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Effects of long-term vegan diet on breath composition

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Abstract

The composition of exhaled breath derives from an intricate combination of normal and abnormal physiological processes that are modified by the consumption of food and beverages, circadian rhythms, bacterial infections, and genetics as well as exposure to xenobiotics. This complexity, which results wide intra- and inter-individual variability and is further influenced by sampling conditions, hinders the identification of specific biomarkers and makes it difficult to differentiate between pathological and nominally healthy subjects. The identification of a "normal" breath composition and the relative influence of the aforementioned parameters would make breath analyses much faster for diagnostic applications. We thus compared, for the first time, the breath composition of age-matched volunteers following a vegan and a Mediterranean omnivorous diet in order to evaluate the impact of diet on breath composition. Mixed breath was collected from 38 nominally healthy volunteers who were asked to breathe into a two-liter handmade Nalophan bag. Exhalation flow rate and carbon dioxide values were monitored during breath sampling. An aliquot (100 mL) of breath was loaded into a sorbent tube (250 mg of Tenax GR, 60/80 mesh) before being analyzed by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). Breath profiling using TD-GC-MS analysis identified five compounds (methanol, 1-propanol, pentane, hexane, and hexanal), thus enabling differentiation between samples collected from the different group members . Principal component analysis showed a clear separation between groups, suggesting that breath analysis could be used to study the influence of dietary habits in the fields of nutrition and metabolism. Surprisingly, one Italian woman and her brother showed extremely low breath isoprene levels (about 5 ppbv), despite their normal lipidic profile and respiratory data, such as flow rate and pCO_2. Further investigations to reveal the reasons behind low isoprene levels in breath would help reveal the origin of isoprene in breath.

Keywords: breath analysis, vegan diet, Mediterranean diet, isoprene, standardization

1. Introduction

Breath analysis has become a promising tool in the field of diagnostic medicine [1], therapeutic drug monitoring [2], toxicology [3], and occupational and environmental exposure [4,5]. Compared to conventional fluids, such as blood and its derivatives, breath can be collected with easy and non-painful procedures that do not require the support of skilled personnel [1]. The simplest breath collection approach is to fill stainless-steel canisters [6] or polymeric bags (e.g., Tedlar [7] and Nalophan [8]) with single or multiple breaths. These methods enable a mixed gaseous sample to be collected containing both dead-space, i.e., the upper airways fraction, and the end-

expiratory air coming from the alveoli, hereafter referred to as the end-tidal fraction.

In a clinical setting, the end-tidal fraction is of primary interest since it reflects the blood-gas exchanges at the alveoli. Commercial or lab-made sampler devices are usually employed to collect the end-tidal fraction in polymeric bags or sorbent traps according to the carbon dioxide levels monitored over time during sampling [9]. After collection, VOCs are extracted from canisters/bags using sorbent phase extraction (SPE) and sorbent phase micro-extraction (SPME) [1] which are then subjected to thermal desorption (TD) coupled with gas chromatography (GC) and mass spectrometer (MS) analysis.

The direct transfer of the sample into sorbent traps-based devices such as sorbent tubes [10] and needle trap devices (NTDs) [11]) simplifies the analytical procedure and prevents the loss of reactive and unstable compounds [12–14], thus improving the reliability of the analytical workflow.

High throughput on-line instruments, such as proton transfer reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), gas chromatography-ion mobility mass spectrometry (GC-IMS), and secondary electrospray ionization high resolution mass spectrometry (SESI-HRMS), have been proposed for the direct analysis of breath [15–18]. These instruments provide similar sensitivity and dynamic range values to most of GC-MS-based methods. However, the limited specificity and the high costs of on-line instruments make the off-line methods the common approach to a comprehensive chemical characterization of breath samples.

In addition to nitrogen, oxygen, carbon dioxide, and water, breath contains volatile organic compounds (VOCs) whose levels may depend on the individual [19]. Lifestyle (e.g., physical exercise [20]), sampling parameters [21], and circadian rhythms [22] are additional variables that can modify the chemical composition of breath. The impact of these factors on the chemical composition of breath is still an open issue and represents one of the main limitations in the clinical diffusion of breath analysis [23].

A series of experiments were recently set up aimed at monitoring the breath composition of individuals after the ingestion of a PEPPERMINT capsule in order to evaluate the benchmark values of selected compounds to be used for comparing breath data among different research groups [24]. The results confirmed the huge variability in the wash-out curves of the PEPPERMINT compounds, thus suggesting the need for standardized protocols to improve the reliability of breath analyses for clinical studies [24].

The impact of diet on the breath composition has only been investigated in a few studies. In 2002, Musa-Veloso *et al*, monitored acetone breath levels in 12 healthy adults who consumed four ketogenic meals over 12 h after 12 hours-fasting [25]. They found a significant increase (3.5-fold) in acetone in breath at the end of the 12-h dietary treatment and highlighted that breath acetone is a good predictor of ketosis. Spanel *et al* [26] found similar results and reported the powerful influence of diet and the change from carbohydrate metabolism to lipid (fat) metabolism on the breath concentration of acetone. In 2012, Filipiak *et al*. analyzed the breath samples of 115 subjects and highlighted that the VOC profile is considerably influenced by exposure to pollution and indoor-air contaminants and particularly by smoking [27]. They suggested diet as one of several factors able to modify individual breath composition. In 2013, Baranska *et al.* monitored the breath composition of 20 healthy volunteers over time while adhering to a gluten-free diet for four weeks prior to adherence to a normal diet [28]. They found that 2-butanol, octane, 2-propyl-1-pentanol, nonanal, dihydro-4-methyl-2(3H)-furanone, nonanoic acid and dodecanal differentiated the samples obtained during a gluten-free diet from those obtained during a normal diet. Their data suggest the reversible impact of the gluten-free dietary period on the breath sample. Raninen *et al.*, monitored the variation in the breath composition of seven healthy male volunteers after the ingestion of a high-fiber meal [29]. They found increased levels of ethanol, 1-propanol, acetoin, propionic acid, and butyric acid and reduced levels of acetone, 1-butanol, diacetylmorphine, and phenol 120 minutes after the ingestion of the test meal.

