Personalised therapeutic management of epileptic patients guided by pathway-driven breath metabolomics

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Abstract

**Background** Therapeutic management of epilepsy remains a challenge, since optimal systemic antiseizure medication (ASM) concentrations do not always correlate with improved clinical outcome and minimal side effects. We tested the feasibility of noninvasive real-time breath metabolomics as an extension of traditional therapeutic drug monitoring for patient stratification by simultaneously monitoring drug-related and drug-modulated metabolites.

**Methods** This proof-of-principle observational study involved 93 breath measurements of 54 paediatric patients monitored over a period of 2.5 years, along with an adult’s cohort of 37 patients measured in two different hospitals. Exhaled breath metabolome of epileptic patients was measured in real time using secondary electrospray ionisation-high-resolution mass spectrometry (SESI-HRMS).

**Results** We show that systemic ASM concentrations could be predicted by the breath test. Total and free valproic acid (VPA, an ASM) is predicted with concordance correlation coefficient (CCC) of 0.63 and 0.66, respectively. We also find (i) high between- and within-subject heterogeneity in VPA metabolism; (ii) several amino acid metabolic pathways are significantly enriched ($p < 0.01$) in patients suffering from side effects; (iii) tyrosine metabolism is significantly enriched ($p < 0.001$), with downregulated pathway compounds in non-responders.

**Conclusions** These results show that real-time breath analysis of epileptic patients provides reliable estimations of systemic drug concentrations along with risk estimates for drug response and side effects.

Plain language summary

The clinical management of conditions such as epilepsy can be challenging. Each person with epilepsy responds differently to antiseizure medication and side effects are common. One approach to address this challenge is therapeutic drug monitoring (TDM), whereby levels of drugs are measured in the blood to follow the response to treatment. However, drug concentrations in the blood do not always reliably predict wanted and unwanted effects of a treatment. Here we show that a simple breath test can provide reliable estimates of circulating concentrations of a widely used antiseizure medication. In addition, the information contained in the breath signature enables us to identify which patients are likely to benefit from the treatment and which ones are likely to suffer from unwanted side effects. Our findings might help clinicians to decide how to treat people with epilepsy and to choose appropriate drug doses.
The concept of personalised medicine revolves around the idea of providing the most effective treatment with the least side effects for a given patient. In this context, the purpose of therapeutic drug monitoring (TDM) is individualising the dose to achieve maximum efficacy and, at the same time, minimise toxicity, for certain drugs with a narrow therapeutic window. Standard-of-care TDM is based on the measurements of plasma/serum drug concentration. TDM has obvious clinical benefits for patients and healthcare systems. However, it also has limitations. First, it relies on blood sampling to determine drug concentrations, which can be cumbersome to perform in infants and children. Second, drug concentrations can often not correlate with improved clinical outcome and/or minimal side effects, due to highly variable, patient-specific drug metabolisms.

Epilepsy is a complex neurological disorder affecting around 50 million people worldwide characterised by recurrent unprovoked seizures. However, treatment with one or more antiseizure medications (ASMs) allows roughly 70% of patients to live seizure free, but in the long run, 40% of those patients relapse and about 25% develop pharmacoresistance. As a result, the overall therapeutic management of epilepsy (especially, in paediatric patients) remains a challenge. Such individualised responses to medication with narrow therapeutic ranges calls for a more comprehensive phenotyping approach, beyond just monitoring systemic drug concentrations. Breath analysis has made substantial progress over the last decade by emerging analytical technologies such as secondary electrospray ionisation-high-resolution mass spectrometry (SESI–HRMS). Breath-metabolome analysis by SESI–HRMS offers a number of advantages, including noninvasiveness, short analysis time, wide metabolic coverage and capabilities to perform actual compound identification of the detected molecules (as opposed to other techniques such as chemical sensors). The latter is key to provide biochemical interpretations, hence gaining insights into the pathophysiology and drug-disease interplay. Over the last decade, a number of efforts have lifted this technology to transition from an interesting analytical platform to a standardised technique with real potential in clinical settings. Based on prior work suggesting that this technology is capable of detecting drugs as well as drug-modulated metabolites in exhaled breath, we hypothesised that this would be the case in a clinical setting, whereby it might contribute to improved phenotyping of patients with chronic epilepsy requiring TDM. Here we show that such breath-metabolomics approach has potential to reliably predict blood levels of valproic acid (VPA, an ASM) and to offer an additional patient screening layer by providing scores for side effects and response to ASMs with minimal interference into routine clinical practice and patient invasiveness.

