On-line breath gas analysis in unrestrained mice by hs-PTR-MS

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Abstract

The phenotyping of genetic mouse models for human disorders may greatly benefit from breath gas analysis as a noninvasive tool to identify metabolic alterations in mice. Phenotyping screens such as the German Mouse Clinic demand investigations in unrestrained mice. Therefore, we adapted a breath screen in which exhaled volatile organic compounds (VOCs) were online monitored by proton transfer reaction mass spectrometry (hs-PTR-MS). The source strength of VOCs was derived from the dynamics in the accumulation profile of exhaled VOCs of a single mouse in a respirometry chamber. A careful survey of the accumulation revealed alterations in the source strength due to confounders, e.g., urine and feces. Moreover changes in the source strength of humidity were triggered by changes in locomotor behavior aS_{Mi}ce showed a typical behavioral pattern from activity to settling down in the course of subsequent accumulation profiles. We demonstrated that metabolic changes caused by a dietary intervention, e.g., after feeding a high-fat diet (HFD) a sample of 14 male mice, still resulted in a statistically significant shift in the source strength of exhaled VOCs. Applying a normalization which was derived from the distribution of the source strength of humidity and accounted for varying locomotor behaviors improved the shift. Hence, breath gas analysis may provide a noninvasive, fast access to monitor the metabolic adaptation of a mouse to alterations in energy balance due to overfeeding or fasting and dietary macronutrient composition as well as a high potential for systemic phenotyping of mouse mutants, intervention studies, and drug testing in mice.

Introduction

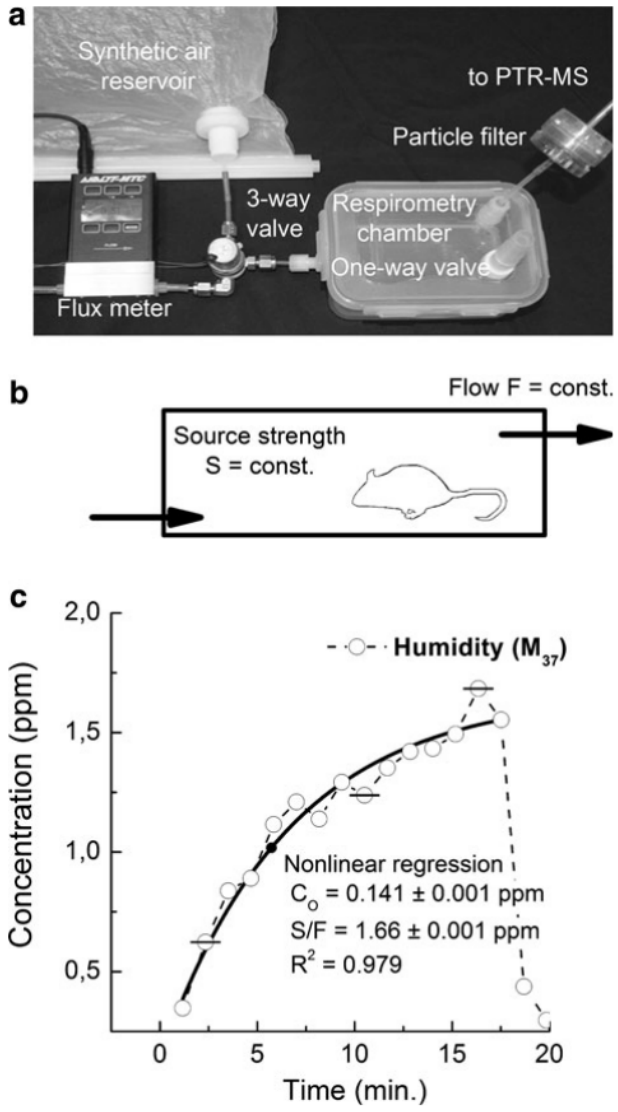
Numerous endogenous volatile organic compounds (VOCs) are exhaled while breathing. VOCs mainly originate from metabolic processes in cells and are distributed via the circulating blood; they pass the alveolar-capillary membrane, enter the volume of the lungs, and are finally exhaled. The composition and abundance of VOCs in breath gas can be used to draw conclusions about metabolic status and specific physiological processes. The analysis of VOCs in exhaled breath gas samples has already been used in studies on human disorders (Whittle et al. 2007), such as cancer (Bajtarevic et al. 2009; Phillips et al. 1999a) or on healthy humans (Smith et al. 2007). By means of gas chromatography and mass spectrometry, a total of about 3500 VOCs was identified in a study comprising 50 healthy humans (Phillips et al. 1999b). Notably, in single subjects of the study, an average number of only about 200 substances could be detected, 27 of which were ubiquitously found in all participants. This distribution suggested (a) that there is a considerable inter-individual variability and (b) that this variability could provide a high diagnostic potential for the analysis of shifts in VOC abundance to draw conclusions about the attributed specific metabolic processes. State-of-the-art breath gas analysis (Cao and Duan 2007) in

humans is based on mass spectrometry in combination with preselection or selective ionization (e.g., gas chromatography mass spectrometry (Miekisch et al. 2004), selected ion flow tube mass spectrometry (Smith et al.2007), ion mobility spectrometry (Vautz et al. 2010), and proton transfer reaction mass spectrometry (PTR-MS) (Hansel et al. 1998; Lindinger et al. 1998)). Breath gas analysis was only rarely applied in small rodents. As early as in 1974, Riely and Cohen reported on the ethane evolution in mice stimulated and diminished by prior injection of drugs (Riely et al. 1974). Further analyses were also restricted to single VOCs, such as ethanol (Cope et al. 2000), ethane (Risby et al. 1999), or $^{13}CO_2$ (Friedrich et al. 2011; Isken et al. 2010). A range of VOCs in the breath of intubated spontaneously breathing mice during anesthesia was provided by ion mobility spectrometry coupled with a multicapillary column (Vautz et al. 2010). Very recently Aprea et al. reported on a fast online method with a nose tube for collection and analysis of exhaled breath in a single rat by PTR–TOF-MS (Aprea et al. 2012). PTR-MS measures a wide spectrum of VOCs online, fast and almost simultaneously over a range of concentrations from ppm (part-per-million) down to ppt (part-per-trillion) levels (Hansel et al. 1998). To our knowledge, the implementation of this method in studies including animal models, such as the laboratory mouse, is not applied even though this species is widely used in biomedical and genome research (Hrabe´ de Angelis and Strievens 2001). Due to the recent technological progress in PTR-MS, the standardized investigation of exhaled VOCs of unrestrained, small animals, such aS_{Mi}ce has the potential to provide large data sets for metabolic profiling. These data contribute to the characterization of the specific metabolic state of individual mice during screening procedures, for example, in comprehensive phenotyping centers for mouse mutants such as the German Mouse Clinic (GMC) (Fuchs et al. 2009). Phenotyping of genetic mouse models for human disorders had been identified as area of highest relevance in genome and biomedical research. Therefore, the aim of our study was to develop a PTR-MS setup suitable for unrestrained screening of exhaled VOCs in mouse models of human diseases. The major challenges of the method described here were to adapt the PTR-MS sampling of breath gas to the dimensions of a mouse, to sort out VOCs that are due to contaminations (urine, feces, food, etc.) confounding the analysis, to identify VOCs that are mainly due to exhaled breath of mice, and to develop a data analysis procedure that is suitable to be integrated into a high-throughput phenotyping screen. In this article, we describe a basic setup—consisting of a respirometry chamber and breath gas analysis (PTR-MS)—to implement this noninvasive and very sensitive online method in the workflow of the energy metabolism module of the GMC.