In the present study, we compared the breath composition of age-matched volunteers following a vegan and a Mediterranean diet in order to assess the usefulness of breath analysis in the fields of nutrition and metabolism. A vegan diet consists of plant foods such as grains, vegetables, fruits, legumes, nuts, seeds, vegetables fats and oils without the consumption of meat and food of animal origin (e.g., eggs and honey) [30]. On the other hand, the Mediterranean diet represents a food and environmental lifestyle typical of the European Mediterranean area which is characterized by the consumption of low processed food, mainly cereals, and fresh or dried fruit and vegetables, with a moderate consumption of fish, meat, and wine [31]. The Mediterranean diet appears to protect against obesity, metabolic disease, and certain types of cancer [32–34].

2. Materials and methods

2.1 Chemicals and materials

A stock liquid mixture of the following were purchased from AccuStandard, Inc. Chemical Reference Standard (USA): *C1-C5 alcohols* (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol, 2-methyl-2-propanol, 1-pentanol, 2-pentanol, 3-pentanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methyl-2-butanol), *C4-C9 ketones* (2-butanone, 2-pentanone, 3-pentanone, 2-hexanone, 4-heptanone, 3-heptanone, 2-heptanone, 3-

octanone, 2-methylcyclohexanone, 3-methylcyclohexanone, 4-methylcyclohexanone, 2-octanone, 5-nonanone, 2-nonanone), *C4-C9 branched ketones* (3-methyl-2-butanone, 3,3-dimethyl-2-butanone, 2-methyl-3-pentanone, 4-methyl-2-pentanone, 2,4-dimethyl-3-pentanone, 2-methyl-3-hexanone, 5-methyl-2-hexanone, 2-methyl-3-heptanone, 5-methyl-3-heptanone, 2,6-dimethyl-4-heptanone, 4-methylpent-3-en-2-one, acetophenone, cyclopentanone, cyclohexanone), *27 VOCs* (certified reference materials, 0.2 mg/mL in methanol) (benzene, bromodichloromethane, bromoform, tetrachloromethane, chloroform, chlorobenzene, dibromochloromethane, 1,2-dichlorobenzene, 1,4-dichlorobenzene, 1,2-dichloroethane, 1,1-dicloroetene, cis-1,2-dichloroethene, trans-1,2-dichloroethene, dichloromethane, 1,2-dichloropropane, ethylbenzene, styrene, tetrachloroethene, toluene, 1,2,4-trichlorobenzene, 1,1,1-trichloroethane, 1,1,2-trichloroethane, trichloroethene, vinyl chloride, m-xylene, o-xylene, and p-xylene).

A stock liquid mixture of *C5-C18 n-alkanes* (pentane, hexane, heptane, octane, nonane, decane, undecane, dodecane, tridecane, pentadecane, hexadecane, heptadecane and octadecane) was purchased from Merck (Italy). Pure methacrolein, acrolein, 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, acetaldehyde, propanal, butanal, hexanal, heptanal, benzaldehyde, isoprene, acetone, 2-methylpentane, 2,3-butandione, 3-hydroxy-2-butanone, dimethyl sulfide, dimethyl disulfide, and carbon disulfide were purchased from Fluka, Sigma-Aldrich (Italy).

In both mixtures, all the compounds had a purity higher than 99% and were used without any purification.

Labelled ^{8}D-toluene was purchased at a purity of 99.8% from ARMAR Chemicals (Switzerland) and used as an internal standard (IS).

Helium 5.6 IP and synthetic air 5.0 IP were purchased from Sol Group Spa (Italy). These gases were further purified with a super clean filter purchased from Agilent Technologies (USA).

Glass sorbent tubes (O.D. 6.4 mm, I.D. 5 mm, 89 mm length), packed with 250 mg of 60/80 mesh Tenax GR phase were purchased from Supelco (USA).

Two-liter handmade Nalophan bags were fabricated with a (film) surface-to-(sample) volume ratio (S/V) of 0.3 cm^{-1} from a roll of Nalophan tube (PET, polyethylene terephthalate, diameter 23.5 cm and film thickness 20 μm), supplied by Kalle (Germany). Bags were assembled according to the procedure described elsewhere [13].

Two-liter glass static dilution bottles equipped with a screw cap Mininert valve were purchased from Sigma-Aldrich (Italy).

A one microliter syringe was purchased from Hamilton (USA).

A mini-BUCK M-5 primary gas flow calibrator, operating in the range 1–6000 mL/min, was purchased from A. P. Buck Inc. (USA).

A thermo-hygrometer, equipped with an immersion probe (o.d. 2 mm, 230 mm length) and operating between 5% and 98% relative humidity (RH), was purchased from Delta Ohm (Italy).

2.2 Preparation of gaseous standard solutions

An aliquot (20 μL) of each pure liquid compound was weighed directly into a 1 mL amber glass vial to prepare a stock solution (MIX19). An aliquot (20 μL) of each stock liquid mixture was injected into pre-evacuated 2 L glass bottles to prepare six stock gaseous mixtures. After evaporation of each solution, the flask pressure was balanced to ambient pressure and then the mixtures were stored at 37 ± 1 °C and 12 ± 2% of RH for 1 week. Table S1 reports the concentration of each compound in the stock gaseous mixtures.

Working gaseous standard mixtures were prepared in 10 L Nalophan bags by diluting appropriate aliquots of stock gaseous mixtures with humidified synthetic air (RH of 85 ± 5%) to simulate real samples. These bags were kept at 37 ± 1 °C and 12 ± 2% of RH for 2 hours and then analyzed as real breath samples (see paragraph 2.5).