Methods

Participants. In total, 66 paediatric epileptic patients (mean ± SD age, 10.7 ± 3.9 years; 37 males and 29 females, Supplementary Data 1) from the University Children’s Hospital Basel (UKBB), under treatment with various ASMs requiring TDM per standard care were enrolled in this study. Furthermore, we also used real-time breath data of 41 adult epileptic patients (mean ± SD age, 51.6 ± 17.1 years; 29 males and 12 females, Supplementary Data 1) from the University Hospital Zurich (USZ) to predict blood concentrations of total and free VPA. All subjects were under steady state of their ASMs at the time of measurements.

Breath real-time mass spectrometry (MRM) analysis was performed in full MS mode (scan range m/z 100–400, AGC target 1e6 and maximum injection time 500 ms) in both positive- (microscans 2 and resolution of 140,000 at m/z 200) and negative- ion mode (microscans 2 and resolution of 70,000 at m/z 200) and maximum injection time 500 ms) in both positive- and negative- ion modes. Q-Exactive Tune software (version 2.9) was used to directly control MS for these measurements. The mass spectrometer was externally calibrated on a weekly basis using a commercially available calibration solution (Pierce™ Triple Quadrupole, extended mass range, Thermo Fisher Scientific, Germany) and
internally calibrated by using common background mass-spectrometric contaminant masses as lock masses (positive mode: m/z 149.02332, 279.15909, 355.06993, 371.10123, and 391.28429; negative mode: m/z 60.99312, 73.0295, 87.04515, 89.02442, 101.0608, 115.07645, 225.23295 and 283.26425).

Serum concentrations of ASMs were measured at the clinical chemistry laboratory of University Hospital Basel (USB) as per their standard operating protocol (Supplementary Table 3).

Procedures. All subjects and/or parents, whichever applicable, signed informed consent to participate in the study in the presence of their neurologist. This study was approved by the Ethics Committee of North–western and Central Switzerland (ID 2017-01537; see supplementary information for complete clinical protocol) and the Cantonal Ethics Committee Zurich (ID 2019-00030). The sample-size calculation included in the clinical protocol is shown in Supplementary Fig. 3. Subjects performed prolonged exhalations directly into SESI–HRMS system following blood draw for TDM (median = 21.2 min; IQR = 38.6 min). Figure 1a shows a representation of a child exhaling into the device (see Supplementary Fig. 2 for a bigger image). During each measurement, the subjects provided 5, 6 replicate exhalations, both in positive- and negative-ion mode (Fig. 1b and Fig. 1c). The concentration of each ion was computed (shaded regions) and normalised by the exhalation time (nAUC). Then, the nAUCs of 5, 6 exhalations were finally averaged to represent mean nAUC of the ion.

This resulted in a 75 × 3252 (measurements × mass spectral features present in at least 10% of total measurements and correlated with exhalations) data matrix (z-score is only used here to ease visual representation; actual downstream analysis was done on raw numbers). Analysis workflow used to predict VPA serum concentration based on drug-related metabolites. The workflow used to predict side effects and drug-response scores based on drug-regulated metabolites. See Methods for more detail about panels e and f. Colour key for heatmaps is shown in-between panels e and f.
total time spent on the breath test was typically in the range of 10–15 min.

