Breath profiles of children on ketogenic therapy

Veronika Ruzsányi1,2, Miklós Péter Kalapos3, Christine Schmidl4, Daniela Karall4, Sabine Scholl-Bürgi4 and Matthias Baumann4

1 Breath Research Institute, University of Innsbruck, Innrain 66, 6020 Innsbruck, Austria
2 Department of Anesthesia and Intensive Care, Medical University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria
3 Theoretical Biology Research Group, Dámvad utca 18, H-1029 Budapest, Hungary
4 Department of Pediatrics I, Medical University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria

E-mail: veronika.ruzsanyi@uibk.ac.at

Keywords: ketogenic diet, modified Atkins diet, ketone bodies, β-oxidation, lipid peroxidation, intestinal flora

Abstract

Ketogenic diets (KD) were introduced to clinical practices as alimentary approaches with the aim to control drug-resistant epilepsies. Over the decades, a large and growing body of research has addressed the antiseizure effect of various KDs, and worked out KD-based dietary regimens, including their acting factors and modes of action. KDs have also appeared in weight loss therapies. Therapy control, particularly at initiation, happens through regular blood analysis and control of urine ketone levels. However, there is a lack of fast, reliable, and preferably non-invasive methods to accomplish this. The detection of exhaled breath constituents may offer a solution. The exhaled breath contains hundreds of volatile organic compounds (VOCs), which can be modified by diet. VOC detection technology has resulted in low-cost sensors that can facilitate the self-monitoring of patients in the future if reliable breath markers are available. Therefore, it is of interest to investigate the composition of exhaled breath in children on KDs. Twenty-two pediatric patients between 4 and 18 years of age were recruited in this study. Eleven of them received a KD and suffered from epilepsy, with the exception of one child, who was admitted to a weight-reduction therapy. The control group involved 11 patients with neurological disorders but not on KD. Breath volatiles were analyzed using gas chromatography mass spectrometry (GC-MS) after preconcentration of the analytes on needle traps (NTs). We found that the breath concentrations of a number of VOCs, namely acetaldehyde, acetone, 2-methylfuran, methyl-vinyl-ketone, and 2-pentanone were significantly elevated in the breath of children on a KD in comparison to their control counterparts. Interestingly, breath ethanol was lower in patients on a KD than in non-KD patients. Association studies revealed an interrelationship among (i) lipid parameters and ketone bodies, (ii) methacrolein, methyl-vinyl-ketone, and high-density lipoprotein, as well as (iii) methyl-vinyl-ketone, acetone, and 2-pentanone, thus raising the possibility of a common metabolic source. The duration of diet was positively and negatively associated with breath acetone and breath ethanol, respectively. Some of the changes were linked to β-oxidation, but there are uncertainties in regard to metabolic sources of other metabolites. Lipid peroxidation and alteration of intestinal microbial composition may also be involved in the changes of VOC profiles during KD. Since lipids used for metabolism during KD originate from external sources, the processes occurring cannot simply be compared to and deduced from changes appearing in starvation; however, lipid mobilization is also evident in starvation. To find reliable and sensitive VOC markers that are linked to the respective ketogenic regimen, further investigations are needed to reveal the metabolic background.

Abbreviations

ALD alcohol dehydrogenase
BMI body mass index
cKD classic ketogenic diet
GC-MS gas chromatography mass spectrometry

© 2018 IOP Publishing Ltd
isopropanol was dedicated to acetone tive anticonvulsants, the seizure-controlling effect of it or its metabolites possess antiseizure activity ketone bodies, it remains unclear whether acetone experiments with seizure-susceptible mice acetone properties of acetone were further substantiated by. Since both isopropanol and acetone were effec- Nonetheless, the possible anticonvulsant properties of acetone were further substantiated by the experiments undertaken with isopropanol in rats. Since both isopropanol and acetone were effective anticonvulsants, the seizure-controlling effect of isopropanol was dedicated to acetone. Although in experiments with seizure-susceptible mice acetone had the strongest anticonvulsant efficacy among ketone bodies, it remains unclear whether acetone itself or its metabolites possess antiseizure activity. Support for the acetone hypothesis comes from a breath acetone investigation in relation to seizure control. A linear relationship between plasma and breath acetone has been reported. Since the classic KD (cKD) is a high fat, low carbohydrate, and low protein diet, the formation of other metabolites related to lipid metabolism can also be expected, and thus should be investigated. Over the decades several types of ketogenic regimens, termed neuroketherapeutics, have been determined to control refractory epilepsies. The modified Atkins diet, often abbreviated as ‘MAD’, is one of these alternatives to the cKD, and is less restrictive in terms of calories. MAD is effective and tolerable in refractory epilepsies.

The aim of the present study was to examine the effect of a KD upon the exhaled air profile of children. This is the first time that the exhaled breath of children on a KD was investigated for metabolites other than acetone (or isopropanol). In the study group, patients having epilepsy or being on weight reduction therapy were included.

Our results show that in addition to acetone other volatile organic compounds (VOCs) are also detectable, and concentrations also change during the course of the KD. Some of these changes are linked to β-oxidation, while the appearance of other metabolites is perhaps due to the alterations in intestinal microbial composition or lipid peroxidation (LPO) evolving as a consequence of ketogenic therapies. The appearance of such metabolites in breath raises the probability of finding indicators more sensitive than acetone for the effectiveness of the KD.

Introduction

Since the 1920s ketogenic diets (KD) as alimentary supplements for controlling seizures have been a valuable approach in the treatment of epilepsies, particularly in children [1]. Over the intervening years a large body of data has been collected to explain the way(s) how a KD may exert its beneficial effect on seizure control. Hypotheses are divided into three classes by their modes of action. Class one suggests a direct anticonvulsant effect of ketone bodies, class two suggests a reduction of neuronal excitability by cerebral ketone body metabolism, and class three suggests a less direct effect of the KD upon seizure control [2, 3]. However, despite scientific endeavors it is still unknown which factors this benefit of KD can be attributed to.

