Real-Time Quantification of Amino Acids in the Exhalome by Secondary Electrospray Ionization–Mass Spectrometry: A Proof-of-Principle Study

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BACKGROUND: Amino acids are frequently determined in clinical chemistry. However, current analysis methods are time-consuming, invasive, and suffer from artifacts during sampling, sample handling, and sample preparation. We hypothesized in this proof-of-principle study that plasma concentrations of amino acids can be estimated by measuring their concentrations in exhaled breath. A novel breath analysis technique described here allows such measurements to be carried out in real-time and noninvasively, which should facilitate efficient diagnostics and give insights into human physiology.

METHODS: The amino acid profiles in 37 individuals were determined by ion-exchange HPLC in blood plasma and simultaneously in breath by secondary electrospray ionization coupled to high-resolution mass spectrometry. Participants were split into training and test sets to validate the analytical accuracy. Longitudinal profiles in 3 individuals were additionally obtained over a 12-h period.

RESULTS: Concentrations of 8 slightly volatile amino acids (A, V, I, G, P, K, F, Orn) could be determined in exhaled breath with a CV of <10%. Exhalome validation studies yielded high accuracies for each of these amino acids, on average only 3% less compared to plasma concentrations (95% CI ±13%). Higher variations were found only for amino acids with a low plasma concentration.

CONCLUSIONS: This study demonstrates for the first time that amino acids can be quantified in the human breath and that their concentrations correlate with plasma concentrations. Although this noninvasive technique needs further investigation, exhalome analysis may provide significant benefits over traditional, offline analytical methods.

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Determination of the concentrations of amino acids is often performed in medicine, including in neonatal screening programs for detecting metabolic disorders, or in adults to monitor disease activity and treatment and for differential diagnosis. In addition to their everyday clinical use, amino acids are also emerging as biomarkers for early detection of a number of common and complex diseases, because of their central role in human metabolism. Accordingly, plasma free amino acid (PFAA)[6] profiles have become a powerful tool for diagnosis over the past few years. The predictive value of amino acid profiles have been demonstrated for diseases such as cancer *(1, 2)*, type-2 diabetes *(3–5)*, and Alzheimer disease *(6)*. Among PFAAs, branched-chain amino acids (BCAA), i.e., L, I, and V, have been studied more extensively because of their unique skeletal muscle metabolism. Strong evidence has been found linking BCAA to type-2 diabetes *(7–9)* and pancreatic adenocarcinoma *(10)*.

Ion-exchange HPLC (IE-HPLC) followed by ninhydrin derivatization for UV detection is the current gold standard for analyzing amino acids in body fluids. However, this method is invasive, time-consuming, and lacks specificity, because metabolites and drugs other than amino acids can react with ninhydrin and coelute during the measurement process. IE-HPLC analysis is also com-

plex, requiring a skilled operator, and generates a substantial amount of solvent waste. Most importantly, to be successful, this method requires robust and standardized sampling, sample handling, and sample preparation.

Analysis of exhaled breath, on the other hand, provides instant results, and may thus offer a simple alternative to traditional laboratory based methods. In this sense, breath analysis is gaining attention as a novel technique for clinical purposes such as diagnostics [11] and pharmacokinetics [12]. Breath analysis can detect differences between individual "breathprints" which are reasonably stable over time [13], can be used to follow diurnal metabolic patterns [14, 15], and is capable of quantifying hitherto unknown compounds in human breath [16, 17]. It also has been proposed for the detection of biomarkers to diagnose lung and sleep diseases [18, 19]. In all of these applications, breath analysis is usually thought of as being restricted to volatile or semivolatile compounds that cross from the blood to the breath via the alveolar compartment [20]. However, a new generation of instruments that employ very sensitive high-resolution mass spectrometry (HRMS) methods, including proton-transfer-reaction TOF [21] MS or secondary electrospray ionization (SESI)–Orbitrap MS, [22–24] now reach limits of detection in the low parts-per-trillion by volume (pptv) range [25], which enables the detection of only partially volatile compounds. This applies to several PFAAs whose reference interval plasma concentrations are in the range of $10–1000$ μmol/L [25–27] and whose Henry's law constants for aqueous solutions are as high as $10^5–10^6$ $mol \cdot m^{-3} \cdot Pa^{-1}$ [28], which results in expected breath concentrations of only around $1–10$ pptv.