Methods

Measuring setup

The basic measuring setup is depicted in Fig. 1a. The respirometry chamber consisted of a transparent polypropylene box (volume: 600 ml). A large top cover opened up easily so that the mouse could be transferred easily into and out of the box. The respirometry chamber was connected to the PTR-MS (air flow 90 ml min^{-1}) and face-to-face to a Teflon bag reservoir (capacity of 10 l, Welch Fluorocarbon Inc., Dover, USA) filled with VOC free, synthetic air (20 % oxygen, 80 % nitrogen, concentration of hydrocarbons B0.1 ppm, Linde AG, Germany). The tube feed-throughs were made of polypropylene and all connecting tubes were made of Teflon and a PFTE membrane filter (pore size 2.0 lm, PALL Corporation, Ann Arbor, USA) cleaned the air stream into the heated inlet capillary of the PTR-MS from particular contaminations. Alternatively, a 3-way valve opened the ventilation path (flow 2.5 l min^{-1}, volume 6 l). Keeping the disturbance of a mouse in the box as small as possible, the jet of synthetic air into the box was deflected through a simple baffle fixed at the outlet of the feed-through used for the ventilation. A one-way mouthpiece (ENVITEC-WISMAR GmbH, Wismar, Germany) mounted on the top cover opened during ventilation to prevent an overpressure in the box. The leak-proof nature of the feed-throughs and of the top cover was carefully and routinely controlled (blank profile). The inlet capillary and the drift tube of the PTR-MS were kept constant at 60 ºC, whereas the respirometry chamber was in equilibrium with ambient temperature comparable to home cage conditions. The repeated flushing of the chamber and continuous diluting of the headspace in presence of a mouse kept the temperature within the chamber at ambient temperature (24 ± 2 ºC). To control the conditions in the respirometry chamber inducing for hypercapnea, we determined typical CO_2 concentrations during a VOC measurement using a portable carbon dioxide analyzer (CA-10, Sable Systems International Inc, Las Vegas, NV, USA). When adjusted to the air flow of 90 ml min^{-1} into the PTR-MS, the concentration of CO_2 was <=3 vol% at the end of the accumulation period, i.e., 20 min after the flushing.



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107 **Fig. 1** a Basic respirometry setup. The respirometry chamber (volume: 600 ml) is connected to the PTR-
108 Quad-MS (flow 90 ml min^{-1}) and face-to-face to a bag reservoir filled with VOC free, synthetic air. The
109 Teflon filter cleans the air stream into the PTR-MS from particular contaminations. Alternatively, the
110 three-way valve opens the ventilation path (flow 2.5 l min^{-1}, volume 6 l). b Description of the dynamic
111 changes in the respirometry chamber by a simple chamber model. The compartment model assumes that a
112 mouse exhales a compound of mass M with a constant source strength S into a head space volume of 600
113 ml. The constant flow of gas F through the chamber dilutes the concentration of this compound. The gas
114 reservoir connected to the chamber maintains equilibrium in pressure. c Accumulation profile of humidity
115 recorded in the respirometry chamber and fitting of the profile with the solution function of the chamber
116 model. The nonlinear regression fit of the solution function of the model equation to a measured profile of
117 humidity determines the values of the two free parameters, the concentration at T = 0, C_0 and the ratio S/F

PTR-MS

In the development of the basic set-up and the pilot nutrition study, a high-sensitivity proton transfer reaction mass spectrometer (hs-PTR-Quad-MS; Ionicon Analytic GmbH, Innsbruck, Austria) was used. Details of the measuring principle and applications have previously been reported in detail (Lindinger et al. 1998). In brief, the breath sample is injected into a drift tube together with a beam of hydronium ions. VOCs with a proton affinity higher than that of the hydronium ion are ionized by the proton transfer reaction. According to the selective ionization, VOCs can be measured with concentrations ranging from ppm down to the ppt level in the presence of high concentration of the main components of exhaled breath (e.g., nitrogen, oxygen, humidity, and carbon dioxide). As a result of the proton transfer reaction, all the mass numbers refer to protonated mass numbers. The PTR-MS was operating with a count rate of the primary ions (H3O+) of *2.0 9 10^7 counts*s^{-1}, and the percentage of the minor precursor ion O_2^+ was kept below 1 % of the count rate of the primaries. The pressure in the drift tube was *2.2 mbar. The drift tube and all connecting tubes from the respirometry chamber to the drift tube inlet were kept at a temperature of 60 ºC for preventing condensation of the humidity. The measured raw count rate of a signal at mass M_i, cps(M_i) was normalized to the count rate of the precursor ion and the water cluster at M_{37}according to (Hansel et al. 1998), denoted as ncps. Other water clusters were neglected because the count rate was at least two orders of magnitude smaller than that of the first water cluster (e.g., cps(M_{55})/cps(M_{37}) ≤ 0.003). The PTR-MS instrument was calibrated with standard gaS_{Mi}xtures with a specified concentration of 100 ± 10 ppb for each compound (Aromatic subset mix, Scott Speciality Gases, Plumsteadville, USA). A daily control of the optimum saturation voltage the SEM was done to detect changes in the sensitivity. For the calculation of the volume ratio (e.g., in part per billion, ppb) for a single compound, the standard formula for PTR-MS concentrations was used (Hansel et al. 1998). The relative uncertainty in the derived concentration in ppb due to the uncertainty in the measured count rate estimated to 10 % or less along within an accumulation measurement. The PTR-MS recorded full mass spectra in the mass range from m/z = 21 to 160. With a chosen dwell time of 500 ms, a full mass scan took 70 s. Therefore, a time series of mass scans recorded one-by-one built up a VOC profile. The used PTR-MS was equipped with a quadrupole mass separator tuned to unit mass resolution. The assignment of a compound to mass was in most cases tentatively especially at higher masses. The assignment of methanol to $M_{33}$was beyond question, and the assignment of M_{59} to acetone was highly supported by the correlation between the count rates of the two acetone isotopes at M_{59} and M_{60}. In the PTR-Quad-MS measurements, the adjoined masses of CO_2 and acetaldehyde were not resolved. In this feasibility study, the validation by GC–MS was not yet available because of the complex mixture in presence of humidity. The monitoring

of the repeatability of exhaled VOCs in separate samples of male C57BL/6J mice were done using a PTR–TOF2000-MS (Ionicon Analytic GmbH, Innsbruck, Austria), which was equipped with a drift-tube common in the hs-PTR-MS. The TOF mass-analyzer principle has been described elsewhere (Jordan et al. 2009).