One candidate molecule responsible for the anti-epileptic action of the KD is acetone [4]. Its possible role in seizure control was already raised in the 1930s [3]. At that time extensive research was made without leading to any appreciated mechanism of action [3]. Nonetheless, the possible anticonvulsant properties of acetone were further substantiated by the experiments undertaken with isopropanol in rats [5]. Since both isopropanol and acetone were effective anticonvulsants, the seizure-controlling effect of isopropanol was dedicated to acetone [5]. Although in experiments with seizure-susceptible mice acetone had the strongest anticonvulsant efficacy among ketone bodies, it remains unclear whether acetone itself or its metabolites possess antiseizure activity [6–8].

Support for the acetone hypothesis comes from a breath acetone investigation in relation to seizure control [9]. A linear relationship between plasma and breath acetone has been reported [10]. Since the classic KD (cKD) is a high fat, low carbohydrate, and low protein diet, the formation of other metabolites related to lipid metabolism can also be expected, and thus should be investigated.

Over the decades several types of ketogenic regimens, termed neuroketherapeutics, have been determined to control refractory epilepsies [11]. The modified Atkins diet, often abbreviated as ‘MAD’, is one of these alternatives to the cKD, and is less restrictive in terms of calories [12]. MAD is effective and tolerable in refractory epilepsies [13].

The aim of the present study was to examine the effect of a KD upon the exhaled air profile of children. This is the first time that the exhaled breath of children on a KD was investigated for metabolites other than acetone (or isopropanol). In the study group, patients having epilepsy or being on weight reduction therapy were included.

Materials and methods

Materials

All compounds listed in table 1 were purchased as pure liquids from Sigma-Aldrich, Merck, and Chem-SampCo companies.

Patients

Twenty-two patients (13/9, m/w) between 4 and 18 years old were recruited in this study. From the ten patients with epilepsy, nine were treated with the MAD and one with the cKD. Additionally, one patient received the MAD in order to lose weight because of obesity. The control group involved 11 patients with neurological disorders but were not on a KD. The study was approved by the Ethical Committee of the Innsbruck Medical University (Study number: AN4315).

Blood samples

Blood samples were collected routinely by the attending physician for determination of blood glucose, liver parameters (GOT, GTP, γ-GT), kidney parameters (urea, creatinine), blood ketone concentrations (whole ketone bodies, acetoacetate, β-hydroxy-butyrate), and drug levels. Blood samples were collected after overnight fasting in 9 of 11 patients in the KD group, and in seven of ten patients in the control group. Blood ketone concentrations were determined in the laboratory of the Pediatric Department of the Medical University of Innsbruck by a cyclic enzymatic test using photometric measuring of the rate of Thio-NADH production (Autokit Total Ketone Bodies; Wako Life Sciences, Mountain View, USA). All other
parameters were measured in the central laboratory using standard procedures.

**Breath sampling protocol**

Alveolar breath and indoor air samples were collected at the pediatric neurology clinic in the Pediatric Department of the Medical University of Innsbruck. As much as possible the same room was used for sampling to minimize the influence of indoor air contamination.

Nine patients in the KD group and seven in the control group gave samples after overnight fasting. The alveolar breath samples were collected manually into glass syringes (250 ml volume, Socorex Isba S.A, Ecublens, Switzerland). The glass syringe was capped with a three-way valve (Discofix, B. Braun Melsungen AG, Melsungen, Germany), and additionally connected via Teflon tube to a single-use mouthpiece (Intersurgical complete respiratory systems, Sankt Augustin, Deutschland) on which, with the help of a single-use Teflon-adapter, a CO₂ sensor (Phasein, Danderyd, Sweden) was mounted. During sampling the breath CO₂ content was monitored using the CO₂ sensor. During exhalation, syringes were filled with exhaled air if the absolute level of CO₂ in the breath exceeded 3%. Ambient air was collected in parallel (also in glass syringes). After sampling the syringes were locked with the three-way valve.

All samples were processed within 2 h. Before use, all syringes were cleaned, washed out with distilled water, and dried overnight at 100 °C to remove any residual contaminants.

**Sample preparation for gas chromatography mass spectrometry (GC-MS) measurements**

Extraction of VOCs was performed using stainless steel needles containing 2 cm Carbopack X and 1 cm Carboxen 1000 sorption materials, a so-called needle trap (NT, PAS Technology, Magdala, Germany). For VOC extraction, the syringe was pierced through a septum by the NT connected at the other end with an electronic mass flow controller (model F-201DV-RAD-11-V, Bronkhorst, Ruurlo, The Netherlands) via a Teflon tube. For the generation of sample flow, a pump (Vacuubrand, Wertheim, Germany) was placed at the end of the sampling system. The collected volume of breath sample was 200 ml, with a total flow of 8 ml min⁻¹ through the NT, which was controlled by means of the mass flow controller. After extraction, NTs were cleaned by thermal heating at 300 °C for 15 min.

**GC-MS analysis**

The sampled analytes were released from sorbents by direct thermal desorption of the NT at 290 °C in splitless mode in the heated injector of the 7890A gas GC equipped with a 5975 C Inert XL mass selective detector (MSD) (both from Agilent Technologies, Waldbronn, Germany). The CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) was modified (by PAS Technology) for application of NTs allowing an automated sample processing.

MS analyses were performed in full scan mode, with a scan range of 20–200 amu. Ionization of the separated compounds was done by electron impact at 70 eV. Chromatographic data was acquired using the Agilent Chemstation Software (GC-MS Data Analysis from Agilent, Waldbronn, Germany), and the mass spectrum library NIST 2008 (Gatesburg, USA) was applied for identification. An RT-Q-Bond capillary column 30 m × 0.32 mm × 10 μm (Varian, Palo Alto, CA, USA) was used. The oven temperature program was as follows: initial 55 °C for 0 min, then ramped 6 °C min⁻¹ up to 120 °C and held for 1 min, then ramped 9 °C min⁻¹ up to 180 °C and held for 1 min, then ramped 8 °C min⁻¹ up to 200 °C and held for 1 min, and finally ramped 8 °C min⁻¹ up to 280 °C and held for 16 min. This was conducted in a constant flow mode (helium at 2 ml min⁻¹).