The aim of this work was to investigate whether amino acids, including BCAA, could be detected in exhaled breath by means of SESI-HRMS. Furthermore, we hypothesized that if even minute concentrations of amino acids can be quantified in breath via SESI-HRMS, this may allow a correlation with plasma concentrations to be established. We suggest that determination of amino acids in breath could become an interesting alternative diagnostic tool compared to traditional plasma amino acid studies.

Materials and Methods

STUDY DESIGN AND PARTICIPANTS
The amino acid profile was synchronously determined by IE-HPLC (blood plasma) and SESI-HRMS (exhalome) in 37 individuals without any known metabolic disease. A power calculation was performed to estimate the required sample size. This calculation used an α of 0.05 and a β of 0.2, and suggested that 12 individuals would be required to detect a mean alanine concentration difference of 50 μmol/L between the IE-HPLC analyzed plasma amino acid concentration and the SESI-HRMS

exhalome analysis. A 50 μmol/L alanine threshold is smaller than interpersonal differences for this amino acid and was chosen based on our previous experience with this technology [29]. Subsequently, participants were split into training (n = 25) and test (n = 12) sets, with no significant differences identified in their baseline characteristics (sex, age, body-mass-index, see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue9). The training set was derived from a cystic fibrosis cohort and used to develop the predictive model. The test set (healthy controls) was used to provide a prospective independent performance validation of the model.

REAL-TIME BREATH ANALYSIS
The methods used for sampling, processing, and analyzing the data in this study have been published in detail elsewhere [19]. In short, study requirements for participants were to refrain from consumption of alcohol and caffeine or use of tobacco and chewing gum 1 hour before assessment. All candidates were in a fasting state before measurement [19]. For SESI-HRMS, a standardized protocol was applied to all participants to exclude the influence of breathing maneuvers on exhaled compounds and to keep artifacts to a minimum concentration [19, 30, 31]. Exhalations via a mouthpiece at a concentration of 10 mbar lasting 15 s were repeated 6 times. Exhaled breath was conducted via a heated (90 °C) Teflon tube connected to the curtain gas port of a TOF mass spectrometer [32] (TripleTOF 5600, AB Sciex) and analyzed in real time (see online Supplemental Fig. 1). The last 6 s of each exhalation (approximately 15 s) were considered for analysis, thus excluding the initial dead volume (air from the upper airways which is inhaled and does not take part in the gas exchange) [19]. Signals obtained for each amino acid, by integrating the HRMS baseline resolved peaks, were normalized against the signal of an internal standard, D_4-alanine (integrated over the same 6 s). This isotopically labeled internal standard was added to the nanoelectrospray solution and proved to be useful for correction of the instrumental shift. For SESI-HRMS/MS analysis, product ion scan experiments were run with a collision energy of 30 (15) V and an isolation window of 1 Da.

PLASMA ANALYSIS
Venous blood samples of 10 mL were obtained within 5 min of breath analyses via vacutainer blood collection tubes (BD) coated with lithium-heparin (17 IU/mL). Blood plasma samples were frozen to −80 °C and stored for further analysis. The amino acids analysis was performed by means of IE-HPLC in an accredited diagnostic laboratory using routine procedures and 2 identical IE-HPLC setups (HPLC 1 and 2). Briefly, 200 μL plasma samples were mixed with 20 μL of a sulfosalicylic