For VPA compound-identification purposes, we collected exhaled breath condensate (EBC) from one patient using an in-house condensation apparatus (containing dry ice and isopropanol). Collected EBC and pure standard of suspected molecules (dissolved in water) were analysed by ultra-high-performance liquid chromatography (UHPLC) system (Vanquish, Thermo Fisher Scientific, Germany) connected to HRMS. Samples were separated on a 50 °C heated pentfluorophenyl (PPF) column (Raptor FluoroPhenyl, 1.8 µm, 150 × 2.1 mm, Restek, USA) at a flow rate of 0.240 ml/min and eluted with a gradient between solvent A (water with 0.1% FA) and solvent B (methanol with 0.1% FA). The gradient profile was 50% solvent B between 0 and 1 min, 50–54% solvent B between 1 and 5 min, 54–95% solvent B between 5 and 5.2 min and 95% solvent B between 5.2 and 8 min followed by column reequilibration to 50% solvent B in a total 10 min run. Mass spectrometer was operated in positive-polarity full MS mode (scan range m/z 100–400, AGC target 1e6, maximum injection time 200 ms and resolution of 140,000 at m/z 200) triggering MS/MS acquisition (AGC target 1e6, maximum injection time 100 ms, resolution of 70,000 at m/z 200, loop count 5, isolation window 0.4 m/z and normalised collision energy 30) if it detects signal higher than 5000.

**Data analysis.** Raw mass spectra data from paediatric training-set patients were converted into mzXML file format using ProteoWizard’s msConvert (version 3.0.11233) and imported into MATLAB (version 2019b, MathWorks Inc., USA) for further analysis. First, each spectrum from all files was aligned using the RAFFT algorithm implemented in MATLAB.14 Then MATLAB’s mspeaks and ksdensity functions were used to appropriately pick and extract the final list of 3252 features. These features were present in at least 10% of all measurements (to avoid noisy features) and were correlated with exhalations ($r_{\text{spearman}} > 0.6$ and FDR < 0.01) in each measurement (to avoid non-breath-related features). Finally, the mean of area under the curve during each exhalation normalised by exhalation time (nAUC) was computed for each of these features in all measurements (Fig. 1c). This resulted in a data matrix of 75 × 3252 (measurements × mass spectral features; Fig. 1d and Supplementary Data 2). This data matrix was then used to develop models (i) to predict drug concentrations (Fig. 1e) and (ii) to predict side effect and drug-response scores (Fig. 1f).

For VPA-concentration prediction (Fig. 1e), first, the full training-set was reduced to 240 features, which were present in at least 80% of the measurements, whereby the patients were receiving VPA (i.e., drug-related features). Later features in the training-set were further reduced to only 11 VPA-related features (Supplementary Table 4). Afterward, time-traces for these 11 features were directly extracted from all paediatric and adult measurements using in-house C# console app based on RawFileReader (version 5.0.0.38), an open-source.Net assembly from Thermo Fisher Scientific. These time-traces were then used to generate nAUC and three different matrices (UKBB training-set, UKBB test-set and USZ test-set). The ComBat15 function from sva (version 3.34.0)16 was then used to remove the known batch effect from these matrices (Supplementary Fig. 4 and Supplementary Data 2). This reduced training-set was finally used to screen for the best regression model (see Supplementary Figs. 5 and 6). Finally, we found Gaussian process regression using exponential kernel (i.e., EGPR) to be best performing on the training-set and hence it was used on an independent test-set containing paediatric and adult patients for final predictions.

In order to gain further insights into the rest of the metabolic signature captured in breath (Fig. 1f), first, the full training-set was reduced to 1005 features present in at least 50% of total measurements and with a CV greater than 30% (i.e., drug-regulated features). Later, two-sample $t$-test was performed followed by false-discovery rate (Supplementary Fig. 7). Afterward, MetaboAnalystR (version 2.0.4)17 was used to add more biological insights into differentially abundant ions, by translating ions to metabolic pathways. The prediction of side effects vs no side effects and non-responders vs. responders in the training-set was conducted using significant metabolites identified by the enrichment analysis using first-principle-component (PC1) score. On this score, using only training-set data, a cutoff was assigned (based on Youden’s index) to separate predicted classes (Supplementary Fig. 8). Later, we projected UKBB test-set data on the training-set PC1 score to complete this analysis.