---

**Table 1. Detection limits and calibration ranges for compounds detected in the study.**

| Compound            | CAS number | Detection limit (ppt) | Calibration range (ppb) | R²  |
|---------------------|------------|-----------------------|-------------------------|-----|
| 1-Pentanol          | 71-41-0    | 770                   | 0–200                   | 0.991 |
| 1-Propanol          | 71-23-8    | 254                   | 0–200                   | 0.982 |
| Isoprene            | 78-79-5    | 153                   | 0–500                   | 0.989 |
| 2-Methylfuran       | 533-22-5   | 233                   | 0–200                   | 0.997 |
| 2-Pentanone         | 107-87-9   | 49                    | 0–200                   | 0.997 |
| Acetaldehyde        | 75-07-0    | 1851                  | 0–200                   | 0.972 |
| Ethanol             | 64-17-5    | 475                   | 0–200                   | 0.974 |
| Acetone             | 67-64-1    | 257                   | 0–1000                  | 0.946 |
| Isopropanol         | 67-63-0    | 169                   | 0–200                   | 0.992 |
| Methacrolein        | 78-85-3    | 323                   | 0–200                   | 0.996 |
| Methyl-vinyl-ketone | 78-94-4    | 193                   | 0–200                   | 0.994 |
| Nonanal             | 124-19-6   | 1663                  | 0–200                   | 0.916 |
| Octanal             | 124-13-0   | 756                   | 0–200                   | 0.976 |
| Pentane             | 109-66-0   | 175                   | 0–200                   | 0.991 |
| 3-Heptanone         | 106-35-4   | 31                    | 0–200                   | 0.998 |
Calibrations
For determination of the retention time of compounds detected in room air and breath samples, gaseous standards were prepared by evaporation of liquid substances into glass bulbs. Each bulb (Supelco, Bellefonte, PA, USA) was cleaned with methanol (Sigma-Aldrich, Steinheim, Germany), dried at 85 °C for at least 20 h, purged with clean nitrogen for at least 20 min, and subsequently evacuated using a vacuum pump (Vacuubrand, Wertheim, Germany) for 30 min. Liquid standards (0.5–1 μl according to desired concentration) were injected through a septum, using a GC syringe. After the evaporation of standards, the glass bulb was filled with nitrogen of purity 6.0 in order to equalize the pressure (to the ambient pressure). Then, the appropriate volume (μl) of vapor mixture was transferred using a gas-tight syringe (Hamilton, Bonaduz, Switzerland) into 250 ml glass syringes previously filled with 200 ml of nitrogen 6.0 (99.9999% purity). Detection limits were evaluated from the calibration curves using a t-distribution with 95% probability (table 1).

Data evaluation
Integration of chromatograms was done by means of Agilent MSD Productivity Chemstation Software (Agilent technologies, Santa Clara, California, 1999) and Breath View (Testversion 1.6 Oncotyrol 2014, Innsbruck, Austria). Mass spectra library NIST 2008 (Gatesburg, USA) was used for peak identification.

Statistical analysis
To compare the treated (MAD and cKD) versus control patient groups we used the Wilcoxon signed rank test as a non-parametric test to calculate the significance of the hypothesis because a normal distribution could not be determined owing to the small number of patients. In the case of a p value < 0.05 mean values can be significantly differentiated, and the value is influenced by the KD.

Correlations between VOC concentrations in breath gas and other parameters were calculated using Spearman coefficients with a significance value of p < 0.05.

Results
Basic characteristics of study and control groups
Table 2 provides a statistical summary of background factors for subjects on and not on a KD. No differences between factors were found. The actual medication of the children is also shown.

Changes in ketone body levels
To control the efficacy of the regimen, the metabolic effects of a KD upon plasma ketone body levels were also determined, and are documented in table 3. As expected, the plasma concentrations of ketone bodies, either the separate or the total concentrations, significantly increased in the KD group in comparison to the non-KD group (table 3).

Ketogenic regimens have been reported to affect serum lipid profiles [14, 15]. To ensure the revelation of correlations among variables, the lipid parameters (cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride) for patients enrolled in a KD were also determined. Their serum lipid profile was similar to that reported in the literature for ketogenic treatments (data not shown).

Measurement of VOCs in breath
The breath profile was examined in both groups. Altogether, 18 VOCs were selected and monitored (table 4). Neither 2-butanol nor hexanal were present at detectable amounts in the breath in either group. Furthermore, ethylacetate was only measurable in the exhaled air of patients on a KD, reaching approximately 0.1 ppb (n = 11), which is very close to the detection limit (0.08 ppb) of the device. In table 4, the values for the other 15 VOCs are shown.

A significant rise in the amount of exhaled acetaldehyde, acetone, 2-methylfuran, methyl-vinylketone, and 2-pentanone was measured in the KD group. Interestingly, the breath ethanol level was lower in patients on KDs in comparison to controls. At the same time, no significantly different levels of isoprene, methacrolein, nonanal, octanal, pentane, 1-pentanol, or 1-propanol were detected (table 4).

As reported earlier, β-oxidation takes place in the breakdown of valproic acid, and 3-heptanone is an end-product of this process [16]. Since the KD also influences the rate of β-oxidation, the level of this metabolite in breath was determined (table 4). Valproic acid levels in plasma for KD and non-KD subjects were also measured, and we found a non-significant difference with values of 52.57 ± 27.84 (mg l⁻¹) (n = 3) and 73.03 ± 41.46 (mg l⁻¹) (n = 3), respectively. As expected, a strong correlation between the levels of 3-heptanone and valproic acid was seen (r = 0.9293, p < 0.001). In topiramate-treated cases, the values of topiramate were 3.7 (mg l⁻¹) and 7.6 (mg l⁻¹) in patients belonging to diet and non-diet groups, respectively.

Association among variables
To determine the interrelationships among variables, we assessed breath and serum variables pair-wise by linear regression analysis (table 5).