Hierarchical cluster analysis

Standard hierarchical cluster analysis (HCA) was carried out using the hclust function in the Stats Package of the statistical computing program R (http://www.R-project.org). Input variables (e.g., times series of the concentration of $masS_{Mi}$) were scaled to the mean and standard deviation of the time series, and the complete agglomeration method was applied. The scaling of the variables to a mean rather than to the median of the time series was supported by the finding that in the former case, the undisturbed isotopes of a compound were represented by a forklike subcluster. Noisy mass variables were excluded from the input matrix of variables. As a rule, all masses, normalized count rates of which fall below a threshold value of 2 ncps for at least half of the values in a time series, were excluded.

Mice housing and nutrition

For the diet intervention study, 12-week-old male C57BL/6N mice were ordered from Charles River Laboratories (Sulzfeld, Germany). The study on repeatability of VOC accumulation profiles was conducted in 8–22-week-old male C57BL/6J mice from the in-house breeding stock. Upon arrival, mice were housed in individually ventilated cages (Ventirack, Biozone, UK) at the GMC. The animal experiments were approved by the Government of the Federal State of Bavaria, Germany. The animal facility was tested for microorganisms according to the FELASA recommendations for mouse health monitoring (Nicklas et al. 2002). Husbandry conditions were as follows: room temperature 20–24 ºC, air humidity 50–60 %, 20 air changes per hour, and light regimen on a 12 h light/dark cycle. Wood shavings (Altromin GmbH, Germany) were applied as bedding material. Mice had access to drinking water and food ad libitum. The mice were fed with a standard lab chow (no. 1314, Altromin, Lage, Germany). For the high-fat diet (HFD) challenge, mice were fed a purified experimental diet containing 60 energy% of fat (E 15741-347, Ssniff, Soest Germany). Mice were weighed in the morning once every week and at the day of the VOC analysis. VOCs in mouse breath gas were monitored immediately before the diet intervention and three weeks after feeding the HFD during daytime. To minimize daily variation in VOC concentrations, mice were measured exactly in the same order and at the same time of the day as in the initial trial.

Minute volume of breath of mice

The minute volume of mice was measured in a whole body plethysmograph (Buxco Electronics, Sharon, Connecticut, USA) according to the principle described by Drorbaugh and Fenn (1955). Through calibration, the pressure swings arising from inspiration and expiration of the mouse are transferred in flow and volume signals. Automated data analysis provides tidal volumes, respiratory rates, and minute ventilation at 10-s intervals. During the measurements, the air in the chamber volume (600 ml) was diluted with clean air at a flow rate of 200 ml min^{-1}. A flushing of the plethysmograph for 2 min at a rate of 3 l min^{-1} minimized the concentration of the VOCs before a profile was recorded. At the same time, the PTR-MS was connected to the chamber, and a constant flow of 47 ml min^{-1} was bypassed to the PTR-MS. To match the time resolution of the pressure measurement, the PTR-Quad-MS recorded only 10 masses with a high count rate in the multiple ion mode with reduced dwell times.

Results

To develop a robust and efficient but also specific and highly automated phenotyping method for mice based on breath gas analysis poses challenges in many respects. Our solution rested upon the accumulation of breath exhaled of a single mouse kept in a clean respiratory chamber to overcome the limitation of minute volume of breath in an online analysis set-up. Accumulation profiles disturbed by confounders from urine and feces were discarded. VOCs in the head space predominantly originating from exhaled breath were described by the dynamic change during the accumulation, i.e., the source strength. Activity levels clearly affect breathing frequency as well as the exhaled minute volume. The adaption to the breath gas analysis device (hs-PTR-MS) and the basics of the strategy are described below.

Accumulation profiles of exhaled VOCs

The disposable gas volume of a single breath of a mouse [tidal volume 0.2–0.3 ml (Reinhard et al. 2002)] was out of scope to be analyzed by PTR-MS. Therefore, the accumulation of exhaled VOCs was monitored within the defined volume of a clean small respirometry chamber and after controlled ventilation with clean air. After placing a mouse into the respirometry chamber, a tenfold ventilation of the volume of the respirometry chamber with synthetic air resets the humidity as well as the concentration of all VOCs to a low basal level. Then, the accumulation of VOCs was monitored for 20 min by recording mass spectra one by one. As an example, the signal for humidity at m/z = 37 (M 37) showed an up to sixfold increase from initially very low levels (Fig. 1c). The signal of M_{37}in an empty respiratory chamber increased only twofold to roughly 10 % of the level of M_{37}in the presence of a mouse.

Determination of the source strength of exhaled VOCs

The quantification of the exhaled concentration of VOCs using the difference between end and starting levels requires the careful monitoring of basal and saturation values. Keeping the residence time of a mouse in the respirometry chamber short resulted in accumulated concentrations which generally were below saturation. Therefore, our quantification scheme was based on the dynamic changes during the accumulation. With the help of a respirometry chamber model (Fig. 1b), the source strength S of an exhaled VOC, given in ppb ml min^{-1}, was determined. In a first approach, we described the concentration of a VOC C_i at time t through a constant source strength S_i which is counterbalanced by the steady flow rate F out of the respirometry chamber.

(1) $$\frac{dC_i(t)}{dt} = \frac{1}{V}\left(S_i - C_i(t) \times F\right)$$

The solution of the equation (1)

(2) $$C_i(t) = C_0 \times e^{-\frac{F}{V}t} + \frac{S_i}{F}\left(1 - e^{-\frac{F}{V}t}\right)$$

includes two free parameters, C_0 and S_i/F. C_0, which is the basal concentration after the ventilation, can be experimentally kept small by sufficiently flushing the respirometry chamber with clean synthetic air. Because F was constant, $S_i$can be calculated from the second free parameter. A nonlinear least-square fitting was applied to the monitored profiles between two ventilations (Fig. 1c). When applied to the undisturbed humidity profiles, the solution of the respirometry chamber model described the profiles sufficiently well (e.g., for humidity R^2= 0.979), suggesting that it was possible to calculate the source strength from the respirometry chamber model as a main factor reflecting the concentrations of specific VOCs in the exhaled breath.