**Results**

**Overview of study pipeline and participants.** During the course of this study, whenever blood-based TDM was performed as per standard of care, the epileptic patients were asked to provide a breath sample (Supplementary Fig. 2). Figure 1 shows the overview of the study pipeline (see Methods section for more details).

In total, 93 successful measurements from 54 paediatric subjects (Supplementary Data 1) covering a wide range of clinical presentations and pharmacotherapies, were performed at the UKBB (Fig. 2 and Supplementary Figs. 9 and 10). This group of patients is a representative real-life sample of unselected hospital outpatients. In addition to paediatric test-set measurements, we also used real-time breath data of 37 adult epileptic patients (Supplementary Data 1) from the USZUSZ to independently predict blood concentrations of VPA, a well-known ASM.

**Predicting drug concentration using drug-related metabolites.** VPA was the most prescribed drug in our cohort (50 out of 93 measurements involved VPA either as monotherapy or in combination with other drugs, see Supplementary Fig. 10), which prompted us to subdivide our dataset into a training and test-set to develop a regression model to predict VPA serum concentrations based on a reduced number of breath signals (Fig. 1e). The reduced number of predictors consisted of 11 mass spectral features (Fig. 3a, b and Supplementary Table 4), which, upon further laboratory investigation (i.e., UHPLC–MS/MS of EBC; Fig. 3c–f and Supplementary Data 3) and also according to the literature18–20, were found to be, as expected, stemming from VPA. The 11 features were assigned to four unique molecules: VPA itself and three metabolites (see VPA metabolic pathway in Supplementary Fig. 11a based on18,19,21). Namely, (i) 3-heptanone, which is a nonenzymatic end product of the β-oxidation pathway of VPA; (ii) 4-OH-γ-lactone, which is an end product of the ω1-oxidation VPA; (iii) a third metabolite with molecular formula C$_7$H$_{12}$O$_2$. Importantly, 4-OH-γ-lactone has been unambiguously identified now, as previous identifications were based on MS/MS only that could not resolve between this lactone and a different potential isomer (i.e., 4-ene VPA), which shows very similar fragmentation pattern (Fig. 3). In addition, to the best of our knowledge, C$_7$H$_{12}$O$_2$ is a novel VPA metabolite not reported in the literature. Based on chemical reasoning and comparison with known VPA-degradation pathways, we hypothesise that it could be either 2,3- or 2,5-heptanedione (Supplementary Fig. 11a; hereafter referred as heptanedione). As expected, even by simple visual inspection of the breath mass...
spectra, one can appreciate that the signal intensity of these 11 mass spectral peaks was overwhelmingly more abundant in the patients taking VPA than in the patients taking other ASMs (Supplementary Fig. 12). However, the differences were less obvious for heptanedione and VPA molecules because non-VPA patients exhale other endogenous compounds (e.g., octanoic acid) that are isomers (i.e., the same exact mass) and hence cannot be resolved by SESI–HRMS22.

We further trained a regression model based on Gaussian process regression using exponential kernel (i.e., eGPR, see Supplementary Figs. 5 and 6 for details about how this model was selected) to predict the total and free serum VPA concentration, based on the signal of these 11 ions detected in exhaled breath. Figure 4 shows the predicted total and free VPA serum concentrations against the actual serum concentrations for the paediatric and adult population test-set (also see Supplementary Data 4). We used Lin’s concordance correlation coefficient (CCC) to evaluate the agreement between actual and predicted serum concentrations23. To build a complete and accurate model, we included patients receiving other drugs but VPA to capture the whole range of concentrations from zero because some VPA-taking patients may actually be well below the therapeutic range. Supplementary Fig. 13 clearly shows that the model predicts accurately the zeros (i.e., patients not receiving VPA). Regarding the prediction of VPA patients (i.e., real-world scenario, Fig. 4), the model could predict reasonably well the systemic total VPA concentrations (CCC of 0.63). For example, in the therapeutic range of total VPA (i.e., 50–100 mg/L for total and 5–10 mg/L for free VPA), the prediction was reasonably accurate; however, some patients were clearly under- or overpredicted (especially those outside the therapeutic range). One explanation for such deviation is that exhaled VPA (and its metabolites) should mirror the free fraction of VPA (rather than the total VPA), as protein-bound VPA cannot be detected in breath and only the free fraction will undergo further metabolism. Despite the limited data availability (mostly because of the difficulty to acquire enough blood sample for free VPA quantification), Fig. 4b shows better free VPA prediction for the paediatric dataset (CCC = 0.84).