acid solution (40% w/v). The precipitated proteins were removed by centrifugation at 6500 rpm and 10 °C for 10 min. Then the supernatant was diluted with an equal volume of citrate loading buffer, and 50 μL of the solution was injected on a Biochrom 30 Plus amino acid analyzer (Biochrom Ltd). Amino acids were separated by IE-HPLC using a lithium high-performance physiological column (Biochrom Ltd) followed by postcolumn derivatization of the eluting amino acids with ninhydrin and detection of the derivatized amino acids at 570 nm and 440 nm *(33)*. In total 46 amino acids could be separated over 153 min by means of a step-gradient elution using 5 distinct lithium citrate buffers of different ionic strength (from 0.20 mol/L to 1.65 mol/L) and pH (from 2.80 to 3.55). The column temperature was also varied step-wise during the run from initial 33 °C to final 78 °C, whereas the flow was kept constant at 20 mL/h. The quantification was performed by means of external calibrators for each amino acid using the EZChrom Elite software (Agilent Technologies Inc). The amino acids were identified according to the retention time and the ratio of the area between the 2 wavelengths (570 nm and 440 nm).

STATISTICS
Breath signals from the training set (n = 25) were normalized against D_4-alanine and used to build a calibration curve for each amino acid (normalized breath signal vs plasma concentration) by means of Passing–Bablok regression *(34)*.

These signals resulted from averaging the last 6 scans (accumulation time per scan = 1 s) of each exhalation (i.e., reflecting mostly the end-tidal fraction). In all cases the same number of scans (i.e., 6) was used for the mean of the breath mass spectra including the internal standard.

Afterward, normalized breath signals from the test set (n = 12) were used to calculate plasma concentrations, by means of the calibration curves previously obtained, and compared with the values obtained from plasma analysis using Bland–Altman plots as a way of assessing agreement between the 2 clinical methods.

QUANTIFICATION OF VAPOR CONCENTRATION
To check our hypothesis of amino acids being exhaled at the low pptv range, alanine was quantified in breath by a method recently developed by Aernecke et al. *(35)* showing "a rapid, near-real-time capability to quantitatively measure low-vapor-pressure compounds." Briefly, the method is based on the generation of known vapor concentrations, calculated from the Clausius–Clapeyron equation, from a solid at different temperatures. A scheme of the setup is shown in Fig. 1. The mass spectrometer was the same used for breath analysis. Tested temperatures were 50.0 °C, 75.0 °C, 100.0 °C, 125.0 °C, and 150.0 °C (±0.1 °C). Vapor concentrations generated by these temperatures were calculated from the Antoine equation

using the following values tabulated for alanine: A= 11.81037, B = 5776.202, and C= −34.143. All experiments were carried out by triplicate and averaged. Final concentrations (Fig. 1) were the result of averaging multiple breath responses.

ETHICS
An independent ethics authority (Cantonal Ethics Committee Zurich, Switzerland) approved this study with the number KEK-ZH-Nr. 2014–0076. Written informed consent was obtained from each participant before participation in the study.

Results

DETECTION AND QUANTIFICATION OF AMINO ACIDS IN BREATH
As stated above, the great sensitivity achieved by SESI-HRMS in the analysis of vapors, which can reach the low pptv range, allows the determination of not only volatile and semivolatile plasma components, but also low volatility compounds if present at sufficiently high concentrations. To check whether this is the case for PFAA, the expected breath concentrations were calculated from known plasma concentrations and tabulated Henry's constant values for aqueous solutions *(28)*. Results (Table 1) suggest that some amino acids may be detectable in breath by SESI-HRMS because they reach breath concentrations above the limit of detection of this technique. Based on this, breath SESI mass spectra were searched for amino acid signals (amino acids are expected to show up as protonated molecules, [M+H$^+$]), based on their accurate mass. Up to 8 amino acids, including those suggested by the calculations presented in Table 1, were clearly detected in breath of all individuals studied (Fig. 2). Their identification was not only supported by the mass accuracy of the instrument (below ±5 ppm), but also further strengthened by obtaining tandem MS spectra. In all cases, a loss of formic acid was found, typical of amino acids, which further confirms that the traces shown in Fig. 2 correspond to the target compounds.

Quantification of vapor concentration by SESI-HRMS is challenging, especially for low-volatility compounds, because of the difficulty of generating known vapor concentrations from chemical standards. Recently, Aernecke et al. have proposed a method *(35)* for quantifying this kind of compound based on the Clausius–Clapeyron equation. This method was applied here to quantify alanine in breath from 9 different participants (Fig. 1), which resulted in breath concentrations from 4 to 24 pptv (Fig. 1, last chart) confirming, in this way, our initial hypothesis, because breath concentration of alanine seems to be in line with predictions in Table 1. The limit of detection for this method, calculated as 3 times the signal-to-noise ratio, was found to be 0.6 pptv.