In the same way, a stock gaseous IS mixture was prepared by injecting five microliters of pure IS into a pre-evacuated 2 L glass bottle. This solution was kept at 37 ± 1 °C and 12 ± 2% of RH for one month. In these storage conditions, the concentration of IS was 600 ppmv.

2.3 Study population

The study population consisted of thirty-eight nominally healthy volunteers (9 males and 29 females) who followed either a vegan (n=18) or a Mediterranean (n=20) diet. The study received approval from the local ethics committee (271/2014).

Exclusion criteria were as follows: pregnancy, medical conditions, and subjects under medication. A food habits questionnaire was used to evaluate the nutritional scheme followed by each volunteer [35]. Each vegan participant was recruited if they had followed the a vegan nutrition scheme for at least two years. Before being included in the study each volunteer signed a written consent explaining all the experimental details.

2.4 Basal metabolic rate evaluation

Subjects underwent an indirect calorimetry to evaluate the basal metabolic rate (BMR) or respiratory exchange ratio (RER) in controlled environmental conditions (temperature of 22-24 °C) at a distance of at least 48 hours from the last sporting engagement. Tests were carried out at the Department

of Clinical and Experimental Medicine of the University of Pisa (Italy). Indirect calorimetry is the gold standard to evaluate BMR and substrate metabolism (Carbohydrates-CHO and Free Fat Acids-FFA) [36]. A paramagnetic O_2 sensor and infrared CO_2 analyzer were purchased by Quark PFT (COSMED, Italy).

Subjects were asked to lie on a couch and were fitted with a Canopy helmet (CANOPY) for gas analysis. Canopy helmet guarantees a tight system and allows the patient to freely breathe -even for long periods- without any discomfort. The air inside the helmet is continuously analyzed by the O_2/CO_2 sensor for calculating base metabolic rate and energy substrates. After an acclimatization period of about five minutes to prevent hyperventilation during exercise, the evaluation began and lasted a total of 20 minutes. A breath-by-breath measurement was used for the gas analysis. Tests were conducted under medical supervision. All technical details are reported elsewhere [37].

2.5 Breath sampling and analysis

Mixed breath samples were collected between 08.00 and 10.00 in resting and fasting (at least 8 hours) conditions using a sampler prototype consisting of a sterile and disposable mouthpiece, a Capnostat 5 CO_2/flow sensors (Philips, Netherlands), a serializable non-rebreathing two-way, three-port valve (Hans Rudolph Inc, USA), a non-return valve (EM-Tecnik GmbH, Germany), and a 2 liters handmade Nalophan bag. Each volunteer wore a nose clip. Sampling lasted two minutes. Room air was also collected to exclude the risk of contamination [5]. Volunteers were asked to avoid smoking 12 hours before the breath sampling.

After collection, Nalophan bags were transported to the laboratory and then were kept at 37 ± 1 °C and 12 ± 2% of RH for half an hour to minimize the amount of water vapor within the bag [13]. To preserve the chemical composition of breath within the bag, the time between sampling and thermal treatment was less than two hours [13]. An aliquot (100 mL) of breath sample was transferred (50 mL/min) into a system composed of a drying tube (packed with 9 g of anhydrous sodium sulfate) (SKC, USA) connected in series with a Tenax GR sorbent tube. Fifty microliters of stock gaseous IS were dispersed in the gaseous flow during the sample transfer. Lastly, the tube was thermally desorbed by an automated two-stage STD 1000 thermal desorption unit (Dani Instruments, Italy) equipped with an internal focusing trap (70 mg of Tenax GR). Primary desorption was performed in splitless mode at 250 °C for 8 minutes applying a He flow rate of 35 mL/min. A cold trap was set at 5 °C. Secondary desorption entailed a rapid increase in the cold trap to 250 °C leading the fast transfer of the analytes through a narrow silica transfer line into the head of the inlet of the Trace GC ultra (Thermo Scientific, USA). Analytes were separated using a DB-624 GC column (60 m length, 0.25 mm internal diameter, 1.4 µm film thickness) (Agilent Technologies, USA) using the following oven temperature profile: 35 °C for 10 min, 4 °C/min to 130 °C (2 min hold), 20 °C/min to 250 °C (10 min hold), and 25 °C/min to 260 °C (15 min hold). The injector temperature and pressure remained stable during the analysis and were set at 200 °C and 210 kPa, respectively. A He split flow of 10 mL/min was used.

Analytes were identified and quantified using a Trace DSQ mass spectrometer (Thermo Scientific, USA) operating in positive electron impact ionization (70 eV). The transfer line, ion source and the quadrupole temperature were set at 260 °C, 250 °C and 150 °C, respectively. Chromatograms were acquired in Total Ion Current (TIC), with an *m/z* range set from 18 to 200, and in Selected Ion Monitoring (SIM). Quantifier (Q) and qualifier (q) ions were monitored with a constant dwell time of 50 ms. TD Manager (DANI Instrument, Italy) and Xcalibur (Thermo Scientific, USA) software controlled the TD unit and GC-MS instrument, respectively.

Relative response factors (RRFs) to IS were calculated at three concentration levels (100-, 1000-, 10000-fold dilution of gaseous stock mixtures) as reported elsewhere [38].

The concentrations of target VOCs were determined based on RRFs and breath volume (100 mL) used for the TD-GC-MS analysis.

CO_2 partial pressure (pCO_2) and inhalation/exhalation flow rate were measured in real time during the sampling. Tidal volume (V_T) and the respiratory rate (RR) were calculated as reported elsewhere [9]. Mixed pCO_2 was also determined by flowing (500 mL/min) breath sample from the Nalophan bag into a Capnostat mainstream CO_2 sensor (Philips, Netherlands).

2.6 Data processing and statistical analysis

Xcalibur software (Thermo Scientific, USA) was used to acquire GC data and to perform quantitative analysis. The presence of each target VOC was evaluated by comparing the retention time and q/Q ratios observed in standard mixtures and in real breath samples (Table S2). Variations within ±0.10 min for the retention time and 10% for the q/Q ratio were accepted. The concentration of each analyte was calculated considering the RRF factors, the amount of IS added during sample treatment and the volume of breath transferred into the sorbent tubes.