Identifying VOCs originating from breath

Every breath of a mouse replaced a small volume of dry air in the respirometry chamber with saturated humidity. Therefore, the accumulation profile of humidity measured at $(H_2O)_2H^+$, M_{37}, was the most distinct breath-driven profile. The visual inspection of the shape of VOC profiles led to a sample of 24 masses which showed an accumulation profile similar to the profile of humidity. Examples are depicted in (cp. Fig. 2a–d). This led to the assumption that a similarity in shape indicated their origin from breath. To systematically identify these VOCs, a HCA was applied to the accumulation profiles of all recorded masses. Noteworthy, the clustering tree started at the lowest level with the mass of humidity M_{37}(Fig. 2e). The members of this cluster treebranch resembled the VOCs with the most similar profiles to the pattern of humidity (e.g., M_{41}, M_{45}, M_{61}).

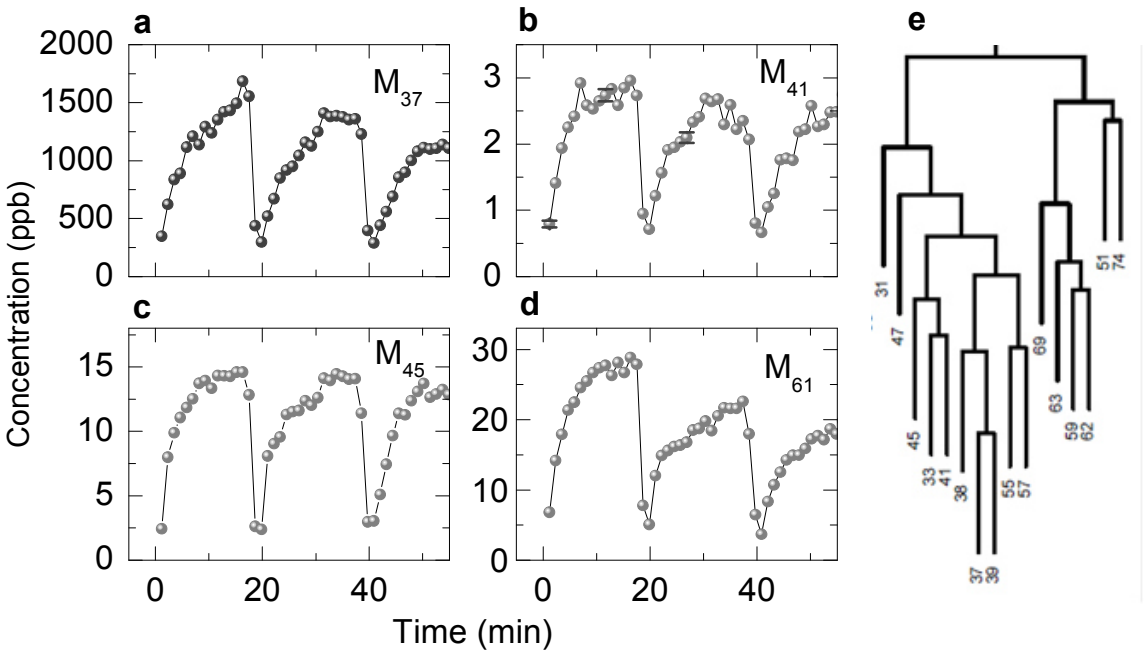


Fig. 2 Selection of similar VOCs profiles and section of the cluster tree of a hierarchical cluster analysis (HCA) applied to the accumulation profiles. a Humidity, detected as protonized water clusters $H_2O{\cdot}H_3O^+$ (M_{37}) is the most prominent constituent in exhaled breath. By visual inspection, b M_{41} (fragment of propanol), c M_{45} (acetaldehyde/CO_2), and d acetic acid (M_{61}) show very similar profiles. The relative uncertainty in the derived concentration in ppb due to the uncertainty in the measured count rate estimated to 10 % or less. Note that the error bars due to the counting statistics were still about the size of the symbols in Fig. 2b, e.g., the profile with a low concentration. e Section of the cluster tree obtained when the HCA is applied to the matrix of profiles of 120 masses of a PTR-MS analysis. Remarkably, the clustering starts with the humidity masse$S_{M37}$and M_{39} without any supposition. The branch includes the masses with the most similar profiles to that of the humidity profile

Sensing environmental confounders

Routinely, four accumulation profiles were recorded in a sequence with every mouse. First, a baseline measurement of the clean, empty box was screened for leakage and contaminations. Then, three accumulation profiles in succession were recorded with a mouse. Within a sequence of profiles, the incidence of confounders resulted in distortions of the profiles, e.g., mice released small amounts of urine

and feces into the respirometry chamber during accumulation profiling which polluted the gas compartment (head space) in the chamber. Detailed head space analysis of these confounders with PTR-MS had shown disturbing effects interfering with exhaled VOCs. A unique urine and feces marker at M_{49}(tentatively assigned to methanethiol) could be detected, which remained at a low level of 0.1 ppb but immediately crossed this threshold concentration in presence of small urine droplets in the chamber. In addition, a droplet of urine added an additional source of humidity, and therefore, clearly increased the concentration of humidity. Based on these observations, profiles of VOCs were regarded as undisturbed and selected for further analysis when the concentration of M_{49} was below 0.1 ppb. Training and adapting of the mouse to the respirometry chamber before the measurement considerably reduced the contamination rates due to urine or feces to roughly to one in four cases.