Fig. 1. Gas-phase quantification of alanine in breath according to the Aernecke et al. method (34).
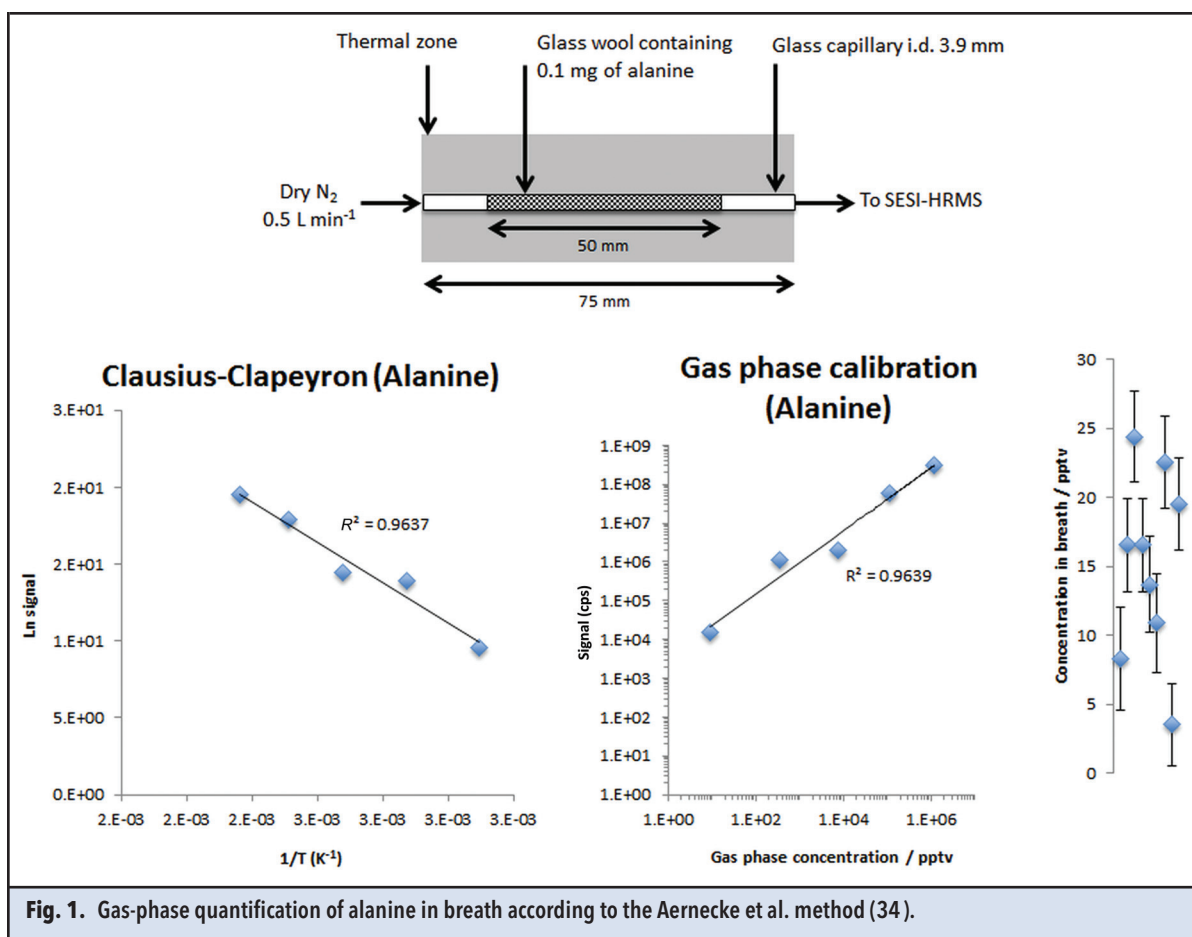
CORRELATION BETWEEN BREATH SIGNAL AND PFAA CONCENTRATION