The resulting data matrix contained 38 rows and 54 columns corresponding to the concentration of VOCs in the breath and to the metabolic data. Some samples showed lower breath GC signals than the detection limit, thus missing values were replaced with half of the minimum positive values observed in the original dataset. Data were log-normalized prior to statistical analysis.

Univariate analysis involved normality testing using the Shapiro-Wilk test as well as calculating the central tendency (mean and median) and dispersion [standard deviation, lower

(25th percentile) and upper quartiles (75th percentile)]. Variables showing a normal distribution were reported as mean ± standard deviation, whereas for a skewed distribution, median with lower and upper quartiles were used. The t-test was used to evaluate the potential differences between groups. Pearson's correlation was carried out to test the statistical relationship between demographic and breath data. A two-tailed p-value of <0.05 was considered statistically significant. Principal component analysis (PCA) was used as the multivariate analysis to obtain an overall view of the entire data-set. All data were analyzed using GraphPad Prism (v. 8.0) from GraphPad Software Inc. (La Jolla, USA) and MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

3. Results and discussion

To the best of our knowledge, there are currently no studies on the impact of veganism on breath composition. The present compared the breath samples collected from 38 volunteers, who were following vegan (n=18) and Mediterranean (n=20) diets.

Table 1 reports the anthropometric measurements, metabolic and respiratory data obtained from the volunteers participating in the study.

Table 1. Characteristics of volunteers following a vegan (n=18) and a Mediterranean (n=20) diet. Data expressed as mean ± standard deviation (min–max). Bold values denote statistical significance at the p <0.05 level.

Variable	Vegan diet	Mediterranean diet	p-value
Gender (male:female)	5:13	6:14	--
Age (years)	34 ± 12 (21–53)	29 ± 8 (22–53)	0.10
Height (cm)	169 ± 10 (150–180)	172 ± 8 (160–186)	0.34
Body weight (Kg)	65 ± 14 (46–86)	70 ± 15 (54–95)	0.32
BMI (Kg/m^2)	22.6 ± 4.0 (17.5–33.6)	23.4 ± 3.1 (19.6–28.7)	0.56
Smokers (n)	7	6	--
Vegan diet (years)	4 ± 1 (2–7)	--	--
Basal metabolic rate (kcal/d)	1310 ± 395 (581–1822)	1673 ± 488 (909–2626)	**0.02**
Respiratory exchange ratio	0.9 ± 0.1 (0.7–1.1)	0.8 ± 0.1 (0.6–1.1)	0.21
Carbohydrates (%)*	49.6 ± 33.1 (0.0–100.0)	33.9 ± 29.0 (0.0–100.0)	0.15
Lipids (%)*	50.6 ± 33.1 (0.0–100.0)	66.5 ± 29.1 (0.0–100.0)	0.15
Respiratory rate (breathes/min)	7 ± 3 (3–14)	7 ± 3 (4–16)	0.80
Tidal volume (mL)	1040 ± 430 (520–1560)	1090 ± 370 (410–1500)	0.62
Exp. Flow rate (L/min)	14 ± 7 (6–30)	12 ± 3 (7–25)	0.27
End-tidal pCO_2 (mmHg)	36 ± 4 (30–44)	39 ± 6 (29–55)	**0.01**

* data are referred to the basal metabolic rate analysis.

Mixed breath composition is particularly prone to be affected by changes of respiratory rate, exhalation flow rate, and tidal volume [39]. As reported in Table 1, both groups showed similar values (p >0.05) of these variables suggesting the minimal impact of mixed breath sampling maneuvers on the chemical composition of breath samples. End-tidal pCO_2 resulted significantly different (p <0.01) between subjects following a Mediterranean diet (39 ± 6 mmHg) with respect to the vegan diet (36 ± 4 mmHg). These results were probably related to the low basal metabolic rate of vegans. Four volunteers showed end-tidal pCO_2 values that did not fall in the normal physiological range (i.e., between 35 and 45 mmHg) likely due to a different capability of volunteers to fill Nalophan bag. In fact, it is well known that filling sampling bags with a single deep breath rather than multiple short breathes leads to increased end-tidal pCO_2 values [40].

All the vegan volunteers had started a plant-based diet two years before their enrollment in the study. Among the vegans,

12 out of 18 volunteers were of normal weight with BMI values ranging from 18.5 Kg/m^2 to 25.0 Kg/m^2. The remaining volunteers were overweight (n=4) (BMI values between 25 and 30 Kg/m^2), underweight (n=1) (BMI <18.5 Kg/m^2), and obese (n=1) (BMI >30.0 Kg/m^2). Likewise, 11 out of 20 volunteers following a Mediterranean diet were of normal weight and 9 out of 18 were overweight. The BMI did not significantly differ among groups. The two groups of volunteers were age-matched and the number of smokers was comparable between groups. Except for five volunteers (2 vegans), most participants had a sedentary lifestyle (≤60 minutes of structured or planned physical activity a week) [41]).

A food habits questionnaire (FHQ) was used to record the nutritional characteristics of the eating habits of each volunteer. Except for subjects Veg1 and Veg17, all the vegan participants were taking vitamin B12 supplements (2.5 µg/d) daily. The Mediterranean diet was composed of 55% carbohydrates, 20% protein and 25% fatty acids, whereas the vegan participants reported a lower relative intake of protein (10-15%), a higher intake of carbohydrate (60-65%), and normal intake of fat (20-30%).