Variability of accumulation profiles

The repeatability of the measured accumulation profiles were analyzed in short- (subsequent profiles), medium-(day-by-day), and long-term (weeks) investigations. The three sequenced accumulation profiles recorded from a mouse revealed a frequent variation (cp. Fig. 2a). The saturation levels of profiles of humidity recorded one after another and therefore the source strength S_{M37} often decreased. By observing the behavior of the mouse during the profile measurement variation could be related to behavioral patterns, in which an initially active mouse settled down from profile to profile. In a sample of 14 male C57 BL/6N mice, the median of S_{M37} of a settled mouse (3rd profile) is roughly 30 % lower than that of an active mouse (1st profile) (Fig. 3a). To evaluate the link between activity and source strength of humidity, we simultaneously measured the respiration frequency in a plethysmograph and the profile of VOCs with the PTR-MS connected to this chamber exemplarily in two male C57 BL/6N mice. The derived source strength increased with respiratory frequency (11.3 ± 2.9 ppm ml min^{-1} per 100 breath$S_{Mi}n^{-1}$) and clustered at low and higher respiration frequencies. The day-by-day repeatability of the source strength was investigated in a sample of 12 male C57 BL/6J mice (mean weight 27.7 ± 1 g). The mean source strength of humidity was roughly 15 % lower at day 3–5 compared with day 1 and 2 (Fig. 3b). Again, day-by-day variation in the source strength of humidity could be related to behavioral patterns especially locomotor activity of the mouse in the respirometry chamber, e.g., a more settled-down behavior at day 3–5 compared to the first 2 days. In a long-term investigation, we repeatedly determined the source strength of VOCs in a sample of 12 male C57BL/6J mice with an age of 8–9 weeks (mean body mass: 25.6 ± 2.3 g), 10 weeks (mean body mass: 25.9 ± 2.2 g), and 20–22 weeks (mean body mass: 31.1 ± 3.4 g), c.p. Fig. 3c. With increasing age and increasing body mass, the source strength of humidity shifted to a higher mean and broadened (weeks 8–9: 1,241 [99] ppm ml min^{-1}, week 10: 1,315 [209] ppm ml min^{-1}, weeks 20–22: 1,590 [450], ppm ml min^{-1}, values in brackets denote the Q3–Q1 interquartile

range). We observed a relation between humidity source strength and body mass following an allometrical mass scaling exponent of 0.69 ± 0.17 (Lighton 2008) that is typically found also for the relation of energy turnover and body mass. The difference in humidity may be related to the age-related increase in body mass and may be seen as a general read-out for body mass-dependent increase in energy expenditure.

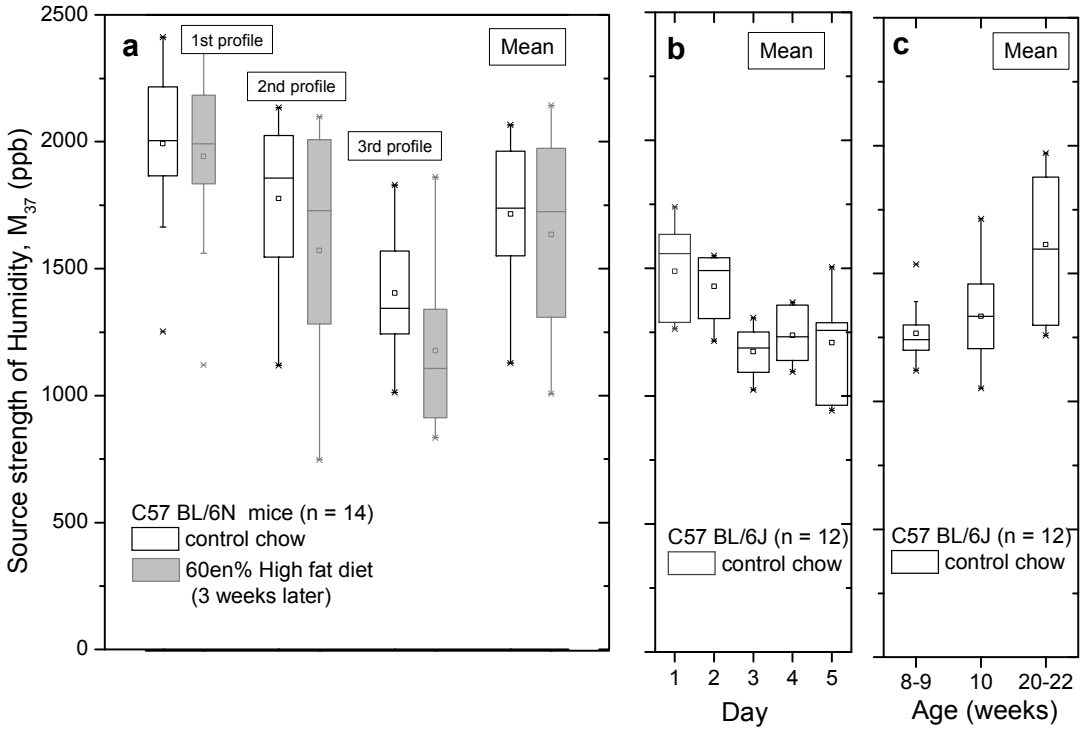
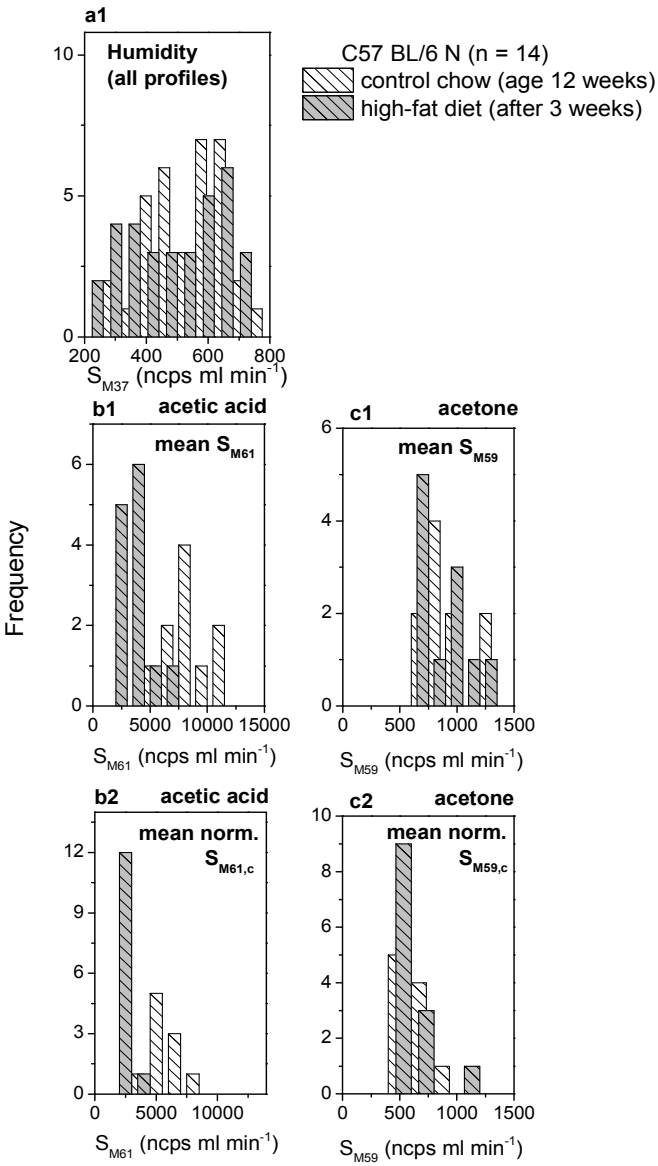