It is known that venous blood concentration and breath concentration for a given metabolite are directly connected, which supports the use of breath analysis as a surrogate for plasma measurements (36, 37). To check this correlation, Passing–Bablok regression curves were built for each amino acid using data from the training set (25 calibration points). These curves (see online Supplemental Table 2) confirmed a high correlation between breath signals and PFAA concentration (quantified by IE-HPLC) with Pearson's r and Spearman's ρ values above 0.85. Variability was checked for breath signals by repeated measurements and compared with IE-HPLC

Table 1. Predicted concentrations of amino acids in breath.

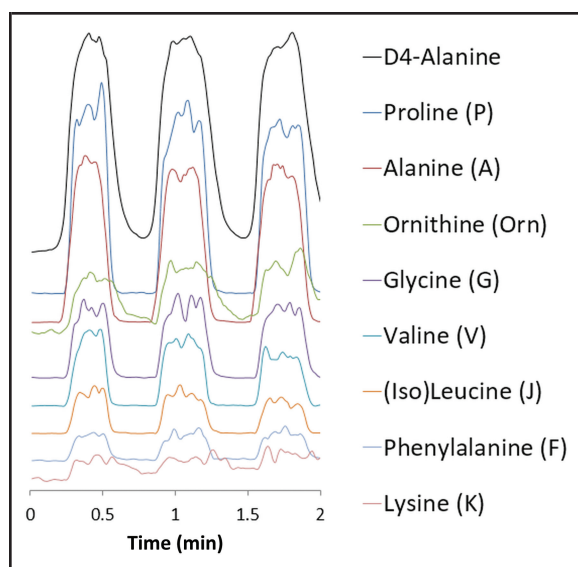| Amino acid	Formula	$[M+H]^+$	Blood, µmol/L	K_H,[a] mol · L^{-1} · atm^{-1}	Breath, pptv
A	$C_3H_7NO_2$	90.0549	419	6.00E+07	6.98
L	$C_6H_{13}NO_2$	132.1019	160	2.00E+07	8.00
G	$C_2H_5NO_2$	76.0393	236	9.00E+07	2.62
R	$C_6H_{14}N_4O_2$	175.1189	89	1.00E+17	8.90E-10
S	$C_3H_7NO_3$	106.0499	114	4.00E+12	2.85E-05
Q	$C_5H_{10}N_2O_3$	147.0764	645	1.00E+13	6.45E-05
N	$C_4H_8N_2O_3$	133.0607	49	1.00E+13	4.90E-06

[a] See Sander (28).

Fig. 2. SESI-HRMS traces for 8 amino acids in 3 consecutive exhalations.

Only the SESI-HRMS flow was recorded and the procedure was well tolerated by all participants. The trace for the internal standard (D4-A) has been scaled down.

method (see online Supplemental Table 3). Values below 10% were found for all amino acids.

To validate these results, signals obtained from the validation set were converted to concentrations by means of Passing–Bablok calibration curves and compared to concentrations obtained by the gold standard IE-HPLC method. Results, as Bland–Altman plots, are shown in Fig. 3.

It should be emphasized that the deviation between the 2 methods for the joint determination of V, L, and I (Σ BCAA, Fig. 3) was low (+5.9%) with an SE of ±12%. Regarding individual amino acids (Fig. 3), deviations were always below 10% with errors <25%, with the exception of F and Orn, that showed errors higher of 50%. This may be the result of their breath concentrations being too close to the limit of detection of the SESI-HRMS instrumentation and some concomitant biological factors that jeopardize the usefulness of the breath test for the quantification of these 2 amino acids. Generally, detected differences between the 2 measurement methods (IE-HPLC vs SESI-HRMS) were smaller than intraindividual differences for all amino acids [29].

LONGITUDINAL MEASUREMENTS OF AMINO ACIDS DURING 12 h

To show the capabilities of the method developed, longitudinal measurements of PFAA by SESI-HRMS breath analysis were run for 3 different participants during 12 h, excluding F and Orn. It is noted that measuring amino acids in exhaled breath is conveniently done and the noninvasiveness presents significant advantages over drawing blood for such time-dependent studies that require frequent sampling.