Predicting side-effect and drug-response scores using drug-modulated metabolites. The second branch of our analysis (Fig. 1f) aimed at identifying endogenous metabolites altered in the training-set measurements of patients suffering from side effects (23/75), or not responding to pharmacotherapy, i.e., non-responders (26/75), or those showing abnormal EEGs (27/75) on the day of consultation (Supplementary Fig. 14). We observed a general trend of exhaled breath metabolites to be (i) upregulated...
in children suffering from side effects as compared with no side effects and (ii) downregulated in non-responders than in responders (Supplementary Fig. 7). In contrast, abnormal EEG showed no significant change in the levels of exhaled metabolites as compared with normal EEG. Subsequent pathway-enrichment analysis using MetaboAnalystR17, revealed significant enrichment ($p < 0.01$) of several amino acid metabolic pathways (Fig. 5 and Supplementary Data 5) in patients suffering from side effects. Whereas, only tyrosine metabolism was found to be significantly enriched ($p < 0.001$) in non-responders (Fig. 5 and Supplementary Data 5).

**Fig. 3 Selected predictors of importance belong to four distinct molecules.** a, b Based on training-set and the total VPA serum concentration, the contribution of each ion (predictor) was assessed by using MATLAB’s relieff and TreeBagger functions. Predictor importance from both methods was combined to generate an overall normalised weight assigned to each predictor from positive (a) and negative (b) modes. Predictors with weight above an empirical cutoff of 0.1 (shown by dashed grey line) were selected for the next steps. c-f Show the compound identification via LC-MS, based on the combination of retention time and MS/MS spectra match. We confirmed that nominal m/z at 115 belongs to 3-heptanone (c, d) and nominal m/z at 143 belongs to 4-OH-$\gamma$-lactone (e, f). This figure shows the comparison of LC-MS chromatograms (c and e) and the average MS/MS spectra (d and f) between the pure standards of suspected molecules and EBC from a VPA taker (subject 10, 3rd visit). Number of averaged MS/MS spectra is denoted by $n$ and the time at which those MS/MS spectra were obtained is denoted by the open circles in the corresponding LC-MS chromatogram.

**Fig. 4 Prediction of systemic drug concentration based on real-time breath mass spectra.** Prediction of total (a) and free (b) VPA serum concentration of independent test-set containing paediatric subjects from UKBB and adult subjects from USZ. Vertical grey box shows the reference therapeutic range of 50–100 mg/L for total VPA and 5–10 mg/L for free VPA. Solid grey line represents the identity ($y = x$) line.
Figure 6a zooms in the altered compounds identified by these analyses (see Supplementary Data 6 for further details about altered compounds). To further complete our analysis, we explored whether the endogenous altered metabolites could be used to predict which patients are likely to respond to pharmacotherapy and to suffer from side effects.

As our UKBB test-set contains a limited number of side-effect cases and non-responders, we used the whole UKBB dataset for this prediction (Fig. 6b and c and Supplementary Data 6). Finally, based on the results presented here, we proposed a clinical decision-making workflow based on real-time breath analysis (Fig. 7).

