumentation

A high-sensitivity PTR-MS instrument (three turbopumps; Ionicon, Innsbruck, Austria) with Teflon rings instead of Viton rings was used for our measurements. The count rate for primary ions (H_3O^+) was approximately 10^7 counts s^{-1}. The dwell time was 0.5 s for each mass-to-charge ratio measured (21–230 m/z). Typical compounds used for determination of transmission coefficients were acetonitrile, acetaldehyde, acetone, dimethylsulphide, 2-butanone, benzene, toluene, p-xylene, benzaldehyde, chlorobenzene, 1,2-dichlorobenzene, and 1,2,4-trichlorobenzene. These compounds do not show fragmentation of their protonated form. Concentrations of these compounds were chosen in a range leading to a ~10% reduction in primary counts, with subsequent observation of recovery of primary ion counts measured at m/z = 21 and the specific mass-to-charge ratio of the corresponding non-fragmenting compound. The length of the drift tube of our PTR-MS is 9.3 cm, with an applied voltage of 600 V. The usual pressure in the drift tube was ~2.3 mbar (with slight variations). We computed concentrations of isoprene by considering both H_3O^+ and the first water cluster $H_2O{\cdot}H_3O^+$ as primary ions. It is notable that our data show not much difference when computing isoprene concentrations considering H_3O^+ as the only primary ion, with a maximum difference of only 11 ppb.

Sample collection and MS analysis

Samples of mixed breath gas were collected in Tedlar bags (SKC Inc., Eighty Four, PA, USA) with parallel collection of ambient air (also in Tedlar bags). Breath gas samples were obtained after the subject had been seated for ~5 min. Each subject provided one or two breath samples using a straw. All samples were processed within 12 h.

The effect of storage in Tedlar bags on isoprene in exhaled breath samples is negligible (apart from water, which quickly diffuses through the walls of Tedlar bags). Acetonitrile seems to diffuse most quickly through the bag walls, with an exponential decay constant of $\tau \approx 31$ h (Herbig J, personal communication). Isoprene diffuses more slowly through the bag walls and its concentration remains almost constant during 12 h.

Isoprene concentrations were determined by PTR-MS using the peak at m/z 69. PTR-MS allows on-line monitoring of VOCs with volume mixing ratios as low as a few parts per trillion (57, 58). In the present study, each sample, including samples of ambient air, was measured at least three times in the range m/z 21–230. Concentrations of isoprene related to m/z = 69 were calculated based on a ''standard'' PTR kinetic rate constant of $k = 2 \times 10^{-9}$ cm^3 s^{-1} and a calibration showing that isoprene fragments to m/z 69 for 44.6% of isoprene molecules (see Results section and Table 2). The underlying calibration series used isoprene (in pure nitrogen, 99.9999%; Linde Gas, Stadl-Paura, Austria) at concentrations of 1.2, 2.4, 12.1, 24.2, 60.6, 121.2 and 242.3 ppb. The nominal concen-

Table 2 Fragmentation pattern of isoprene in PTR-MS measurements.

m/z	Slope of the calibration line	Percentage of fragment	Remarks
39	0.1	10.1	m/z 39 might appear through expulsion of (neutral) ethane from protonated isoprene
40	0.003	0.3	m/z 40 represents the ^{13}C isotopes of m/z 39
41	0.40	39.5	m/z 41 might appear through expulsion of (neutral) ethene from protonated isoprene
67	0.017	1.7	m/z 67 might appear by reaction of the parasitic precursor ion of nitric oxide (NO^+) with isoprene with H^- abstraction
68	0.005	0.5	m/z 68 represents the ^{13}C isotopes for m/z 67
69	0.4469	44.6	m/z 69 is the main fragment used for quantification in the present study
70	0.026	2.6	Amount of m/z 70 corresponds to 5.88% of m/z 69, in accordance with the fact that isoprene has 5 carbon atoms (with an expected percentage of 5.5% related to 5 carbon atoms with 1.1% ^{13}C)
137	0.007	0.7	Might be a dimer (isoprene-isoprene-H^+)
Sum	1.003	100	