Indirect calorimetry showed that subjects following a Mediterranean diet had a higher mean (range) basal metabolic rate than the vegan group [1673 kcal/d (581–1822 kcal/d) *vs.* 1310 kcal/d (909–2626 kcal/d), p <0.02]. These data can be due to the lower protein intake of the vegan participants, thus confirming the data discussed by Nadimi *et al*. [42]. Subjects following a Mediterranean diet showed a slight increased mean (range) consumption of fatty acids [66.5% (0–100%) *vs.* 50.6 (0–100%), p >0.15] and a slight decreased mean (range) carbohydrate value [33.9% (0–100%) *vs.* 49.6 (0–100%), p >0.15]. Although these differences were not significant, our data suggest that the diet of most of the vegan participants was not properly balanced, increasing the risk of metabolic diseases such as insulin resistance.

Principal components analysis (PCA) (Figure 1) was used to explore the entire dataset of indirect calorimetry and breath analysis data.

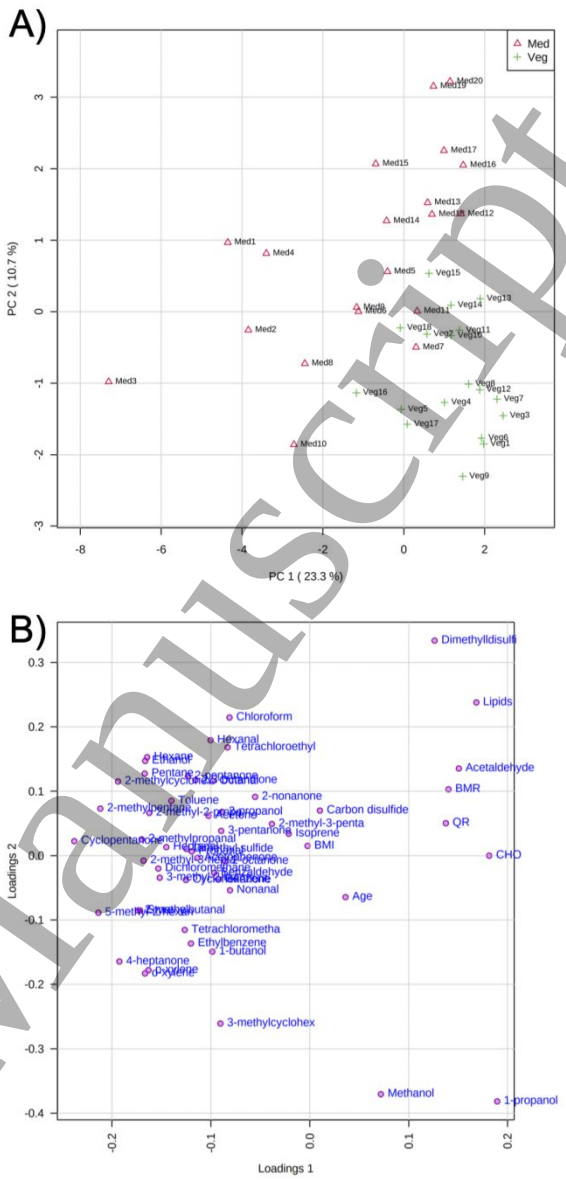

Figure 1. PCA of metabolic and breath data. A) score plot, B) loadings plot. Legend: carbohydrate metabolism (CHO), body mass index (BMI), free fatty acids metabolism (FFA), base metabolic rate (BMR), and respiratory exchange rate (QR).

The PCA score plot (Figure 1A) shows a clear separation among groups along PC1 and PC2 which explains 34% of the total variance. The vegan participants are grouped in the bottom-right corner of the score plot and show a high mean (range) concentration of methanol [56 ppbv (6–220) *vs.* 13 ppbv (5–22 ppbv), p <0.05] and 1-propanol [4350 pptv (875–23200 pptv) *vs.* 425 pptv (150–875 pptv), p <0.05] as well as low levels of a panel of VOCs such as pentane [890 pptv (510–1170 pptv) *vs.* 1370 pptv (890–2910 pptv), p<0.01], hexane [160 pptv (100–280 pptv) *vs.* 600 pptv (130–1520 pptv), p <0.01], and hexanal [200 pptv (110–280 pptv) *vs.* 360 pptv (170–780 pptv), p <0.001]. Figure 1A clearly shows that the

spread of the subjects following a Mediterranean diet is wider than the vegan group, in fact they are located along the main diagonal of the score plot. This is probably related to the heterogeneous food intake of the Mediterranean diet leading to larger variations in exhaled volatile products. In fact, the Mediterranean diet includes a wider range of food sources, such as wholegrain cereals, vegetables, fruit, pulses, foods of animal origin (e.g., meat, fish, and eggs). Two subjects following a Mediterranean diet (i.e., Med7 and Med11) showed a similar profile as those from the vegan group. These data could be related to the limited consumption of animal foods of these two volunteers, as highlighted in the questionnaire and interview.

Variations in the VOC profile of subjects following different diet habits were reported by Baranska *et al*. [28]. They found significant changes in the VOC profile of the subjects on a gluten-free diet compared with that of (the same) subjects on a normal diet. In our case, the high levels of methanol of the vegan participants may be due to the degradation of pectin, a soluble fiber mainly found in fruits and vegetables. In vivo studies have shown that pectin administration induces a significant increase in methanol in the blood and breath of humans [43]. In the same way, increased levels of 1-propanol may be related to bacterial fermentation in the colon of two essential amino acids (i.e., threonine and isoleucine) which are present at high levels in several legumes and vegetables [44,45]. These results are in accordance with those reported by De Filippis *et al* [46], highlighting a clear difference between the salivary metabolome profiles of omnivores, vegans and ovo-lacto-vegetarians. Omnivore individuals showed higher salivary levels of formate, urea and uridine, whereas levels of hexanoic acid, proline, 1-propanol and 5-methyl-3-hexanone were higher in subjects following vegan and ovo-lacto-vegetarian diets. The high levels of pentane, hexane, and hexanal could be explained considering the high daily consumption of fruits and vegetables of vegans. It is well-known that this food restriction leads to reduced saturated fatty intake and to increased fiber and vitamin intake on a daily basis, improving the antioxidant status of the vegan population [47].