Measurements were started with participants in the fasting state and included breakfast and lunch. Results are shown in Fig. 4. Individual and grouped amino acids showed similar results, starting with high concentrations in fasting conditions that decreased after meals. Similar profiles were found by Fernstrom et al. [38] for medium protein diets by analyzing plasma samples.

Discussion

In this work, a novel method for quantifying amino acids in the human exhalome has been presented. This approach is distinct from traditional laboratory based blood plasma analytics in several important aspects. In direct comparison with the current gold standard IE-HPLC, amino acid analysis in the exhalome via SESI-MS offers a noninvasive, less time-consuming, and less complex alternative. Furthermore, this technique theoretically has the potential to become a fully automated real-time process. Most importantly, traditional offline measurements of amino acids (i.e., IE-HPLC) suffer from preanalytic disruptions e.g., diverse supply chain management issues, storage/transport chain problems, and human factors influencing sample acquisition. Although this study was not designed to assess these issues, we suggest that exhalome analysis is characterized by much simpler (and more robust) preanalytics by allowing sample handling steps to be skipped as a result of its real-time computation of the results at comparable quality.

POTENTIAL APPLICATIONS OF SESI-MS

Generally, a method for amino acid analysis should have the following properties: (a) high sensitivity to identify amino acids present even at very low concentrations, (b) high specificity to enable distinction from interfering compounds, and (c) accuracy and high test–retest reliability to allow for longitudinal monitoring strategies for therapies. The current data of this proof-of-principle study suggest that SESI-MS is especially powerful in its accuracy and high test–retest reliability. It still has problems with amino acids with a low plasma concentration and/or a very low volatility (i.e., F and Orn) generating data with standard errors higher than 50% when compared to plasma analysis. Not a single participant with an IE-HPLC result in the reference interval showed an abnormal exhalome analysis result. Because this study was limited to healthy participants and did not include patients with known metabolic diseases, it is unknown whether the current technique is sufficiently accurate to diagnose patients with metabolic diseases, especially for diseases that result in abnormally low plasma concentrations of some amino acids. It should be noted, however,
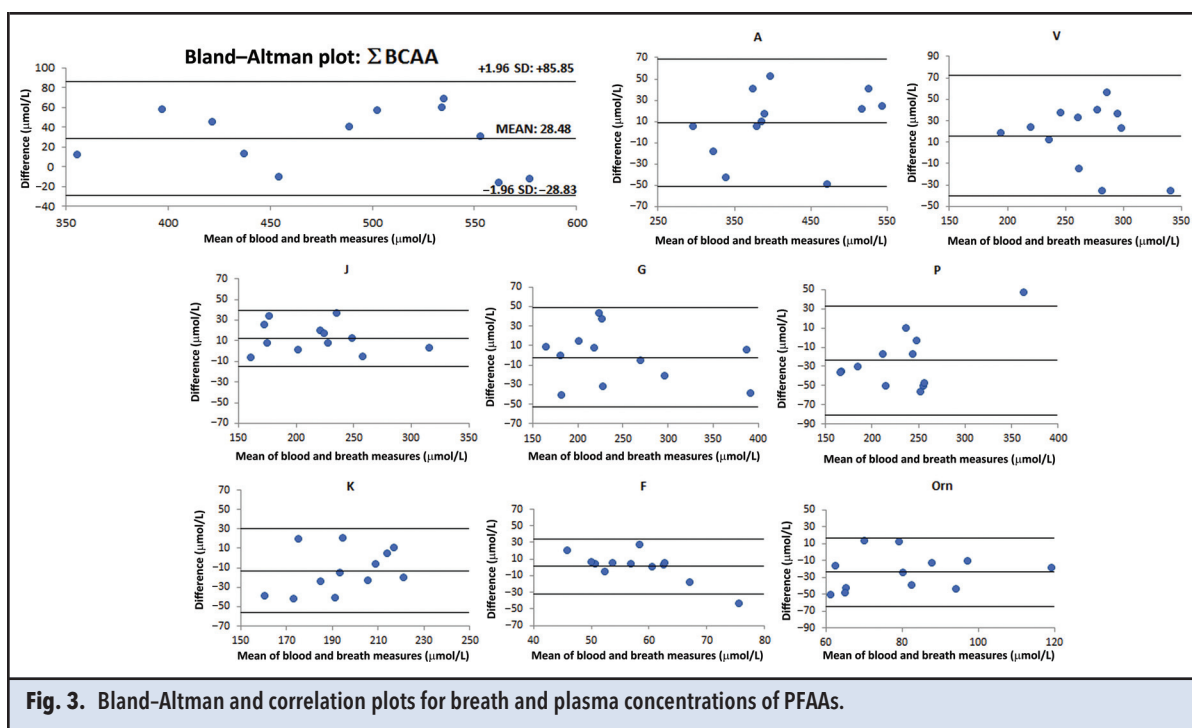
Fig. 3. Bland-Altman and correlation plots for breath and plasma concentrations of PFAAs.