The underlying calibration series used isoprene (in pure nitrogen) at concentrations of 1.2, 2.4, 12.1, 24.2, 60.6, 121.2 and 242.3 ppb. Isoprene in exhaled breath was quantified using the peak at m/z 69 and multiplying the nominal concentration (based on a kinetic rate constant of 2×10^{-9} cm^3 s^{-1}) by a factor of $1/0.4469 = 2.24$.

tration (based on a kinetic rate constant of 2×10^{-9} cm^3 s^{-1}) at m/z = 69 was multiplied by a factor of 2.24 to obtain absolute concentrations of isoprene.

Blood cholesterol testing was undertaken at the same time as breath testing using a commercially available cholesterol test (Menarini Diagnostics, Vienna, Austria) requiring a 12-h fasting prior to blood sampling. The study was approved by the local Ethics Committee.

Data pre-processing and statistical analysis

The concentrations of isoprene (ppb) measured were log-transformed ($\log_{10}$) before any further analyses. This generates a symmetric concentration distribution and suppresses the dependence of standard deviation of within-bag concentrations measured on the mean within-bag concentration. Thus, a within-bag standard deviation may be determined. Previous studies revealed that log-transformation of VOC concentrations in breath yielded probability distributions close to a normal (Gaussian) distribution (31, 59).

The isoprene concentration of a particular sample (Tedlar bag) was calculated as the median value of all replicate values measured for that sample. The isoprene concentration for a particular subject was calculated as the median value of all available sample concentrations.

The following data pre-processing was also performed: only samples with isoprene concentrations at least twice as high as in ambient air were taken into consideration, otherwise the sample concentration may not reflect the real level of the compound in blood (59). Using this approach, only one sample had to be omitted from our data set. Moreover, one sample with an extremely large geometric standard deviation (GSD) of 3.93 was excluded from further considerations owing to possible non-stability of the measurement process (estimated within-sample GSD 1.04).

We also omitted from the data set one observation, namely, a 29-year-old male with an unusually high cholesterol level of 316 mg/dL (8.2 mmol/L) for his age. Since the study cohort was supposed to comprise adult subjects without pathologies, we excluded this subject as being non-healthy, regarding such high cholesterolaemia to be a result of an undiagnosed lipid disorder.

When examining correlation between isoprene concentrations and cholesterol or BMI, we used the partial correlation coefficient (PCC) to assess the true correlation between these quantities after eliminating the possible effect of age. PCC is used to measure the true correlation between two variables of interest (X and Y) when these are influenced by one or more other variables. In such a situation, the value of the ''simple'' correlation coefficient for X and Y may not reveal the true relationship between them. PCC can be thought of as a measure of the correlation between X and Y when keeping the values of the influencing variables fixed.

Results

The relationships between isoprene concentrations, age and gender were examined using breath samples collected from 205 adults (the smoking status of the cohort is given in Table 1). A total of 217 breath samples were analysed (12 volunteers provided 2 samples each). Only one value for m/z 69 was used for each subject (in the case of more than 1 sample per patient, a within-subject median value was used in further considerations).

Isoprene concentrations in all subjects ranged from 5.8 to 274.9 ppb, with an overall geometric mean (GM) 99.3 ppb (Figure 1). The within-sample (within-bag) GSD was 1.04 and the within-subject GSD was 1.27.

Identification of isoprene

Figure 2 plots the concentration of ions at m/z 70 against the concentration of ions at m/z 69 for 217 exhaled breath samples. The correlation coefficient is $R^2 = 0.92$. If m/z = 69 were only due to isoprene (with 5 carbon atoms) and if m/z = 70 referred only to ^{13}C isotopes of m/z = 69, we would expect a straight line with a slope of 0.055 (due to 5.5% ^{13}C isotopes). Since practically all exhaled breath samples fulfil the condition $\text{concentration}_{m/z\ 70} \geq 0.055 \times \text{concentration}_{m/z\ 69}$, we conclude that the peak at m/z = 69 does not represent any 4-carbon compounds. The Figure indicates, nevertheless, that m/z = 69 does not correspond exclusively to isoprene: a certain amount of other com-