Fig. 3 The repeatability of the measured accumulation profiles in short- (subsequent profiles), medium- (day-by-day), and long-term investigations. a Source strength S_{M37} in a sample of 14 male C57 BL/6N fed with control chow (age 12 weeks, mean body weight 32 ± 3 g) and after a HFD (age 15 weeks, mean body weight 40.8 ± 3.8 g). As a rule, the 1st profile belonged to an active, and the 3rd profile to a settled down mouse. b The day-by-day repeatability of the mean source strength S_{M37} in a sample of 12 male C57 BL/6J mice (mean body weight 27.7 ± 1 g). c Long-term investigation of the mean source strength S_{M37} in a sample of 12 male C57 BL/6J mice: age of 8–9 weeks (25.6 ± 2.3 g), 10 weeks (25.9 ± 2.2 g), and 20–22 weeks (31.1 ± 3.4 g). The mean body mass is given in parentheses

Significant changes in the source strength in response to a dietary intervention

Our main interest was whether a dietary intervention, i.e., feeding a HFD resulted in a statistically significant shift in the source strength of exhaled VOCs. The source strength of 24 breath-driven VOCs was determined in a sample of 14 mice before and after feeding a purified HFD (60 % of the energy from fat). The mean body mass of the C57BL/6N mice initially fed with a control chow diet increased from 32 ± 3 g to 40.6 ± 3.8 g within three weeks of HFD. After HFD challenge, the S_{M37} again depended on behavioral characteristics (i.e., calming down) during the measurement of the subsequent accumulation profiles (Fig. 3a) as could be shown before. Evaluating the frequency distribution of S_{M37} from all humidity profiles suggested a bimodal shape (Fig. 4a). This bimodal distribution reflects the observation that mice showed rather high levels of locomotor activity just after the transfer into the chamber, but calmed down when it was kept for a longer time in the chamber. Therefore, S_{M37} can be regarded as a monitor of locomotor activity, whereas other VOCs were clearly influenced by HFD. Irrespective of the behavioral link, e.g., S_{M61} showed distinctly lower levels after feeding the HFD compared with initial levels (Fig. 4b1), whereas other VOCs, e.g., S_{M59}, did not (Fig. 4c1). The obvious changes due to varying levels of locomotor behavior should be accounted for before comparing the derived source strengths of exhaled VOCs. In a first approach, we decided to normalize source strengths to the mean source strength $S_{M37,c}$ = 300 ncps ml min^{-1} of obese mice at rest. Hence, with S_{M37} /$S_{M37,c}$, a correction factor was derived, and the S_{Mi} values of the other breath-driven VOCs were corrected, i.e., $S_{Mi,c}$. The distribution of the corrected $S_{M61,c}$ and $S_{M59,c}$ are depicted in Fig. 4b2–c2. Again, $S_{M61,c}$ showed distinctly lower levels after HFD compared with initial levels, whereas $S_{M59,c}$ did not. Several breath-driven VOCs showed distinctly lower levels after HFD compared with initial levels when fed with the control chow, especially M_{33} (methanol), M_{43} (propanol), M_{61} (acetic acid), and M_{75} (propionic acid) (Fig. 5a). The tentatively mass-to-substance assignment is given in parentheses. In the sample of 12 male C57 BL/6J mice S_{M33} of methanol—the substance showing strongest effects of HFD—did not show age-related changes (Fig. 5b). The outcomes of the two feed modes of diets, i.e., control chow and HFD, were compared using the Mann-Whitney-test (significance level 0.05, two-tailed). Table 1 summarizes the statistical significance of the separation of the two samples dependent of the derived source strength: the mean source strength S_{Mi} of three accumulation profiles in series, source strength of the (3rd) accumulation profile of the settled mouse S_{Mi},3, and the mean normalized source strength $S_{Mi,c}$. In the case of mean S_{Mi}, a subset of seven masses (M_{33}, M_{43}, M_{45}, M 47, M_{61}, M_{75}, and M_{93}) was significantly separated (P \ 0.001). As a rule, the separation improved if normalized $S_{Mi,c}$ were used. With the $S_{Mi,3}$, the source strength of the last profile in a mouse measurement, the separation was still significant, but with lower P values in comparison with results for the mean S_{Mi} and $S_{Mi,c}$.



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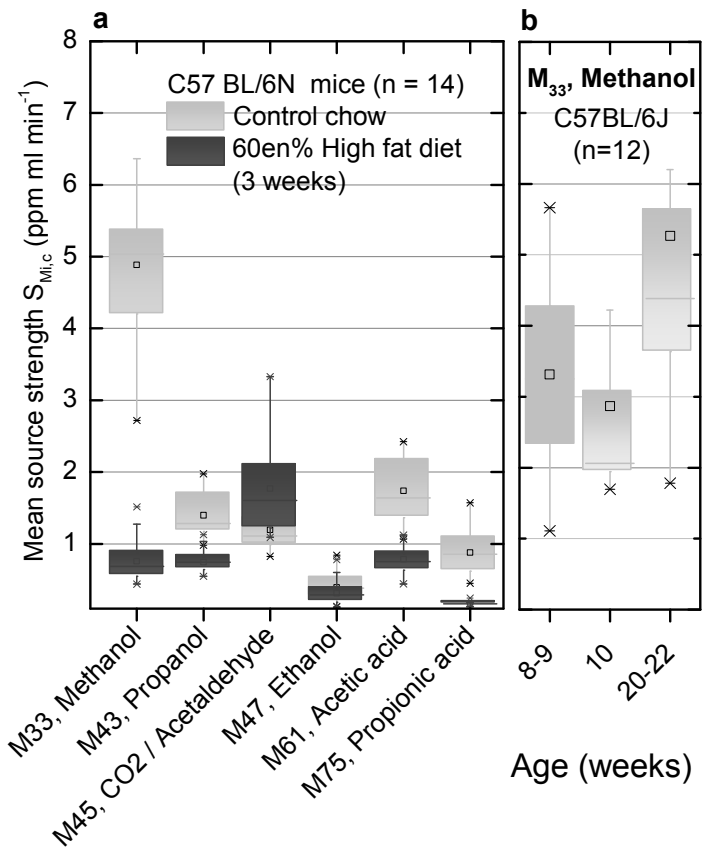
343 **Fig. 4** Distributions of the source strengths S_{M37}, S_{M61}, and S_{M59}, evaluated from C57 BL/6N mice initially
344 fed with a control chow and after a three-week HFD. a1 The bimodal distributions of S_{M37} reflect the
345 observation that just after its transfer into the box, an active mouse comes to rest with the increasing
346 residence time in the box. In addition, the histogram of the source strength of obese mice shows a shift
347 to lower source strengths. b1, c1 The HFD reduced significantly the source strength of acetic acid, S_{M61}
348 but not of acetone, S_{M59}. b2, c2 The normalized $S_{Mi,c}$ to obese mice at rest as a reference. Ncps denotes
349 the count rate normalized to count rate of the precursor ion and the water cluster at M_{37}according to
350 (10) (c.p. see ‘‘PTR-MS’’ section)