**Discussion**

In this translational study, by combining real-time, noninvasive and rapid breath analysis with sophisticated bioinformatics tools, we showed that systemic VPA concentrations can be accurately predicted (Fig. 4). Earlier work with exhaled breath measurements of 3-heptanone and 4-OH-γ-lactone showed promising results regarding the use of these molecules as potential breath-based markers for therapeutic monitoring of VPA\(^1\),\(^2\),\(^4\),\(^9\),\(^19\),\(^20\),\(^24\).

However, none of those studies performed any independent prediction of blood concentration of VPA. We also confirmed that separately protonated 3-heptanone (CCC of 0.06 and −0.16 in total and free VPA, respectively) and 4-OH-γ-lactone (CCC of 0.40 and 0.19 in total and free VPA, respectively) underperform (Supplementary Fig. 15a–d) compared with our proposed model with 11 predictors (CCC of 0.63 and 0.66 in total and free VPA, respectively).

Interestingly, we observed that including covariates such as age, gender and number of ASMs as predictors, does not necessarily make the VPA prediction any better (Supplementary Fig. 15e and f). Hence, to reduce the complexity, we used the model with only exhaled VPA-related ions as predictors. Notably, the signal-intensity distribution for these drug-related ions was similar across adult and paediatric populations (Supplementary Fig. 16). For this reason, we attempted to predict also adults’ VPA concentrations using the model created with children (Fig. 4).

Not surprisingly, the model performed better predicting the children’s subpopulation (free VPA CCC = 0.84 for children vs. 0.52 for adults). More accurate predictions are expected when creating a regression model using an adult population as training dataset. Overall, these results show the feasibility of estimating systemic VPA via breath analysis in a clinical context.

It has also been shown that free VPA is physiologically active and clinically relevant, which stresses the importance of measuring free VPA concentration\(^2\),\(^5\),\(^26\). In spite of this, current clinical practice relies most often on total VPA blood levels, perhaps due to the fact that determination of free VPA requires relatively large blood volumes, lengthy and laborious mass spectrometric analyses requiring hours-to-days of laboratory work (Supplementary Table 3). In our paediatric dataset, 9 out of 42 (21%) requests to determine free serum VPA failed, seven of them due to lack of enough material (Supplementary Data 1).

Additionally, we observed that keeping total VPA within therapeutic range does not guarantee that ultimately free VPA will also do so (Supplementary Fig. 17).

Perhaps even more importantly, conventional TDM presents fundamental shortcomings. The lack of clinical correlation between the efficacy outcomes or side effects with ASM concentrations due to high inter- and intra-individual variation, decreases the value of TDM. This limitation discourages practitioners to use TDM, except perhaps in specific circumstances (e.g., pregnancy and known pharmacokinetic interactions). The limitation that keeping the drugs’ concentrations in the
therapeutic range, does not assure an optimal clinical response of no side effects, was also evidenced in our cohort. For example, in 32% of the paediatric measurements (40 out of 123), the patients were not responding to the therapy. However, of these 40 measurements, only in 18, at least one drug was outside its reference range.

Similarly, in 37 paediatric measurements, the patients were suffering from side effects. However, blood concentration of at least one drug was outside its reference range in only 14 of these cases. Take for example visits 5 and 6 of subject 3 (Fig. 2 and Supplementary Data 1). In both visits, the patient was under the same therapeutic regime, with similar total VPA and lamotrigine (LTG) levels in blood. However, while the child was responding properly, not suffering from side effects and presented normal EEG in visit 5, in visit 6, the situation was dramatically reversed. Such individual-specific response to the medication further stresses the importance of adopting a more comprehensive and personalised stratification approach.