As expected, correlations were found among lipid parameters (cholesterol, HDL, LDL, ketone bodies) (table 5). Furthermore, the length of therapy and breath acetone showed a positive correlation, while the length of therapy and breath ethanol showed a negative correlation (table 5). Associations were revealed among methacrolein, methyl-vinyl-ketone, and HDL, as well as methyl-vinyl-ketone, acetone, and...
Changes of metabolism during KD in humans
The KD involves a special composition of food. The cKD is a high-fat, low-carbohydrate, and low-protein diet that, in principle, mimics starvation, and is known for its beneficial effect on seizures since Biblical times (see Matthew 17:21). Over the decades, the cKD has been modified to avoid its side effects and starvation, 2-pentanone, thus suggesting the possibility of a common metabolic source (table 5). However, this assumption has to be clarified in the future.

Discussion
The central issue in this research has been to identify VOCs that are intrinsic to ketogenic regimens, and can serve as biomarkers for treatment efficacy. A biomarker reflects biological processes and responses to therapeutic interventions in patients; therefore, the excretion of VOCs via breath is presented in this paper with regard to their appearance in the course of diet, along with a set of VOCs for which changes in their concentrations are linked to the respective ketogenic regimen.

The biochemical backgrounds of these changes are quite different. There are metabolites whose origins are not clearly identifiable at present. However, despite uncertainties the events can essentially be divided into two main groups of metabolic conversion: changes due to metabolic events inside the body that result from the introduction of a lipid-rich diet, and those seen as a consequence of the rebuilding of the structure of human gut microbiota.

Changes of metabolism during KD in humans
The biochemical backgrounds of these changes are quite different. There are metabolites whose origins are not clearly identifiable at present. However, despite uncertainties the events can essentially be divided into two main groups of metabolic conversion: changes due to metabolic events inside the body that result from the introduction of a lipid-rich diet, and those seen as a consequence of the rebuilding of the structure of human gut microbiota.

2-pentanone, thus suggesting the possibility of a common metabolic source (table 5). However, this assumption has to be clarified in the future.

Table 2. Basic statistics of KD and non-KD groups.

| Parameter                  | KD group | non-KD group |
|----------------------------|----------|--------------|
| Demographic parameters: mean ± S.D. (range) |          |              |
| Age (year)                 | 10.55 ± 3.98 (4–17) | 12.27 ± 3.17 (7–18) |
| BMI (kg m⁻²)               | 20.10 ± 7.84 (13.7–40.4) | 19.45 ± 4.21 (13.7–28.1) |
| Body weight (kg)           | 47.52 ± 33.9 (16.5–132.3) | 51.56 ± 21.26 (17.5–83) |
| Body weight percentile     | 49 ± 35 (0–100) | 52 ± 38 (0–99) |
| Height (m)                 | 1.45 ± 0.25 (1.07–1.81) | 1.59 ± 0.25 (1.13–1.94) |
| Height percentile           | 49 ± 31 (0–99) | 56–33 (1–100) |
| Routinely detected laboratory values: mean ± S.D. (range) |          |              |
| GOT (U l⁻¹)                | 28 ± 5.4 (18–36) | 26.18 ± 10 (11–41) |
| GPT (U l⁻¹)                | 19.55 ± 5.73 (8–29) | 16.55 ± 3.42 (10–21) |
| γ-GT (U l⁻¹)               | 14.82 ± 5 (9–27) | 16.73 ± 5.08 (10–27) |
| Creatinine (μM)            | 502.0 ± 196.1 (310–820) | 629.0 ± 125.7 (430–810) |
| Urea (mM)                  | 34.44 ± 9.65 (17.6–48.1) | 25.21 ± 4.25 (18.5–34.0) |
| Glucose (mM)               | 4.39 ± 0.94 (3.22–6.72) | 5.03 ± 0.32 (4.61–5.67) |
| Medication                 |          |              |
| Valproic acid              | 3        | 3            |
| Lamotrigine                | 1        | 1            |
| Topiramate                 | 1        | 1            |
| Clobazam                   | 1        | 0            |
| Methylphenidate            | 1        | 0            |
| Potassium bromide          | 1        | 0            |
| Levetiracetam              | 0        | 1            |
| Sulthiame                  | 0        | 1            |
| Potassium bromide          | 0        | 1            |

2-pentanone, thus suggesting the possibility of a common metabolic source (table 5). However, this assumption has to be clarified in the future.

Table 3. Ketone body levels in the plasma in μM. Data are presented in minimum (min), 25th percentile (Q1), median, 75th percentile (Q3), and maximum (max) values.

| Compound                         | Diet group (μM) | Non-diet group (μM) |
|----------------------------------|-----------------|---------------------|
|                                  | Min  | Q1   | Median | Q3   | Max  | Min  | Q1   | Median | Q3   | Max  | p value |
| β-hydroxy-butyrate               | 96   | 513  | 1199   | 2995 | 4160 | 14   | 18   | 23    | 47.5 | 117  | <0.01   |
| Acetoacetate                     | 60   | 198.5| 439    | 932  | 1024 | 7    | 13   | 15    | 22.5 | 72   | <0.01   |
| Total ketone body level          | 156  | 791.5| 1638   | 3838 | 5158 | 24   | 30   | 40    | 66.5 | 189  | <0.01   |
The core biochemical change in starvation is an increase in β-oxidation accompanied with a decrease in glycolysis, leading to the elevation of plasma levels of all the three members of the ketone body family [17–19]. In general, plasma concentrations of acetoacetate, β-hydroxybutyrate, and acetone increase, and exceed the mmolar range in starved humans [20]. In cKD-treated patients, the levels of these compounds reach a similar level [10, 21]. Acetone concentration may exceed the level of 4 mM, and correlates with breath acetone [10]. Patients successfully treated with a cKD had elevated levels of acetone in their brains [22]. In our study, β-hydroxybutyrate and acetoacetate concentrations were as high as 4.1 mM and 1.0 mM, respectively, with a concomitant increase in breath acetone and isopropanol levels (tables 3 and 4). In this sense, our findings are in accordance with the data reported in the literature [10, 21]. The duration of the KD also correlated well with breath acetone level (table 5), which is in contrast to the finding of others [23]. However, this difference may be due to the fact that the cKD and MAD are not the same. Nevertheless, there is another opportunity for the outflow from the cycle at the level of 3-ketoacyl-CoA (figure 1). The leakage of the cycle at this level may result in the formation of both 2-pentanone and 2-heptanone but has remained rich in lipids and restricted in carbohydrates [11, 12, 14].