that most metabolic diseases are associated with abnormally increased rather than decreased concentrations of amino acids in blood, e.g., phenylketonuria and hyperphenylalaninemias (increased F); maple syrup urine disease (increased *a*Ile); lactic acidosis, pyruvate metabolisms disorders and in mitochondriopathies (increased alanine); or propionic and methylmalonic acidemias (increased G). In these disorders, the corresponding amino acids can easily reach concentrations in the mmol/L range. Although SESI-MS has the potential to diagnose these diseases, the question remains of whether it will be sensitive enough to detect pathological concentration of notoriously low abundance and/or less volatile amino acids like citrullin, *a*Ile, argininosuccinate, or homocysteine, which are specific markers of some metabolic disorders.

We conclude that possible areas of clinical application of the current technique include (*a*) monitoring of disease activity and treatment/dietary compliance in patients; (*b*) large epidemiological studies investigating amino acid profiles (*3*); and (*c*) experimental studies for the identification of new pathways and biomarkers of disease. For example, based on this study, the monitoring of dietary compliance in patients suffering from phenylketonuria (a disease affecting 1 in 8000 individuals, who need regular monitoring of their blood F concentration) with SESI-MS seems like a promising and feasible clinical application.

PHYSIOLOGICAL CONSIDERATIONS
The described method also raises questions about the underlying physiological principles. It is unclear whether these results can be generalized to patients of different ages, patients with comorbidities, and children. Furthermore, breathing dynamics, temperature, and concentration gradient along the respiratory tree may influence the results the same way they do in traditional sample acquisition (temperature, blood stasis, venous/arterial gradient etc.). All these factors may contribute to an alteration of the final results. It is important to keep in mind that exhaled breath concentrations of substances only represent a proxy of the plasma concentration. Furthermore, there is evidence that the end-tidal breath concentration of substances and the underlying alveolar concentrations may be not identical, a factor which may have been revoked by data from our training set (*39*). In the light of these unknowns, our results were surprisingly accurate and allowed longitudinal measurements of high quality. Future studies are needed to confirm and expand the possible applications and performance characteristics of this method. For example, we measured only free amino acid patterns of blood plasma. These blood plasma patterns correlate strongly with those of muscles and erythrocytes, therefore new insights into human physiology with this technology could be hypothesized (*40*).