Here we showed the possibility of predicting free VPA concentrations in 15 min by a simple noninvasive breath test. But perhaps more importantly, the prediction is based on VPA and its downstream metabolites; hence, further insights could be gained through real-time breath analysis offers a noninvasive window into altered metabolic pathways in patients not responding to pharmacotherapy and/or suffering from side effects. A SESI-HRMS breath analysis detected alteration in the levels of several amino acids and associated compounds in epileptic patients. The figure shows a simple (unweighted, undirected, no loops, or multiple edges) graph of amino acid metabolism (based on KEGG map01230: biosynthesis of amino acids). Each node is a compound, where node-fill colour represents the mean log₂-scaled fold change in the side effects (yes vs. no), and drug-response (nonresponder vs. responders) dataset from the training set. Node-border colour represents whether the compound was assigned to significant or background list via MetaboAnalystR. Only compounds from the significantly enriched pathways (top-right quadrant in Fig. 5) are coloured. The rest are shown as grey. Node with two colours (node split) denotes that the compound was present under significantly enriched pathway of both datasets. b, c Density plot for the predicted score and classes of having side effects (b) and drug response (c). Density curves are accompanied with the actual data points, where each point represents one measurement from UKBB dataset, coloured based on clinically observed side effects (b) and clinically observed drug response (c). On predicted scores, a cutoff was assigned (based on Youden’s index calculated using only training-set data) to separate predicted classes.
on how the drug is metabolised at an individual level. For example, based on 3-heptanone and 4-OH-γ-lactone signal-intensity ratios, we could estimate inter- and intraindividual variability in the activity of β- and ω1-oxidation pathways of VPA metabolism. We found considerable heterogeneity in metabolic pathway patterns within the sampled population and even within subjects during the course of this study (Supplementary Fig. 11b).

Moreover, because the breath test captures a large number of metabolites (well beyond the drug-related metabolites just described), we explored whether some of these may be associated with the actual clinical outcome. We observed a significant enrichment ($p < 0.001$) of the tyrosine metabolism, where pathway compounds were downregulated in the subset of patients not responding to medication (Fig. 6a and Supplementary Data 6). The association between downregulation of tyrosine metabolism and increased number of seizures (i.e., not responding adequately to medication) may be rationalised by the fact that neurotransmitter dopamine, which is known for its anti-epileptic action$^{27}$, is also downregulated, since it is synthesised from tyrosine and phenylalanine$^{28}$. In addition, previous studies have suggested that administration of D-leucine$^{29}$, glutamic acid$^{30}$ and tyrosine$^{31}$, can reduce seizure frequency, which also correlates with detecting lower levels of glutamic acid and tyrosine in the non-responders of this study. Additionally, multiple earlier studies have reported on the alteration in the amino-acid concentrations in the cerebrospinal fluid and the plasma during epilepsy$^{32,33}$. Previously we have also shown that, at least for some of the amino acids mentioned here, breath levels correlate with blood concentrations$^{34}$. Moreover, recently several studies based on animal models and humans have reported about the effects of branched-chain amino acids on epilepsy$^{35}$ and the importance of having amino-acid-balanced diet$^{36}$. Taken all these observations together, it seems worth exploring different intervention routes to rebalance lower amino-acid levels found in non-responders.

Furthermore, we also found a strong enrichment of urea cycle/ amino acid metabolism, where pathway compounds were mainly upregulated in measurements with side effects (Fig. 6a and Supplementary Data 6). Previously, different randomised crossover trials have shown that glycine improves sleep quality$^{37}$, which correlates with our observation of detecting lower levels of glycine in patients with side effects (such as somnolence). Additionally, several other amino acids, such as aspartic acid, glutamic acid and γ-aminobutyric acid (GABA) have been shown to be increased in blood of subjects with aggressive behaviour$^{38}$, this is also consistent with observing a higher level of these amino acids in patients with side effects (such as irritability). VPA is known to increase blood levels of proline$^{39}$ and GABA$^{40}$. Since the majority of our population were taking VPA, higher levels of proline and GABA observed in side-effects case can also be attributed mostly to VPA. Overall, we believe that these findings open new routes for metabolic pathway-guided drug monitoring and management, by providing risk estimates for side effects as well as drug-therapy effects (Fig. 6b and c)$^{41}$. Pathway analysis may not only be relevant for the dosage of the ASMs, but also may potentially be useful for the choice of the ASMs (e.g., to rebalance amino-acid metabolism).