Table 4. Levels of VOCs in breath in ppbV. Data are presented in minimum (min), 25th percentile (Q1), median, 75th percentile (Q3), and maximum (Max) values.

| Compound          | Diet group (ppbV) | Non-diet group (ppbV) |
|-------------------|-------------------|-----------------------|
|                   | Min   | Q1    | Median | Q3    | Max   | p value |
| 1-Pentanol        | 0.0   | 0.0   | 0.0    | 1.3   | 5.8   | 0.0     |
| 1-Propanol        | 8.3   | 20.7  | 52.3   | 121.8 | 422.4 | 2.5     |
| Isoprene          | 23.4  | 38.4  | 69.4   | 102.2 | 126.5 | 25.5    |
| 2-Methylfuran     | 2.3   | 2.4   | 2.6    | 3.2   | 4.0   | 2.0     |
| 2-Pentanone       | 1.5   | 1.6   | 1.9    | 2.1   | 2.2   | 1.2     |
| Acetaldehyde      | 94.0  | 115.3 | 143.1  | 194.5 | 226.1 | 16.4    |
| Ethanol           | 0.0   | 11.6  | 26.0   | 116.8 | 140.1 | 46.6    |
| Acetone           | 226.1 | 571.2 | 1298.8 | 2119.6| 3359.5| 58.0    |
| Isopropanol       | 53.7  | 78.2  | 135.2  | 217.9 | 550.0 | 63.8    |
| Methacrolein      | 0.0   | 0.0   | 0.1    | 3.5   | 12.0  | 0.0     |
| Methyl-vinyl-ketone| 1.9   | 3.1   | 6.8    | 15.7  | 33.5  | 0.0     |
| Nonanal           | 0.0   | 0.0   | 54.1   | 126.8 | 139.1 | 0.0     |
| Octanal           | 0.0   | 5.9   | 26.5   | 51.5  | 74.2  | 3.9     |
| Pentane           | 0.0   | 0.0   | 0.2    | 0.5   | 11.7  | 0.0     |
| 3-Heptanone       | 0.0   | 0.0   | 0.0    | 14.6  | 19.2  | 0.0     |

As noted in the text, neither 2-butanol nor hexanal were detectable in the breath of either group, and ethylacetate was only measurable in the breath of patients on a KD.

Metabolite of valproic acid.

Table 5. Significant interrelations (Pearson’s) between evaluated parameters in the KD group. Since p < 0.05 was accepted as significant only these data are shown.

| Variable 1         | Variable 2         | r    | p value |
|--------------------|--------------------|------|---------|
| Total ketone body  | Acetocetate        | 0.954| <0.001 |
|                    | β-hydroxy-butyrate | 0.997| <0.001 |
|                    | Acetone            | 0.827| 0.003  |
| Acetoacetate       | β-hydroxy-butyrate | 0.928| <0.001 |
|                    | Acetone            | 0.862| 0.001  |
| β-hydroxy-butyrate | Acetone            | 0.806| 0.0049 |
| Acetone            | Duration of KD     | 0.742| 0.009  |
|                    | 2-Pentanone        | 0.657| 0.027  |
| Triglyceride       | 1-Pentanol         | 0.832| 0.003  |
| Cholesterol        | BMI                | −0.695| 0.026 |
|                    | HDL                | 0.657| 0.038  |
| HDL                | Methacrolein       | 0.680| 0.030  |
|                    | Methyl-vinyl-ketone| 0.655| 0.041  |
| LDL                | BMI                | −0.771| 0.009 |
|                    | Cholesterol        | 0.982| <0.001 |
| 1-Pentanol         | Ethanol            | 0.755| 0.007  |
|                    | Glucose            | 0.747| 0.008  |
|                    | γ-glutamyl transferase| −0.737| 0.01 |
| Isopropanol        | 1-Propanol         | 0.948| <0.001 |
| Nonanal            | Octanal            | 0.915| <0.001 |
| Pentane            | 1-Propanol         | 0.931| <0.001 |
|                    | Isopropanol        | 0.880| <0.001 |
| Ethanol            | Duration of KD     | −0.605| 0.048 |
|                    | Glucose            | 0.722| 0.012  |
|                    | γ-glutamyl transferase| 0.610| 0.046 |
|                    | GPT                | 0.627| 0.039  |
| Methacrolein       | Methyl-vinyl-ketone| 0.835| 0.001  |
| 3-Heptanone        | Valproic acid      | 0.929| <0.001 |

In the course of β-oxidation, every turn of the cycle shortens the carbon chain by two carbons, leading to the production of one molecule of acetyl-CoA, NADH + H⁺, and FADH₂ (figure 1). Nevertheless, there is another opportunity for the outflow from the cycle at the level of 3-ketoacyl-CoA (figure 1). The leakage of the cycle at this level may result in the formation of both 2-pentanone and 2-heptanone...
2-Pentanone was well detectable in the breath of children of both groups, with a significant elevation in patients on a KD in comparison to controls (table 4); it also showed a positive correlation to breath acetoacetate, β-hydroxy-butyrate, and total ketone body levels, which were all related to β-oxidation (table 5). The duration of the KD was also correlated with 2-pentanone levels (table 5). Development of more sensitive detection methods may enable the monitoring of 2-heptanone, thus giving further evidence for the role of 3-ketoacyl-CoA. However, an alternative way to achieve 2-pentanone formation is through an incomplete β-oxidation of medium-chain fatty acids, probably in peroxisomes; this is a mechanism similar to that used in filamentous fungi [24].