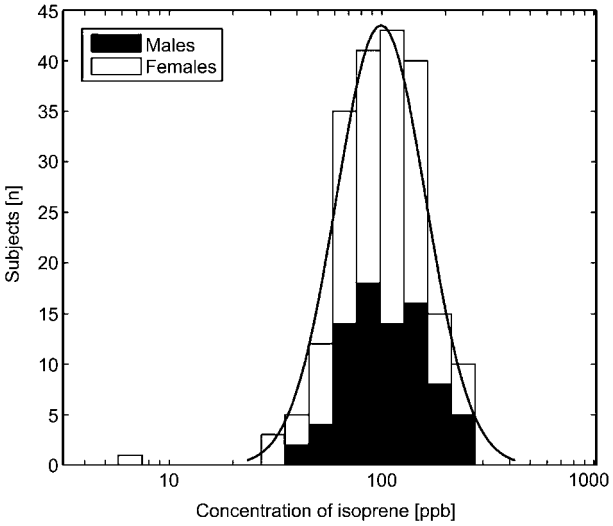

Figure 1 Isoprene concentrations (ppb) in the study cohort with fitted log-normal distribution curve.

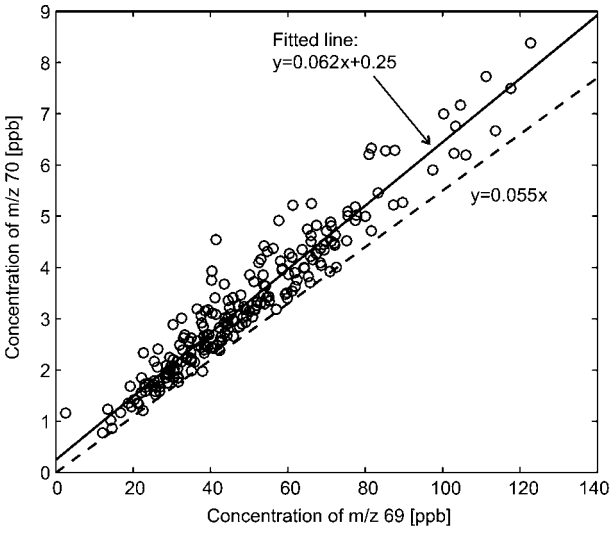

Figure 2 Concentration of ions with m/z 70 against concentration of ions with m/z 69.
The correlation coefficient is $R^2 = 0.92$. If m/z 69 were only due to isoprene (with five carbon atoms) and if m/z 70 were only due to ^{13}C isotopes of m/z 69, we would expect a straight line with a slope of 0.055 (5.5% ^{13}C isotopes). The results presented here indicate that the peak at m/z 69 does not represent any 4-carbon compounds.

pounds or fragments must also correspond to m/z = 69. Additional candidates for compounds appearing at m/z = 69 in PTR-MS measurements are cyclopentene, furan and pyrazole. These compounds are not usually observed in high concentrations (of approx. 100 ppb) in GC-MS analysis of exhaled breath. We conclude that a large proportion of the peak intensity observed at m/z = 69 is due to isoprene.

Calibration for isoprene

The fragmentation pattern of isoprene for PTR-MS measurements is presented in Table 2. The underlying calibration series used isoprene concentrations (in pure nitrogen) of 1.2, 2.4, 12.1, 24.2, 60.6, 121.2 and 242.3 ppb. The slope of the fitted calibration line for m/z = 69 vs. the prepared calibration concentration of isoprene is 0.4469. Therefore, we quantified exhaled breath measurements using m/z = 69 by multiplying the nominal concentration (based on a kinetic rate constant of 2×10^{-9} cm^3 s^{-1}) by a factor of 1/0.4469 = 2.24.