Breath pentane, hexane and hexanal are well-established markers of oxidative stress as they are end-products of the reaction of reactive oxygen species with various biological compounds such as lipid and DNA [48]. It is thus reasonable to hypothesize that there is a direct link between the low breath levels of these compounds and the reduced oxidative stress of the vegan population, as discussed elsewhere for oxidative stress levels in blood [47].

The oral microbiota metabolism may also play a role in the release of VOCs in breath, although a recent study indicated no effect of dietary habit on salivary microbiota [46]. Oral microbiota would thus not seem to contribute to the metabolomic differentiation of the two groups through breath samples.

Interestingly, one participant (Med2) (a 22-year old Italian woman) showed a very low concentration level of isoprene in the breath (6 ppbv). We thus decided to replicate the breath analysis twice a week for one month and found a mean (range) isoprene concentration of 5 ppbv (2–9 ppbv). We also extended the breath analysis to her relatives, i.e., father and brother as well as maternal grandmother, maternal grandfather, maternal aunt, and her daughter (cousin). Only her brother showed a similar isoprene concentration (4 ppbv), whereas the other members of her family showed a normal isoprene concentration range (90–150 ppbv). All her family members showed similar lipid-profiling (i.e., cholesterol, lipoproteins, high density lipoproteins, low density lipoproteins, and triglycerides) and respiratory data (i.e., flow rate and end-tidal pCO_2). Unfortunately, we were not able to perform any genetic measurements on these volunteers aimed at evaluating any potential differences in mevalonate pathway enzymes.

Isoprene has a clear endogenous origin [20,21,49-50] and is not produced within the upper airways [51]. Scientific evidence in this field suggests that isoprene is a by product of cholesterol biosynthesis [52] as well as being produced by the peroxidation of squalene [53]. Several papers have thus investigated the potential application of breath isoprene as a non-invasive diagnostic tool to assess blood cholesterol levels or cholesterol synthesis rate [54,55] as well as pulmonary gas exchange patterns [20]. The first study on low isoprene breath levels was reported in 1999 by Spanel *et al.* [56], who were unable to determine a cause as the blood cholesterol values of the subjects were similar to the controls. Nearly ten years later, Harshman *et al.* found another subject with no isoprene in their breath [57]. They were not able to perform additional experiments due to ethical restraints, however they hypothesized some differences in the genetic profile. Recently, Sukul *et al.*, found a German woman with low isoprene levels in her breath [58]. They reported an absolute isoprene concentration (repeated measurements mean ± SD) in her blood-relatives, i.e., sibling sister, mother, father and infant daughter (<1 year old), ranging between 5 ± 2 ppbv and 39.25 ± 6 ppbv. Her husband showed an isoprene concentration of 111.46 ± 15 ppbv which is similar to those measured in the same study in 532 adults (137.52 ± 48.2 ppbv). The reduced levels of isoprene of the German woman were not related to physio-metabolic changes during the menstrual cycle, pregnancy or after childbirth nor to any alterations in the genes of mevalonate pathway enzymes.

In summary, we found significant variations in the breath composition due to the different food habits of 38 nominally healthy volunteers following a vegan (n=18) and a Mediterranean (n=20) diet. Methanol, 1-propanol, pentane,

hexane, and hexanal were found to be the most promising VOCs for differentiating between the two groups.

These data support the idea that performing breath analysis to study the influence of dietary habits could be used in the fields of nutrition and metabolism to better understand their implication on chronic diseases. Interestingly, an Italian woman and her brother showed low isoprene levels in breath, confirming a few cases already reported in literature.

We believe that researchers in this field should set-up a task force to further investigate the low levels of isoprene aimed at addressing the correct origin of isoprene in breath. Such results are fundamental for proposing isoprene as a biomarker of disease in breathomics.

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References

[1] Lourenço C and Turner C 2014 Breath Analysis in Disease Diagnosis: Methodological Considerations and Applications *Metabolites* **4** 465-498

[2] Neerincx A H, Vijverberg S J H, Bos L D J, Brinkman P, van der Schee M P, de Vries R, Sterk P J and Maitland-van der Zee A H 2017 Breathomics from exhaled volatile organic compounds in pediatric asthma *Pediatr. Pulmonol.* **52** 1616-1627

[3] Pleil J D 2016 Breath biomarkers in toxicology *Arch. Toxicol.* **90** 2669-2682

[4] Wallace M A G, Pleil J D, Oliver K D, Whitaker D A, Mentese S, Fent K W and Horn G P 2019 Targeted GC-MS analysis of firefighters' exhaled breath: Exploring biomarker response at the individual level *J. Occup. Environ. Hyg.* **16** 355-366

[5] Ghimenti S, Tabucchi S, Bellagambi F G, Lomonaco T, Onor M, Trivella M G, Fuoco R and Di Francesco F 2015 Determination of sevoflurane and isopropyl alcohol in exhaled breath by thermal desorption gas chromatography-mass spectrometry for exposure assessment of hospital staff *J. Pharm. Biomed. Anal.* **106** 218-223

[6] Lindstrom A B and Pleil J D 2002 A review of the USEPA's single breath canister (SBC) method for exhaled volatile organic biomarkers *Biomarkers* **7** 189-208

[7] Beauchamp J, Herbig J, Gutmann R and Hansel A 2008 On the use of Tedlar® bags for breath-gas sampling and analysis *J. Breath Res.* **2** 046001

[8] Ghimenti S, Tabucchi S, Lomonaco T, Di Francesco F, Fuoco R, Onor M, Lenzi S and Trivella M G 2013 Monitoring breath during oral glucose tolerance tests *J. Breath Res.* **7** 017115

[9] Salvo P, Ferrari C, Persia R, Ghimenti S, Lomonaco T, Bellagambi F and Di Francesco F 2015 A dual mode breath sampler for the collection of the end-tidal and dead space fractions *Med. Eng. Phys.* **37** 539-544

