Acute liver failure and acute kidney injury: Definitions, prognosis, and outcome
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Chapter 7

Exhaled breath analysis with electronic nose technology for detection of acute liver failure in rats


Submitted for publication
Abstract

Background & Aim: Exhaled breath analysis using electronic nose (e-Nose) technology is potentially useful in clinical diagnostics and monitoring because of the real time and non-invasive measurement. The aim of this study was to assess the classification accuracy of an e-Nose in detecting acute liver failure (ALF) in rats.

Methods: Exhaled breath from 14 rats was repeatedly sampled by e-Nose (8 sensors) and an additional external CO₂ sensor at three stages: healthy period; portacaval shunt; and during the development of ALF due to surgically induced complete liver ischemia. We performed principal component analysis (PCA) on the (grouped) sensor data in each stage and the classification accuracy of the first two principal components was assessed by the leave-one-out approach. In addition we performed gas chromatography - mass spectrometry (GC-MS) analysis of the exhaled breath from 3 rats.

Results: The first and second principal components from the PCA analysis of e-Nose data accounted for more than 95% variance in the data. Measurements in the ALF stage were contrasted with the measurements in the control stage. Leave-one-out validation showed classification accuracy of 96%. This accuracy was reached after 3 hours of ALF development, and was reached already after 2 hours when data of an external CO2 sensor were also included. GC-MS identified 2-butanol, 2-butanone, 2-pentanone and 1-propanol as most elevated in the ALF stage.

Conclusion: This is the first study to demonstrate that ALF in rats can be detected by e-Nose data analysis of the exhaled breath. Confirmation of these results in humans will be an important step forward in the non-invasive diagnosis of ALF.
Introduction

Acute liver failure (ALF) is a syndrome with a high mortality estimated 60 to 80% \(^1\). In most cases the only life saving treatment for ALF is liver transplantation (LT). An early and exact diagnosis of ALF together with a prediction of its further development is crucial in order to determine the further management and prevent a delay of listing the patients for LT.

A number of prognostic criteria have been proposed for ALF. However, they do not have sufficient predictive performance capacities \(^2\) and the search for alternative methods continues. Current prognostic criteria are mostly based on blood biochemical analysis. Ideally a predictive method should be non-invasive, available at the bed-site, cheap, and providing real-time results.

In recent years, exhaled breath analysis has been increasingly studied as an alternative to the invasive method of blood testing. In clinical practice they have been performed mainly by gas chromatography and mass spectrometry (GC/MS) \(^3, 4\). These methods, however, do not find widespread application due to their expensiveness, time consuming measurement and requirement of high expertise for the interpretation of the results \(^5\).

To overcome this, the electronic nose (e-Nose) – a device to mimic the discrimination of the mammalian olfactory system for smells – was introduced in 1982 \(^6\). E-Nose technology has become commercially available and is potentially useful in clinical medicine because of the non-invasive testing, real-time, rapid results and portability. The underlying technology varies among the e-Noses \(^7\) but the general principle is based on pattern recognition of complex mixtures, which is comparable to smelling by the human nose.

Human breath is largely composed of nitrogen, oxygen, carbon dioxide, water vapour and additionally carbon monoxide, nitric oxide and nobel gases (ppm’s) and of very little amounts of numerous volatile organic compounds (VOCs (ppb’s)) \(^8\). Generally an e-Nose is built of an array of chemical sensors that react to the different fractions of the VOCs. When these sensor responses are combined, so-called “breath print” specific for a disease can be sought and be useful for diagnostic and monitoring purposes. When using GC-MS in parallel, identification of individual VOCs may allow exploration of the underlying pathophysiological pathways.