351

352 **Fig. 5** Change in the corrected source strength $S_{Mi,c}$ of selected VOCs exhaled by C57 BL/6N mice
353 subjected to a diet challenge and long-term variability of methanol in C57 BL/6J mice fed with control
354 chow. a The source strength $S_{Mi,c}$ of selected VOCs in the exhaled breath of 14 male C57 BL/6N mice
355 shift significantly to smaller values after a 3-week-long feeding up with 60 energy% HFD. In the case of
356 methanol, S_{M33} decreased down to 10 % of the initial value of chow-fed mice. The boxes show the
357 medians, Q1 and Q3, and the symbols denote 5 and 95 percentiles. b In a control group of C57 BL/6J
358 mice, the exhaled methanol level did not drop when fed with the control chow for more than 10 weeks

359

360 **Table 1** Statistical significance of the separation of the source strength distributions of two samples of
361 mice fed the control chow and HFD in dependency of the derived source strength

362 Outcome of the two feedings were tested using the Mann-Whitney Test (significance level 0.05, two-
363 tailed) using (1) mean source strength S_{Mi} (n1 = 10, n2 =13), (2) source strength of the 3rd profile $S_{Mi,3}$
364 (n1 = 11, n2 =10) and (3) the normalized $S_{Mi,c}$ (n1 = 10, n2 =13) to obese mice at rest as a reference

threshold. The sample size of mice is noted in parentheses and the masses (substances) whose distributions of the two groups differed significantly ($P < 0.001$) were shaded.

Protonated mass number, MH^+	Substance (tentatively assigned)	P-value		
		mean S_{Mi}	$S_{Mi,3}$	mean$S_{Mi,c}$
M_{31}		0.059	0.42	0.4
M_{33}	methanol	$6.3\ 10^{-5}$	$1.2\ 10^{-4}$	$6.3\ 10^{-5}$
M_{37}	water cluster	0.78	0.098	1
M_{41}		$2.4\ 10^{-3}$	0.038	$3.2\ 10^{-3}$
M_{43}	propanol fragment	$3.6\ 10^{-4}$	$3.2\ 10^{-4}$	$6.3\ 10^{-5}$
M_{45}	CO_2/acetaldehyde	$6.3\ 10^{-5}$	$1.2\ 10^{-4}$	$3.2\ 10^{-3}$
M_{47}	ethanol	$3.8\ 10^{-4}$	0.045	0.18
M_{59}	acetone	0.31	0.062	0.44
M_{61}	acetic acid	$1.06\ 10^{-4}$	0.062	$6.3\ 10^{-5}$
M_{75}	propionic acid	$6.3\ 10^{-5}$	$1.2\ 10^{-4}$	$6.3\ 10^{-5}$
M_{93}		$7.0\ 10^{-3}$	0.027	0.088

Discussion

Methodological aspects

We developed a respirometry setup adapted to PTR-MS and suitable for the screening of exhaled VOCs in the breath of unrestrained laboratory mice. We hypothesized that VOC signatures in exhaled breath reflect the metabolic status of mice investigated in experimental studies to decipher causes and pathogenesis of human metabolic disorders. Therefore, the access to this group of substances—VOCs—previously not addressed by other technologies (approaches such as metabolomics or proteomics, etc.) may provide valuable additional information regarding dysfunctional metabolic pathways related to disease. Here, we report results of initial feasibility studies evaluating the practicability, robustness, and usefulness of VOC breath gas analysis in mice. Contrary to directly sampling the breath gas via intubation or specifically adapted mouthpieces, our setup is based on head space measurements of unrestrained mice making use of the accumulation of VOCs in a respirometry cage. While implementing this setup, it cannot be ruled out that VOCs do not only originate from the blood stream being exchanged and exhaled via the lung. Therefore, we aimed to specify which other sources of VOCs may confound the analysis and which

sources of variability in VOCs can be identified. Mouse urine contains a considerable number of VOCs, which are unique to this species, and some of them strongly depend on sex and endocrine status (Schwende et al. 1986). With PTR-MS, the signal at, tentatively assigned to methanethiol, was identified as a unique monitor for urine and feces. This signal was at low levels of around 0.1 ppb if the mouse in the respirometry chamber did not urinate or defecate. By controlling this sensitive and immediate monitor marker, VOC profiles that did not satisfy specific criteria like smoothness of the profile and concentration of a marker below a threshold value were rigorously excluded. The evaporation rate of VOCs from urine varies with the urine sources (i.e., droplet size, spots and wet fur), and this does not allow an estimation of the contribution to the concentration in the headspace. In this feasibility study, the link of particular masses to chemical substances was not evaluated by means of other methods (e.g., GC–MS). However, in PTR-MS, a correlation plot of isotopes of a compound may support a tentative assignment of the compound behind a mass. The assignment of methanethiol to $M_{49}$was supported by the finding that the concentration of M_{51} corresponded with 5 % of the count rate of M_{49}roughly to the second-most abundant isotope of methanethiol, M_{51}. During the measurement of the accumulation profiles, a mouse is likely to vary in physical activity levels. After being transferred to the respirometry chamber, the mouse exhibited exploratory behavior and grooming, but it settles down after habituation. Such changes in physical activity levels resulted in variation of the minute volume of exhaled breath. Lung mechanics is profoundly affected by breathing frequency (Bates and Irvin 2003). Reinhard et al. (2002) reported a great diversity between inbred mice strains, including the C57BL/6J strain, for lung function parameters. Among other differences, the total lung capacity and the pulmonary diffusing capacity for carbon monoxide varied by 50 % and the static lung compliance by a factor of 2 between the strains. On the other hand, there was no simple allometric relationship of lung size with body mass. These results were based on defined respiratory maneuvers in anesthetized mice. In our unrestrained plethysmography test in consciousS_{Mi}ce, the changing of physical activity resulted in a decrease in S_{M37} by roughly 30 % when the mouse settled down. The normalization to a VOC which relates to a reference behavioral state, e.g., the source strength of humidity of a settled mouse, may improve the statistical significance of the difference in the source strengths of exhaled VOCs due to a nutrition challenge. However, the impact of this normalization on the distribution of S_{Mi} has to be carefully reviewed in order not to distort the dependence on other physiological parameter. The measuring set-up was intended to analyze the headspace under conditions which do not massively induce stress in mice. The mice were handled with great care and adapted to the chamber before breath gas analysis. Increased activity, urination and defecation are early visible markers for stress. By definition, measurements with urine and feces contamination were discarded. Still, we found a fluctuation of the sources strength of 30 % during habituation in this study, which may partly be related to stress. Published data on GC-analysis of the headspace of urine showed that mouse urinary

biomarkers provided, among others, signature typical for stress (Schaefer et al. 2010). Therefore, a careful evaluation of changes in the CO_2 and humidity source strength in combination with a PTR-MS search for stress biomarkers evaporating from urine should be addressed to further refine the method.