[10] Wilkinson M, White I R, Goodacre R, Nijsen T and Fowler S J 2020 Effects of high relative humidity and dry purging on VOCs obtained during breath sampling on common sorbent tubes *J. Breath Res.* **14** 046006

[11] Biagini D, Lomonaco T, Ghimenti S, Bellagambi F G, Onor M, Scali M C, Barletta V, Marzilli M, Salvo P, Trivella M G, Fuoco R and Di Francesco F 2017 Determination of volatile organic compounds in exhaled breath of heart failure patients by needle trap micro-extraction coupled with gas chromatography-tandem mass spectrometry *J. Breath Res.* **11** 1-14

[12] Kim Y H, Kim K H, Jo S H, Jeon E C, Sohn J R and Parker D B 2012 Comparison of storage stability of odorous VOCs in polyester aluminum and polyvinyl fluoride Tedlar® bags *Anal. Chim. Acta* **712** 162-167

[13] Ghimenti S, Lomonaco T, Bellagambi F G, Tabucchi S, Onor M, Trivella M G, Ceccarini A, Fuoco R and Di Francesco F 2015 Comparison of sampling bags for the analysis of volatile organic compounds in breath *J. Breath Res.* **9** 047110

[14] Lomonaco T, Romani A, Ghimenti S, Biagini D, Bellagambi F G, Onor M, Salvo P, Fuoco R and Di Francesco F 2018 Determination of carbonyl compounds in exhaled breath by on-sorbent derivatization coupled with thermal desorption and gas chromatography-tandem mass spectrometry *J. Breath Res.* **12** 04600

[15] Herbig J, Müller M, Schallhart S, Titzmann T, Graus M and Hansel A 2009 On-line breath analysis with PTR-TOF *J. Breath Res.* **3** 027004

[16] Španěl P and Smith D 2011 Progress in SIFT-MS: Breath analysis and other applications *Mass Spectrom. Rev.* **30** 236-267

[17] Westhoff M, Litterst P, Freitag L, Urfer W, Bader S and Baumbach J I 2009 Ion mobility spectrometry for the detection of volatile organic compounds in exhaled breath of patients with lung cancer: Results of a pilot study *Thorax* **64** 744-748

[18] Bregy L, Nussbaumer-Ochsner Y, Martinez-Lozano Sinues P, García-Gómez D, Suter Y, Gaisl T, Stebler N, Gaugg M T, Kohler M and Zenobi R 2018 Real-time mass spectrometric identification of metabolites characteristic of chronic obstructive pulmonary disease in exhaled breath *Clin. Mass Spectrom.* **7** 29-35

[19] Kim K H, Jahan S A and Kabir E 2012 A review of breath analysis for diagnosis of human health *TrAC - Trends Anal. Chem.* **33** 1-8

[20] 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

[21] Miekisch W, Kischkel S, Sawacki A, Liebau T, Mieth M and Schubert J K 2008 Impact of sampling procedures on the results of breath analysis *J. Breath Res.* **2** 026007

[22] Martinez-Lozano Sinues P, Tarokh L, Li X, Kohler M, Brown S A, Zenobi R and Dallmann R 2014 Circadian variation of the human metabolome captured by real-time breath analysis *PLoS One* **9** e114422

[23] Risby T H 2008 Critical issues for breath analysis *J. Breath Res.* **2** 030302

[24] Henderson B, Ruszkiewicz D M, Wilkinson M, Beauchamp J D, Cristescu S M, Fowler S J, Salman D, Di Francesco F, Koppen G, Langejürgen J, Holz O, Hadjithekli A, Moreno S, Pedrotti M, Sinues P, Slingers G, Wilde M, Lomonaco T, Zanella D, Zenobi R, Focant J F, Grassin-Delyle S, Franchina F A, Malásková M, Stefanuto P H, Pugliese G, Mayhew C and Thomas C L P 2020 A benchmarking protocol for breath analysis: The peppermint experiment *J. Breath Res.* **14** 046008

[25] Musa-Veloso K, Likhodii S S and Cunnane S C 2002 Breath acetone is a reliable indicator of ketosis in adults consuming ketogenic meals *Am. J. Clin. Nutr.* **76** 65-70

[26] Španěl P, Dryahina K, Rejšková A, Chippendale T W E and Smith D 2011 Breath acetone concentration; Biological variability and the influence of diet *Physiol. Meas.* **32** 23-31

[27] Filipiak W, Ruzsanyi V, Mochalski P, Filipiak A, Bajtarevic A, Ager C, Denz H, Hilbe W, Jamnig H, Hackl M, Dzien A and Amann D A 2012 Dependence of exhaled breath composition on exogenous factors, smoking habits and exposure to air pollutants *J. Breath Res.* **6** 036008

[28] Baranska A, Tigchelaar E, Smolinska A, Dallinga J W, Moonen E J C, Dekens J A M, Wijmenga C, Zhernakova A and Van Schooten F J 2013 Profile of volatile organic compounds in exhaled breath changes as a result of gluten-free diet *J. Breath Res.* **7** 037104

[29] Raninen K J, Lappi J E, Mukkala M L, Tuomainen T P, Mykkänen H M, Poutanen K S and Raatikainen O J 2016 Fiber content of diet affects exhaled breath volatiles in fasting and postprandial state in a pilot crossover study *Nutr. Res.* **36** 612-619

[30] Smith A M 2006 Veganism and osteoporosis: A review of the current literature *Int. J. Nurs. Pract.* **12** 302-306

[31] Burlingame B and Dernini S 2011 Sustainable diets: the Mediterranean diet as an example. *Public Health Nutr.* **14** 2285-2287

[32] Trajkovska Petkoska A and Trajkovska-Broach A 2021 Mediterranean diet: a nutrient-packed diet and a healthy lifestyle for a sustainable world *J. Sci. Food Agric.* **101** 2627-2633