However, this study has several limitations. First, the inclusion criteria allowed for a quite heterogeneous patient population, which may explain the limited false-discovery rates found in the comparison between responders and non-responders. Second, chemical identification of endogenous metabolites associated with non-responders and side effects could only be postulated based on the database matching of measured accurate masses (within 2 ppm). Chemical identification with the highest degree of confidence would require further UPLC–MS/ MS analysis using chemical standards, as done in this work for VPA metabolites. For this reason, although the enrichment analysis algorithm used here analyses at a collective level the behaviour of groups of metabolites (assuming random errors at the individual peak level), the biochemical/metabolic interpretation should be taken cautiously, until unambiguous chemical identification is provided. Third, the reported altered pathways in non-responders and patients suffering from side effects could not be completely tested in an independent cohort. Fourth, the prediction of (total and free) VPA concentrations in the adult dataset was done using regression model trained on paediatric dataset, due to limited size of the adult dataset. Most of these limitations can be overcome by ongoing recruitment of more participants; however, the results shown here provide sufficient evidence on the feasibility of applying breath metabolomics in such clinical context.

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Fig. 7 Proposed concept of extension of current TDM methods to monitor noninvasively drug-related and drug-modulated metabolites in exhaled breath. The workflow starts with screening the breath metabolome of a new patient requiring TDM. Then, drug serum concentrations are predicted based on drug-related metabolites, whereas drug-modulated metabolites can be used to estimate side effect and drug-response scores. This information is available within 15 min on the same day of consultation. Based on this information, the responsible clinician decides whether to continue treatment, adjust the dose, or change medication. Once the decision is made and implemented, the patient’s breath metabolome is reassessed in the next visit to repeat the whole process.
In conclusion, based on the evidence presented here, we propose that SESI–HRMS breath testing may serve as a companion diagnostic approach to potentially minimise drug side effects and help to choose the seizure-specific treatment. Key advantages that make it ideal for the hospital in- and outpatient setting include noninvasiveness and real-time results. It is thus ideally suited for chronically ill patients and children in real time during an outpatient’s consultation. This, along with the ability to predict serum concentration of drugs, allows us to propose a clinical decision-making workflow based on real-time breath analysis (Fig. 7). Furthermore, we believe the data presented here will serve as the foundation that may transform today’s antiseizure therapy selection approach to a more objective pathway-based personalised approach. Future randomised controlled drug trials may profit from SESI–HRMS breath metabolomics analysis at study entry to better characterise responding/nonresponding patients. We finally envision that this concept has potential to be deployed in drug monitoring of other chronic diseases.

Data availability
All the data generated and analysed that support the findings in this study are within the article and its supplementary information files and are available from the corresponding author upon reasonable request. Additionally, the RAW and mzXML files of the real-time breath measurements are available from the MetaboLights (https://www.ebi.ac.uk/metabolights) repository (accession number MTBLS2400).

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Author contributions

K.S., V.C.Z., J.v.d.A., A.N.D. and P.S. designed the study. K.S., M.O. and M.A. did the breath mass spectrometric measurements. K.S. and P.S. developed the pipeline to analyse the breath mass spectrometric data. K.S. collected EBC and performed LC–MS/MS measurements. K.S., M.K., D.G.G., J.v.d.A., U.F., A.N.D. and P.S. interpreted the data. K.S., U.F., A.N.D. and P.S. wrote the manuscript. V.C.Z., J.U., L.L.I., M.K., D.G.G. and J.v.d.A. contributed further to the writing of the manuscript. A.N.D. and L.L.I. assessed the clinical status of the paediatric and adult epilepsy patients, respectively.

Competing interests

PS and MK are cofounders of Deep Breath Intelligence AG (Switzerland), which develops breath-based diagnostic tools. KS is consultant for Deep Breath Intelligence AG (Switzerland). All other authors declare no competing interests. The breath-analysis concept of predicting drug concentrations and risk scores, and the data-processing pipeline discussed here have been incorporated in the European patents 20186274.5, filed on July 16th 2020 and 21185400.5, filed on July 13th 2021, respectively.

Additional information

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