Breath isoprene, age and gender

We found no significant difference in mean levels of (log-transformed) breath isoprene between male and female subjects (GM 105.4 and 95.5 ppb, respectively). The ageing process caused a decrease in isoprene concentrations in men, with an estimated slope of the regression line for log-transformed isoprene concentrations of –0.0049, but did not significantly influence the isoprene level in women (Figure 3 and Table 3). The slope of –0.0049 on a logarithmic scale corresponds to a change by a factor of $10^{-0.0049} = 0.9888$ per year, or $10^{-0.0049 \times 10} = 0.8933$ per 10 years.

Figure 4 shows boxplots of isoprene levels for smokers and non-smokers. The isoprene GM was 113.8 ppb for smokers and 99.5 ppb for non-smokers (computed after omitting the outlier for a female non-smoker described in the legend to Figure 3). However, the difference in mean levels of (log-transformed) isoprene in these two groups was not significant (5% level; two-sample t-test, $p = 0.0789$).

Breath isoprene and total serum cholesterol

The level of total cholesterol in blood serum was measured in 79 healthy adults (Figure 5). We observed that the degree of cholesterolaemia gradually increased with age in females ($p < 0.001$). An increasing tendency was also observed in males, but it cannot be confidently stated to be significant: it was significant at the 5% level ($p = 0.0126$), but a line with a zero trend is included in the 95% confidence band around the regression line in the age range considered (see Figure 5).

We did not observe any significant correlation between breath isoprene content and the degree of cholesterolaemia (Figure 6). As both isoprene levels (at least for males) and cholesterol levels seem to depend on age, the PCC was used to assess the true correlation between (log-transformed) isoprene and cholesterol after eliminating the possible influence of age. For both males (PCC = 0.2982, $p = 0.1095$) and females (PCC = 0.1974, $p = 0.1886$), the PCC was not significantly different from zero, after excluding the outlier of 5.8 ppb for the latter, as described in the legend to Figure 3. These PCC values demonstrate that the lack of correlation between breath isoprene and serum cholesterol is not a result of the effect of age.

Breath isoprene and BMI

There was no significant correlation (5% level) between concentrations of breath isoprene and BMI. In addition, PCC calculation revealed that the behaviour of both parameters is not due to the influence of age.

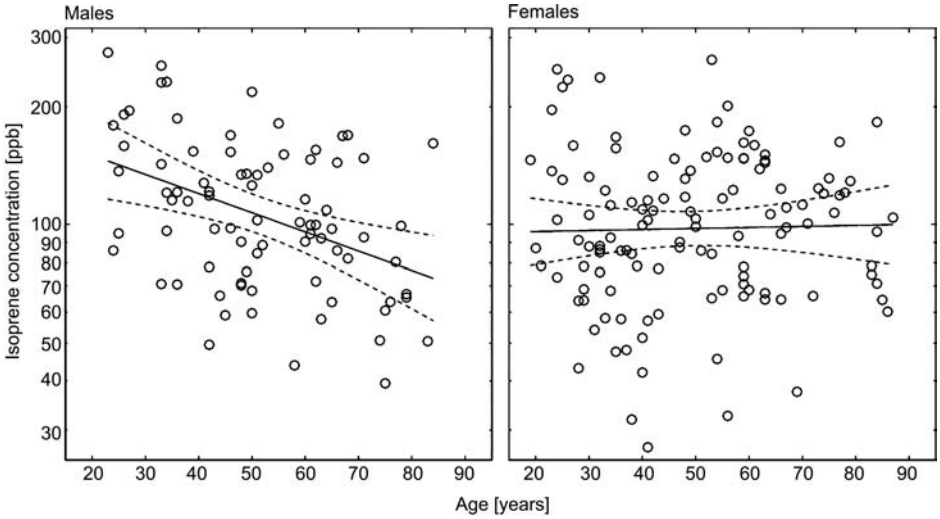

Figure 3 Isoprene concentrations (ppb) in the breath of male and female volunteers as a function of age (plotted on a logarithmic scale). The fitted regression lines are shown, together with their 95% confidence limits.
The female outlier observed with an unusually low isoprene concentration of 5.8 ppb does not influence the statistical significance of the regression (the hypothesis of the zero trend cannot be rejected at the 5% level for both cases; $p = 0.431$ if the outlier is included in the data set and $p = 0.792$ if it is omitted). A decreasing trend in isoprene with increasing age in males is significant at the 5% level, $p < 0.001$.