In cholesterol synthesis, acetyl-CoA is a precursor molecule for mevalonate formation that is transformed into dimethylallyl pyrophosphate, a compound from which isoprene (2-methyl-1,3-butadiene) is produced [25]. In children, exhaled isoprene levels show an age-dependent character, whereas this correlation is not seen in adults [26–28]. In this study, an increase in exhaled isoprene levels was detected in patients on a KD compared to the those not on a KD, but it did not reach the level of significance (table 4). King and associates [29] assumed that isoprene is stored in muscles, and can be released fast during movement. Therefore, breath isoprene sampling analysis requires carefully rendered breath sampling, and patients should avoid movement, e.g. remain at rest for at least 5 min, and sitting before sampling. This criterion is difficult to achieve in the case of children. Despite the lack of significance, the trend in breath isoprene level might reflect a metabolic pressure built up by the rise of acetyl-CoA and NADH + H⁺ (figure 2). The latter is known to slow down the TCA cycle, resulting in a reduced acetyl-CoA breakdown, thus creating a situation that may enhance the operation of the mevalonate pathway. This note is supported by the fact that isoprene may be the source of methyl-vinyl-ketone and methacrolein formation in the course of LPO by the hydroperoxyl pathway, and a specificity of oxygen free radicals is involved in the process [30, 31]. Although the present study revealed a correlation between the amounts of methyl-vinyl-ketone and methacrolein (tables 4 and 5), any correlation to isoprene was not seen (data not shown).

Figure 1. The network of β-oxidation. Abbreviation: HMG = hydroxymethyl-glutaryl.
LPO
LPO is the oxidative degradation of lipids. This process proceeds by a free radical chain reaction. A wide variety of compounds can be detected in the course of LPO, among others ketones, such as 2-pentanone and 3-pentanone, or alkanals, such as hexenal or octanal [30]. We detected compounds (e.g. pentane) that are related to LPO, while others also known as LPO products (e.g. hexanal) were undetectable.

There seems to be an agreement that LPO does not have a pathological role in the KD because LPO-related events are not mentioned among the late-onset complications [12, 32]. Nevertheless, this optimistic opinion might have to be questioned. Although there are reports having found the KD protective against oxidative stress, only short-term investigations are available [33–35]. In clinical practice, KDs are long-term treatments, e.g. in our case the longest therapy had lasted 54 months. However, the studies from which the conclusion regarding safety has been drawn are comparatively short. Assuming that KDs are mainly used in the case of intractable seizures, one can suggest that a long-term treatment has to occur; therefore, from this point of view it would be relevant to see whether LPO contributes to side effects.

Although our present results do not fundamentally oppose the above view, they provide some reasonable doubts. An example is pentane. Breath pentane is used as an index to monitor LPO in animal models [36, 37]. Its analysis in breath may provide an early, rapid, non-invasive, and real-time assessment of LPO [37]. In addition, its measurement is advised in clinical practice to assist in the treatment of LPO-associated disorders in preterm infants [38]. In our case, breath pentane levels increased in the KD group, almost reaching the level of significance (table 4). Perhaps a larger sample size would have provided a significant difference between groups.

Several arguments that may support the aforementioned skepticism, and direct attention to the possible vascular adverse effects of fat-rich regimens, should also be addressed. Firstly, note that the NADH + H⁺ burden may also enhance LPO, in which Fe²⁺ ions play a role [39]. Secondly, note that acetoacetate, but not β-hydroxy-butyrate, has been reported to cause an elevation in LPO in cultured human venous endothelial cells through an Fe²⁺ ion-dependent oxygen free radical generation [40]. Thirdly, note that acetoacetate leads to methylglyoxal production via reactive oxygen species generation in the presence of molecular oxygen; in the reaction either myoglobin, or, less effectively, hemoglobin takes part [41, 42]. Fourthly, note that the KD leads to a higher arterial stiffness (an early marker of vascular damage), and elevated cholesterol and triglyceride levels in treated young subjects in comparison to controls [43]. Fifthly, note that seizures themselves raise LPO [44]. Finally, note that an impairment of the glutathione system has been reported in patients with epilepsy independent from seizure frequency [45]. Therefore, a modest speculation is appropriate here. A KD for patients, seizure-free or not, may open the way for LPO with a proposed mechanism as depicted in figure 2.

Metabolites in which their production can be related to changes connected to rebuilding gut flora in the course of diets
Since ethanol is oxidized to acetaldehyde, a reaction catalyzed by alcohol dehydrogenase (ALD), one would expect the changes in the levels of these compounds in breath to run parallel. However, this is obviously not
the case. The levels of ethanol and acetaldehyde have changed in the opposite direction in these two patient groups; thus an elevated acetaldehyde and a lower ethanol concentration could be observed in the KD group compared to the control group (table 4).

One possible explanation of these findings is based on two arguments. First is the auto-brewery syndrome, which is a well-known phenomenon leading to ethanol production by gut flora [46]. Ethanol generated in the gut is mainly converted to acetaldehyde to produce NADH + H^+ by bacterial ALD_b (figure 3). Both ethanol and acetaldehyde can be secreted into the gut lumen and taken up by enterocytes. In human tissues, a large amount of NADH + H^+ is generated as a result of ketogenic therapies and inhibits ethanol—acetaldehyde and acetaldehyde—acetate conversion by human ALD_b and aldehyde dehydrogenase, respectively; however, this makes the reverse reaction thermodynamically favorable [46]. At the same time, acetyl-CoA levels are also elevated, which is an obstacle to the further oxidation of acetaldehyde. This is the case for enterocytes as well (figure 3). In the case of non-KD subjects, ethanol is fairly oxidized to aldehyde. This argument explains why changes moved in different directions.

The second argument is the rebuilding of gut flora. The relationship between humans and their intestinal microbial community is complex and mutually favorable under physiological conditions. The gut ecosystem provides beneficial products (e.g. vitamins) for the host, while the host grants a suitable environment for microbiota [47, 48]. The structure of the gut microbiome varies as a function of adaptation to the intestinal environment depending on diet, diseases, or antibiotics taken [47–50].Ethanol production of gut flora depends on microorganisms and nutritional conditions [51]. The cKD reduces total intestinal flora, which results in changes in the metabolites produced [52]. Nevertheless, this is a field that warrants further investigation.