LIMITATIONS OF THE STUDY
This study comes with the limitations of a proof-of-principle study, namely, a small sample size, the lack of established performance characteristics for the novel technology (e.g., interday imprecision, trueness, specificity, interferences), and the fact that the remaining uncer-
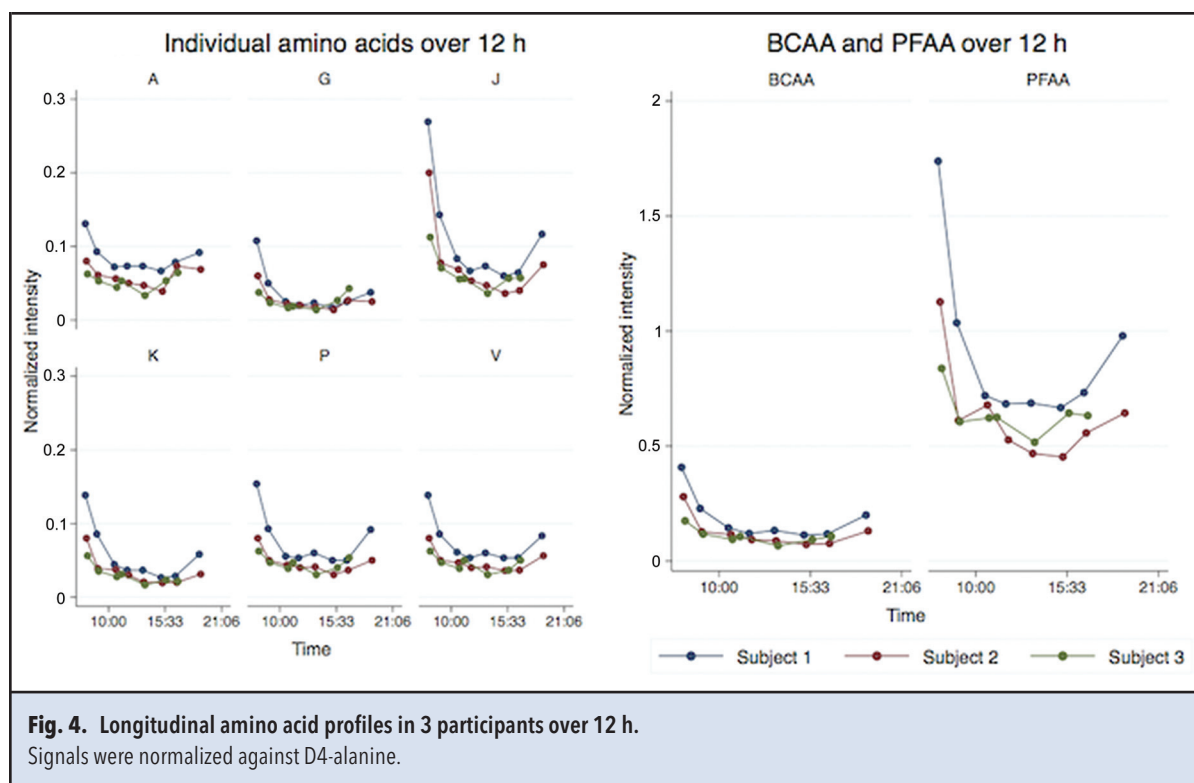
Fig. 4. Longitudinal amino acid profiles in 3 participants over 12 h.
Signals were normalized against D4-alanine.

tainties between the 2 methods were not fully elucidated. Thus, further research is needed to address the remaining inaccuracies between the 2 methods with a focus on preanalytics for the IE-HPLC method and performance characteristics. Inconsistencies within the preanalytic part of the IE-HPLC method could partly explain small disagreements between the 2 methods. Amino acids are subject to degradation when they leave the body and to a certain extent even at freezing temperatures. For example, arginase released by the erythrocytes can convert plasma arginine into Orn, which would artificially increase the concentration of the latter amino acid. This is supported by the fact that Orn measurements by SESI-MS are biased negatively from the IE-HPLC measurements (-33%). Much more robust preanalytics for the SESI-MS measurements or the fact that real-time exhalome analysis skips downstream sample handling steps entirely supports our hypothesis that SESI-MS measurements reflect true amino acid concentrations well.

In conclusion, it has been shown for the first time that amino acids can be quantified in human breath in real time, and that their concentrations correlate with plasma concentrations. These results suggest that this diagnostic method has the potential to become an alternative to the traditional determination of amino acids in plasma. Although SESI-HRMS is still under development, it may establish itself as a useful and efficient broad-spectrum clinical method, which is capable of automation, unlimited iterations, and parallel measurement of other substances. The noninvasive approach combined with real-time analysis that skips traditional preanalytic obstacles (i.e., sample storage/treatment), suggest a broad area of clinical applications in the future.

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