Table 3 Estimated slope of the regression lines describing the dependence of log-transformed isoprene on age for males and females (computed without the outlier of 5.8 ppb; see Figure 3), together with p-values obtained when testing if the slope is zero.

	Males	Females
Estimated slope	−0.0049 ($p < 0.001$)	0.0003 ($p = 0.792$)

Discussion

Breath isoprene, age and gender

At present there is no consensus in the literature on the relationships between breath isoprene and age or gender. For example, several studies revealed no age- and/or gender-related differences in exhaled isoprene profiles in adults (11, 30, 31, 39, 53). In contrast, Senthilmohan et al. (54) reported that older subjects had higher concentrations of breath isoprene than younger individuals (it should be noted that this study involved only eight subjects of each gender, and therefore it is quite possible that the age effect was a misinterpretation of individual variations in isoprene levels). Some investigators found that isoprene levels were significantly lower in young people and children than in older adults (11, 53, 55).

It should be mentioned that in all the above studies, the age-related variation of isoprene was analysed in cohorts not separated by gender. We observed a decrease in isoprene concentrations with ageing only in men, whereas there were no significant changes for men and women grouped together ($p > 0.05$).

Breath isoprene and total serum cholesterol

The use of breath isoprene measurement in monitoring of cholesterol status would require a correlation between breath isoprene concentrations and the degree of cholesterolaemia, either positively or inversely. However, we found no significant correlation between breath isoprene content and the degree of cholesterolaemia, even after adjusting for the possible effect of age.

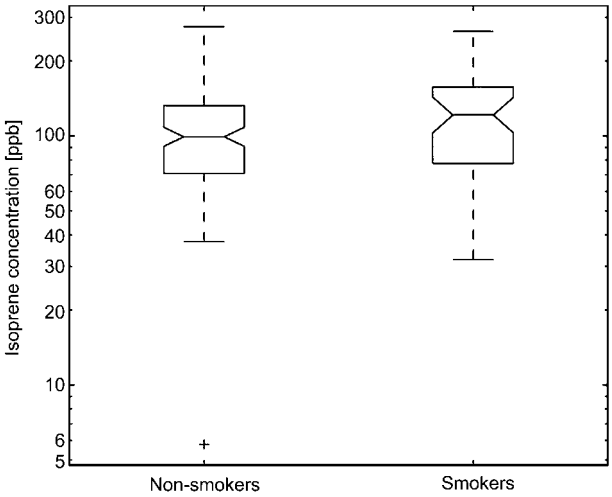

Figure 4 Boxplots of isoprene concentration (on a logarithmic scale) in the breath of smokers (45 values) and non-smokers (125 values).

The age-dependent increase in cholesterol levels in our study cohort reflects typical changes in lipid metabolism during ageing and is in full accordance with much earlier population studies (60, 61). The prevalence of obesity and an increase in hypercholesterolaemia with age is well known. For the use of breath isoprene measurement in monitoring serum cholesterol levels and the efficiency of lipid-lowering therapy, we would expect to see similar age-related variations in isoprene and cholesterol, i.e., parallel increases in breath isoprene and serum cholesterol concentrations