### Effect of food

There are compounds, e.g. furan and its metabolite 2-methylfuran, that are present in food [53]. It is, however, a question to what extent food can be responsible for elevated levels of 2-methylfuran in breath. Despite this uncertainty, 2-methylfuran was detected at a significantly elevated level in the KD group in comparison to the non-KD group (table 4). The reason could be its higher intake from food, but this suggestion needs further verification.

### Limitations of the study

In this study the number of patients enrolled was relatively low. This may explain why the p value did not reach the level of significance in some cases. A further limitation of this study is to what degree group unification was successfully undertaken. Two
points need to be mentioned here. Firstly, the age range of children was wide (4–18), and this made the interpretation difficult because of the broader concentration range of the exhaled volatiles. Secondly, exploring the family environment did not extend to factors that might have potential importance for the study, for example, the smoking of family members. Methyl-vinyl-ketone in the breath of KD group patients was significantly elevated in comparison to controls (table 3). However, methyl-vinyl-ketone is not only a major oxidation product of isoprene [31], but is also among the hundreds of metabolites found in cigarette smoke [54]. Although isoprene, methacrolein, and methyl-vinyl-ketone levels changed in similar ways, a child living with smoking parents may present higher levels than one living with non-smokers. This rule also applies to other compounds abundant in urban environments, e.g. furan [54].

It is also rational to suggest that different neurological conditions in the control group (non-diet group) might have an effect on the metabolic network. This is part of the problem of group unification, which is a function of patient recruitment and sample size. Nevertheless, we are short of literature data that either supports or opposes this suggestion.

Conclusions

In conclusion, several volatile metabolites were detected in the breath of children. Changes in the levels of metabolites could be explained in the majority of cases, and were related to biochemical changes caused by ketogenic therapy. Figure 4 depicts the basic biochemical mechanisms and changes that were observed. It is, however, a question of future research which mechanism explains the clinical findings and which metabolite can be used as a biomarker in ketogenic therapies.

As it now looks, besides the acetone/isopropanol pair, the detection of 2-pentanone and the monitoring of acetaldehyde and ethanol together may be more promising as a marker due to the fact that their concentrations change in opposite directions, thus making it possible to distinguish the ketogenic state occurring in a KD from other states, such as alcoholism and probably diabetes mellitus.

Finally, one obvious and crucial question has to be addressed in future studies. Although both starvation and ketogenic regimens are characterized by high levels of lipids and ketone bodies, the fundamental difference between them is that in the former state the body mobilizes its own metabolic sources (switching from mainly glucose to lipid-based energy provision [55]), while in the latter case lipids arise from an external source. If this difference is significant then the metabolic and cellular events cannot be one and the same.

Acknowledgments

We acknowledge Dr Christopher A Mayhew for his critical reading of the manuscript. VR gratefully acknowledge financial support from the Austrian Research Promotion Agency (Forschungsförderungsgesellschaft (FFG)) for the program KIRAS Security Research under grant DHS-AS. Furthermore, we thank the government of Vorarlberg (Austria) for its generous support, and the European Union’s Horizon 2020 Program for research, technological development, and demonstration (grant agreement No. 644031). MPK was supported by the Theoretical Biology Research Group (Hungary).
References