Short-term changes in physical activity level during the accumulation of a profile modulate the shape of the VOC profiles. Therefore, the assumption of constant source strengths had to be refined by an extended respirometry chamber model with a source strength which takes into account sudden changes. When exemplarily applied to a selection of smooth and modulated profiles, the additional time dependent term had only marginal effects on the fit but essentially improved the fit in disturbed, modulated profiles. As a consequence, the whole profile need not be discarded because the undisturbed part of the accumulation profile can be used. In PTR-MS, breath gas is not being processed prior to analysis. The quantitative determination of the concentration of some VOCs at dry and low CO_2 condition just after the flushing of the respirometry chamber with dry synthetic air may differ from the humid and CO_2 -enriched conditions after a few minutes of exhalation (Keck et al. 2008; Schwarz et al. 2009). The count rate of humidity cps (M_{37}) of 0.5–2.5 % of the count rate of the primary ions [cps(M_{19})] at the end of an accumulation profile was low compared to that of the saturated humidity of (human) breath (cps(M_{37})/ cps(M_{19}) = 60 %). Nevertheless, the suggested normalization of the measured count rate, which includes the consumption of primaries due to water clusters, was applied (Lindinger et al. 1998). Common VOCs in (human) breath show substantial fragmentation depending on the humidity and CO_2 content (Keck et al. 2008). For selected VOCs, a correction due to this fragmentation should be considered in future.

Intervention study

Investigations of exhaled VOCs of small animals such aS_{Mi}ce were previously restricted to single VOCs such as ethanol. Cope et al. (2000) reported on ethanol production in 20-week-old lean mice. After a 30-min equilibration time in a respirometry chamber, they recorded concentrations of about 10 ppb ml^{-1} CO_2. If we assume 3 vol% of CO_2 in air, a breathing rate of 130 min^{-1} at rest, and 0.3 ml tidal volume, the source strength of ethanol can be estimated as 0.26 nl min^{-1}. This is comparable to the source strength of 1.8 nl min^{-1} of a mouse at rest fed with a standard laboratory chow in our pilot study, especially if taking into account that the ethanol production underlies a daily rhythm (Cope et al. 2000). In this feasibility study, we applied breath gas analysis for metabolic profiling in mice initially fed with a standard laboratory chow diet and then a HFD containing 60 energy% of fat. We identified a set of VOCs that were significantly reduced after the HFD intervention of three weeks, especially the source strength of methanol. In recent years, the importance of mammalian gut microbiota on the metabolism of their host organism has been widely acknowledged. A possible correlation between microbiota metabolism and

obesity is under discussion (Cani and Delzenne 2009; Tilg and Kaser 2011; Vrieze et al. 2010). Aprea et al. (2012) reported that the methanol concentration in the breath of rats fed with a HFD which was half of the concentration when fed with a standard diet. These authors suggested that the difference can be related to the digestion of fibers by the gut microflora; however, the link to microbial activity was only rarely addressed experimentally. Future nutrition challenge experiments have to be carefully designed to distinguish between matrix characteristics and the fat composition of the diets. Acetic acid (M_{61}) and propionic acid (M_{75}) are important endogenously produced and circulated metabolites in mice. As their abundance was reduced in the breath after HFD, we hypothesize that these compounds were utilized for gluconeogenesis in the liver (propionic acid) or as substrate for the synthesis of cholesterol and lipids in the peripheral tissues (acetic acid) (Samuel et al. 2008). However, alcohols and short chain fatty acids (SCFA), such as acetic acid and propionic acids, are products of gut bacteria, too. These metabolites can be easily absorbed from the colon into the blood and may influence carbohydrate and lipid metabolism and thus may profoundly interact with the metabolism of the mouse.

Conclusion

In conclusion, we could show that online breath gas analysis by PTR-MS can be adapted to the dimensions of a mouse regarding the small exhaled minute volume. Further progress will be enabled by the recently implemented commercial PTR–TOF-MS in the breath gas screen which records full mass spectra within a second. In combination with higher mass resolution, extended mass range, and higher sensitivity in mass range above m/z * 100 compared to the PTR-MS with a quadrupole mass analyzer, the refinement of the compartment model, and the development of a multiplexed set-up, become feasible thereby increasing study capacities in a high throughput phenotyping screen such as the GMC.

In our feasibility study, despite the intra-individual variability in the source strength induced by behavioral characteristics of an unrestrained mouse, a set of VOCs was found to be significantly altered in response to the dietary intervention. These VOCs can be related to physiological functions and metabolic pathways and may be useful as markers for metabolic phenotyping. It remains to be clarified whether these changes are related to the metabolism of the mouse, to metabolites originating from gut microbiota, or to an interaction of microbiota metabolites with mouse metabolism. Further studies addressing this aspect are in progress. Animal studies could improve our understanding of the metabolic pathways and correlated breath gas components in mouse models for human disorders. Standardized housing conditions, a defined genetic predisposition to diseases and the possibility to investigate the interaction of lifestyle factors (e.g., nutrition, physical activity) and targeted metabolic alterations in established challenge experiments provide the possibility to decipher subsets of target VOCs in the exhaled breath of

mice. A promising perspective is their use and the search for these target VOCs in human breath which may otherwise not be identified as biomarkers in human breath due to the human intra- and inter-individual variability. This has the potential of being clinically useful for the early noninvasive diagnosis of diseases, physiological disorders, and therapeutic monitoring. In this sense, the use of breath tests as a diagnostic tool for human disorders will greatly benefit from animal models.

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Author contributions

W. S. and J.R. conception and design of methodology.

W.S., D.P., S.K., M. Kistler., M. Kneipp and H.S. performed experiments and data evaluation.

W.S., J.R. and V.H. interpreted results of experiments.

W.S. and J. R. drafted manuscript.

M. Klingenspor, C.H. and M.H.A. edited manuscript.

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