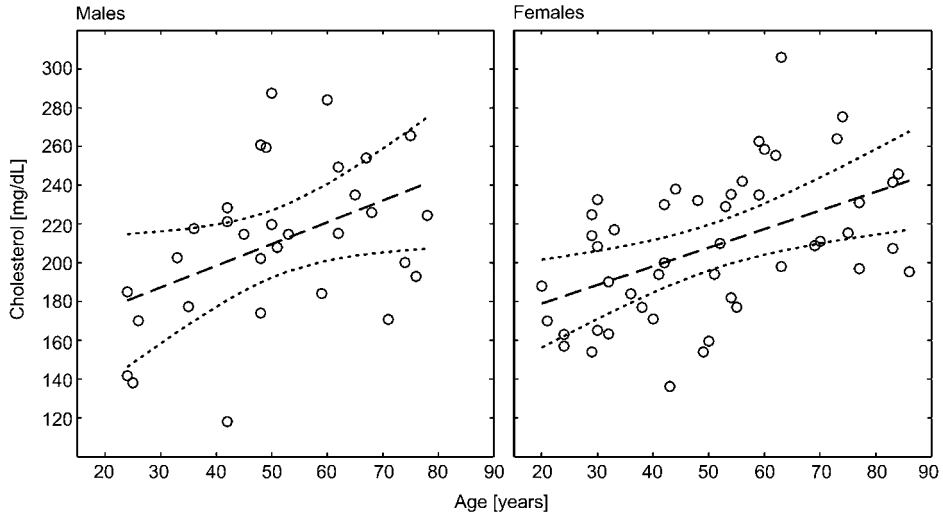

Figure 5 Levels of total serum cholesterol (mg/dL) in males and females as a function of age (years).
The fitted regression lines are shown, together with their 95% confidence limits. To convert cholesterol in mg/dL to the SI unit of mmol/L, multiply by the conversion factor 0.026.

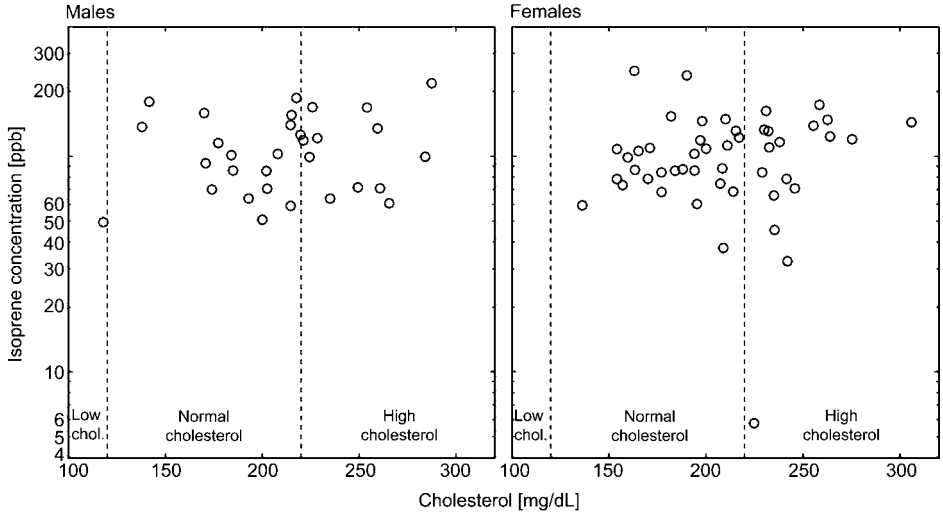

Figure 6 Concentrations of breath isoprene (ppb) on a logarithmic scale and total serum cholesterol (mg/dL) do not show a statistically significant correlation.
To convert cholesterol in mg/dL to the SI unit of mmol/L, multiply by the conversion factor 0.026.

with age. However, in our study and in all but one of the previous investigations by other authors, no such tendency was observed (11, 30, 31, 39, 53); see the comment about the study of Senthilmohan et al. (54) above.

In view of our findings and taking into consideration earlier studies, the current isoprene-cholesterol situation can be summarised as follows:

- There are no indications that isoprene and cholesterol levels are directly related.
- The role of multiple sources of isoprene is likely underestimated, and other metabolic sources of this compound may essentially contribute to the whole pool and negate direct relations with serum cholesterol (32, 34, 54, 62–64).
- Since isoprene is expected to reflect the cholesterol synthesis rate (and not the cholesterol concentration), it could provide information complementary to cholesterol status.
- Isoprene is not expected to correlate with cholesterol concentrations, but rather to the rate of synthesis of cholesterol.

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