[1] Wheelis J W (ed) 2004 History and origin of the ketogenic diet *Epilepsy and the Ketogenic Diet* ed C E Stafstrom and J M Rho (Totowa, NJ: Humana Press) pp 31–50
[2] Morris A A 2005 Cerebral ketone body metabolism *J. Inherit. Metab. Dis.* 28 109–21
[3] Hartman A L et al 2007 The neuropharmacology of the ketogenic diet *Pediatr. Neurol.* 36 281–92
[4] Kalapos M P 2007 Possible mechanism for the effect of ketogenic diet in cases of uncontrolled seizures—the reconsideration of acetonemia theory *Med. Hypotheses* 68 1382–8
[5] Driver R L 1947 Isopropyl alcohol, other ketogens, and miscellaneous agents on thresholds for electrical convulsions and diphenylhydantoin *Proc. Soc. Exp. Biol. Med.* 64 248–51
[6] Rho J M et al 2002 Acetoacetate, acetone, and dibenzylamine (a contaminant in L- (+)-beta-hydroxybutyrate) exhibit direct anticonvulsant actions in vivo *Epilepsia* 43 358–61
[7] Gasior M et al 2007 The anticonvulsant activity of acetone, the major ketone body in the ketogenic diet, is not dependent on its metabolites acetyl, 1,2-propanediol, methylglyoxal, or pyruvic acid *Epilepsia* 48 793–800
[8] Kalapos M P 2007 What is the antiseizure activity of acetone due to? *Epilepsy* 48 2002–3
[9] Musa-Veloso K 2004 Non-invasive detection of ketosis and its application in refractory epilepsy *Prostaglandins, Leukotrienes Essent. Fatty Acids* 70 329–35
[10] Musa-Veloso K et al 2006 Breath acetone predicts plasma ketone bodies in children with epilepsy on a ketogenic diet *Nutrition* 22 1–8
[11] Koppel S J and Sverdlov R H 2017 Neuroketherapeutics: a modern review of a century-old therapy *Neurochem. Int.* 17 30227–9
[12] Kossoff E H, Zupec-Kania B A and Rho J M 2009 Ketogenic diets: an update for child neurologists *J. Child Neurol.* 24 979–88
[13] Sharma S and Jain P 2014 The modified Atkins diet in refractory epilepsy *Epilepsy Res. Treat.* 2014 404202
[14] Bergqvist A G 2012 Long-term monitoring of the ketogenic diet: Do’s and Don’ts *Epilepsy Res.* 108 261–6
[15] Zamani G R et al 2016 The effects of classic ketogenic diet on serum lipid profile in children with refractory seizures *Acta Neurol. Belg.* 116 529–34
[16] Erhart S et al 2009 3-Heptanone as a potential new marker for valproic acid therapy *J. Breath Res.* 3 016004
[17] Cahill G F Jr 1970 Starvation in man N Engl. J. Med. 282 668–75
[18] Sugden M C, Holmes M J and Palmer T N 1989 Fuel selection and carbon flux during the starved-to-fed transition *Biochem. J.* 263 313–23
[19] McCue M D 2010 Starvation physiology: reviewing the different strategies animals use to survive a common challenge *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* 156 1–18
[20] Reichard G A et al 1979 Plasma acetone metabolism in the fasting human *J. Clin. Invest.* 63 619–26
[21] 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
[22] Seymour K J et al 1999 Identification of cerebral acetone by 1H-MRS in patients with epilepsy controlled by ketogenic diet *MAGMA* 8 53–42
[23] Musa-Veloso K et al 2002 Epilepsy and the ketogenic diet: assessment of ketosis in children using breath acetone *Pediatr. Res.* 52 443–8
[24] Walker V and Mills G A 2014 2-Pentanone production from hexanoic acid by *Pentillumium roqueforti* from blue cheese: is this the pathway used in humans? *Sci. World J.* 2014 215783
[25] Karl T et al 2001 Human breath isoprene and its relation to blood cholesterol levels: new measurements and modeling *J. Appl. Physiol.* 91 762–70
[26] Mendis S, Sobota P A and Euler D E 1994 Pentane and isoprene in expired air from human: gas-chromatographic analysis of single breath *Clin. Chem.* 40 1485–8
[27] Nelson N et al 1998 Exhaled isoprene and acetone in newborn infants and in children with diabetes mellitus *Pediatr. Res.* 44 363–7
[28] Smith D et al 2010 Isoprene levels in the exhaled breath of 200 healthy pupils within the age range 7–18 years studied using SIFT-MS *J. Breath Res.* 4 011017
[29] King J et al 2012 Measurement of endogenous acetone and isoprene in exhaled breath during sleep *Physiol. Meas.* 33 413–28
[30] Burcham P C 1998 Genotoxic lipid peroxidation products: their DNA damaging properties and role in formation of endogenous DNA adducts *Mutagenesis* 13 287–305
[31] Liu Y et al 2013 Production of methyl vinyl ketone and methacrolein via the hydroperoxyl pathway of isoprene oxidation *Atmos. Chem. Phys.* 13 5715–30
[32] Kang H C et al 2004 Early- and late-onset complications of the ketogenic diet for intractable epilepsy *Epilepsia* 45 1116–23
[33] Dashi H M et al 2004 Long-term effects of a ketogenic diet in obese patients *Exp. Clin. Cardiol.* 9 200–5
[34] Nazarewicz R R et al 2007 Effect of short-term ketogenic diet on redox status of human blood *Rejuvenation Res.* 10 435–9
[35] Rhyu H S, Cho S Y and Roh H T 2014 The effects of ketogenic diet on oxidative stress and antioxidant capacity markers of Taekwondo athletes *J. Exerc. Rehabil.* 10 362–6
[36] Allerheiligen S R, Ludden T M and Burk R F 1987 The pharmacokinetics of pentane, a by-product of lipid peroxidation *Drug Metab. Dispos.* 15 794–800
[37] Li P et al 2009 Breath pentane: an indicator for early and continuous monitoring of lipid peroxidation in hepatic ischaemia-reperfusion injury *Eur. J. Anaesthesiol.* 26 513–9
[38] Nycky J A, Drury J A and Cooke R W I 1998 Breath pentane as a marker for lipid peroxidation and adverse outcome in preterm infants *Arch. Dis. Child.* 79 F67–9
[39] Takayanagi R, Takeshige K and Minakami S 1980 NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. dependence on the rate of electron flow in the respiratory chain and an antioxidant role of ubiquinol *Biochem. J.* 192 853–60
[40] Jain S K, Kannan K and Lim G 1998 Ketosis (acetooacete) can generalize and cause increased lipid peroxidation and growth inhibition in human endothelial cells *Free Radiol. Biol. Med.* 25 1083–8
[41] Milligan L P and Baldwin R L 1967 The conversion of acetooacete to pyruvaldehyde *J. Biol. Chem.* 242 1095–101
[42] Takayama K et al 1976 Generation of electronic energy in the myoglobin-catalyzed oxidation of acetoacetate to methylglyoxal *Arch. Biol. Chem.* 176 663–70
[43] Coppola G et al 2014 The impact of the ketogenic diet on arterial morphology and endothelial function in children and young adults with epilepsy: a case-control study *Seizure* 23 260–5
[44] Masino S A and Rho J M (ed) 2012 Mechanism of ketogenic diet action *Jasper's Basic Mechanisms of the Epilepsies* (Bethesda, MD: Oxford University Press) pp 1–28
[45] Mueller S G et al 2001 Brain glutathione levels in patients with epilepsy measured by in vivo (1H-MRS) *Neurology* 57 1422–7
[46] Logan B K and Jones A W 2000 Endogenous ethanol ‘auto-brewery syndrome’ as a drunk-driving defence challenge *Med. Sci. Law* 40 206–15
[47] Albenberg L G and Wu G D 2014 Diet and the intestinal microbiome: associations, functions, and implications for health and disease *Gastroenterology* 146 1564–72
[48] Llorente C and Schnabl B 2015 The gut microbiota and liver disease *Cell. Mol. Gastroenterol. Hepatol.* 1 275–84
[49] Selkirk J et al 2014 Metabolic tinkering by the gut microbiome: implications for brain development and function Gut Microbes 5 369–80
[50] Kirpich I A, Parajuli D and McClain C J 2015 The gut microbiome in NAFLD and ALD Clin. Liver Dis. 2015 55–8
[51] Elshaghabee F M et al 2016 Ethanol production by selected intestinal microorganisms and lactic acid bacteria growing under different nutritional conditions Front Microbiol. 7 47
[52] Newell C et al 2016 Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder Mol. Autism 7 37
[53] Fromberg A et al 2014 Furan and alkylated furans in heat processed food, including home cooked products Czech J. Food Sci. 32 443–8
[54] Vickroy D G 1976 The characterization of cigarette smoke from Cytrel® Smoking products and its comparison to smoke from flue-cured tobacco. 1. vapor phase analysis Beiträge zur Tabakforschung 8 415–21
[55] Soeters M R et al 2012 Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation Am. J. Physiol. Endocrinol. Metab. 303 E1397–407