[33] Grosso G, Pajak A, Mistretta A, Marventano S, Raciti T, Buscemi S, Drago F, Scalfi L and Galvano F 2014 Protective role of the Mediterranean diet on several cardiovascular risk factors: EVIDENCE from Sicily, southern Italy *Nutr. Metab. Cardiovasc. Dis.* **24** 370-377

[34] Verberne L, Bach-Faig A, Buckland G and Serra-Majem L 2010 Association between the mediterranean diet and cancer risk: A review of observational studies *Nutr. Cancer* **62** 860-870

[35] Moradi S, Pasdar Y, Hamzeh B, Najafi F, Nachvak S M, Mostafai R, Niazi P and Rezaei M 2018 Comparison of 3 Nutritional Questionnaires to Determine Energy Intake Accuracy in Iranian Adults *Clin. Nutr. Res.* **7** 213-222

[36] Mtaweh H, Tuira L, Floh A A and Parshuram C S 2018 Indirect calorimetry: history technology and application *Front. Pediatr.* **6** 1-8

[37] Vandarakis D, Salacinski A J and Broeder C E 2011 A comparison of Cosmed metabolic systems for the determination of resting metabolic rate *Res. Sports Med.* **21** 187-194

[38] States U and Protection E 1999 Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air - Second Edition Compendium of Methods Determination of Toxic Organic Compounds in Ambient Air *Environ. Res.*

[39] Lomonaco T, Salvo P, Ghimenti S, Biagini D, Bellagambi F, Fuoco R and Di Francesco F 2015 A breath sampling system assessing the influence of respiratory rate on exhaled breath composition *Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBS* vol 2015-November

[40] O'Hara M E, O'Hehir S, Green S and Maywhew C A 2008 Development of a protocol to measure volatile organic compounds in human breath: a comparison of rebreathing and on-line single exhalations using proton transfer reaction mass spectrometry *Physiol. Meas.* **29** 309-330.

[41] Piercy K L, Troiano R P, Ballard R M, Carlson S A, Fulton J E, Galuska D A, George S M and Olson R D 2018 The physical activity guidelines for Americans *JAMA - J. Am. Med. Assoc.* **320** 2020-2028

[42] Nadimi H, Yousefinejad A, Djazayery A, Hosseini M and Hosseini S 2013 Association of vegan diet with rmr, body composition and oxidative stress *Acta Sci. Pol. Technol. Aliment.* **12** 311-318

[43] Lindinger W, Taucher J, Jordan A, Hansel A and Vogel W 1997 Endogenous production of methanol after the consumption of fruit *Alcohol. Clin. Exp. Res.* **21** 939-943

[44] Janssen P H 2004 Propanol as an end product of threonine fermentation *Arch. Microbiol.* **182** 482-486

[45] Lamsen E N and Atsumi S 2012 Recent progress in synthetic biology for microbial production of C3-C10 alcohols *Front. Microbiol.* **3** 00196

[46] De Filippis F, Vannini L, La Storia A, Laghi L,

Piombino P, Stellato G, Serrazanetti D I, Gozzi G, Turroni S, Ferrocino I, Lazzi C, Di Cagno R, Gobbetti M and Ercolini D 2014 The same microbiota and a potentially discriminant metabolome in the saliva of omnivore, ovo-lacto-vegetarian and vegan individuals *PLoS One* **9** e112373

[47] Kim M K, Cho S W and Park Y K 2012 Long-term vegetarians have low oxidative stress, body fat, and cholesterol levels *Nutr. Res. Pract.* **6** 155-161

[48] Aghdassi E and Allard J P 2000 Breath alkanes as a marker of oxidative stress in different clinical conditions *Free Radic. Biol. Med.* **28** 880-886

[49] DeMaster E G and Nagasawa H T 1978 Isoprene, an endogenous constituent of human alveolar air with a diurnal pattern of excretion *Life Sci.* **22** 91-97

[50] Sukul P, Schubert J K, Zanaty K, Trefz P, Sinha A, Kamysek S and Miekisch W 2020 Exhaled breath compositions under varying respiratory rhythms reflects ventilatory variations: translating breathomics towards respiratory medicine *Sci. Rep.* **10** 14109

[51] Lärstad M A E, Torén K, Bake B and Olin A C 2007 Determination of ethane, pentane and isoprene in exhaled air - Effects of breath-holding, flow rate and purified air *Acta Physiol.* **189** 87-98

[52] Buszewski B, Kesy M, Ligor T and Amann A 2007 Human exhaled air analytics: Biomarkers of diseases *Biomed. Chromatogr.* **21** 553-566

[53] Kohlmüller D and Kochen W 1993 Is n-pentane really an index of lipid peroxidation in humans and animals? A methodological reevaluation *Anal. Biochem.* **210** 268-276

[54] 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** 762-770

[55] Kushch I, Arendacká B, Štolc S, Mochalski P, Filipiak W, Schwarz K, Schwentner L, Schmid A, Dzien A, Lechleitner M, Witkovský V, Miekisch W, Schubert J, Unterkofler K and Amann A 2008 Breath isoprene - Aspects of normal physiology related to age, gender and cholesterol profile as determined in a proton transfer reaction mass spectrometry study *Clin. Chem. Lab. Med.* **46** 1011-1018

[56] Španěl P, Davies S and Smith D 1999 Quantification of breath isoprene using the selected ion flow tube mass spectrometric analytical method *Rapid Commun. Mass Spectrom.* **13** 1733-1738

[57] Harshman S W, Pitsch R L, Davidson C N, Lee E M, Scott A M, Hill E M, Mainali P, Brooks Z E, Strayer K E, Schaeublin N M, Wiens T L, Brothers M C, Drummond L A, Yamamoto D P and Martin J A 2020 Evaluation of a standardized collection device for exhaled breath sampling onto thermal desorption tubes *J. Breath Res.* **14** 036004

[58] Sukul P, Richter A, Schubert J K and Miekisch W 2021 Deficiency and absence of endogenous isoprene in adults, disqualified its putative origin *Heliyon* **7** e05922