VOCs can give different odour to the breath. Specific VOCs have been characterised for gastrointestinal and liver diseases \(^3, 4, 9-12\). Recently, e-Nose has gradually been used in medicine for the diagnosis of diabetes \(^13-15\), renal disease \(^15-17\), urinary track infections \(^18\), asthma and chronic obstructive pulmonary disease \(^19-22\), pneumonia \(^23, 24\), Alzheimer and Parkinson disease \(^25\), lung cancer \(^21, 26-28\), breast, colorectal, prostate cancers \(^29\) and others \(^5, 7\).
Our hypothesis is that e-Nose is able to detect ALF in early stage. We assume that ALF features a distinguishable “breath print” derived from principal components of e-Nose sensor data. The objective of this study is to assess the classification accuracy of using such principal components for distinguishing ALF measurements from other measurements. As proof of concept an established animal model of ALF in rats was studied in which each rat was its own control. Finally, GC-MS of exhaled air was used to explore which VOCs might contribute to the ALF breath prints.

**Methods**

*Experiment*

All procedures were conducted in accordance with the institutional guidelines of the Animal Ethical Committee of the AMC (protocol number BEX 102522) for supervision of animal experiments and all efforts were made to minimize suffering. The experiment was performed in a rat-model developed for the purpose of this study (Figure 1). A picture of our experimental set-up is presented in Supplement Figure 1. In our model the rats served as their own control. 14 male Wistar rats (Charles Rivers), weighing 250-280 g were used. They were kept in a temperature-controlled room and fed standard rat chow and water ad libitum.

**Figure 1.** A schematic presentation of the experimental set-up
**Induction of ALF**

To resemble the clinical ALF characterized by complete loss of liver function and massive liver cell necrosis, complete liver ischemia (LIS) was obtained in a 2-steps procedure (adapted from 30). In the first step at day 3 pre-LIS an end-to-side portacaval shunt was performed between the portal vein and the inferior caval vein (PCS). For this purpose, rats were anesthetized with isoflurane 2-3% and O2/air 1:1 and buprenorphine (Temgesic) 0.03mg/kg and placed on a temperature-regulated table (37 °C) to maintain the body temperature. Rats were allowed to recover for 3 days to exclude bleeding from the shunt anastomosis. In the second step complete ischemia of the liver was performed by ligation of the hepatic artery and common bile duct. For this purpose, rats were anesthetized with isoflurane 2-3% and O2/air 1:1 and placed on a temperature-regulated table (37 °C). Both procedures were conducted through a midabdominal incision. During the LIS period, if necessary, 5% glucose mixture was applied intraperitoneally in a dosage depending on the blood glucose level. Following the surgical procedures, rats were allowed to recover in their cages. During the LIS period severity of hepatic encephalopathy (HE) was assessed for each rat (Table 1) 31.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal behaviour</td>
</tr>
<tr>
<td>1</td>
<td>Mild lethargy</td>
</tr>
<tr>
<td>2</td>
<td>Poor posture control; decreased motor activity</td>
</tr>
<tr>
<td>3</td>
<td>No spontaneous righting reflex; severe ataxia; diminished response to pain</td>
</tr>
<tr>
<td>4</td>
<td>No righting reflex on pain stimulus</td>
</tr>
<tr>
<td>5</td>
<td>Deep coma; no reaction on pain stimulus</td>
</tr>
<tr>
<td>6</td>
<td>Death</td>
</tr>
</tbody>
</table>

**Blood collection**

Blood samples were collected from the rat tail vein during the LIS period every two hours for glucose maintenance and at three time points (at the start, after 2 hours and at the end) for ammonia measurement. Rats at stage 5 of HE were sacrificed under isoflurane by heart puncture and blood was collected for aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT).

**Breath collection**

An electronic nose (e-Nose) (Comon Invent, The Netherlands), a handheld portable continuous chemical vapour analyzer of semiconductor gas sensors was used. Breath samples were collected for 15 minutes. The raw data are stored as the changes in electrical resistance of each of the 8 sensors in the on-board database, copied into an offline database and used for further analysis.
Along with e-Nose measurement, the CO$_2$ sensor (Agilent M1460A, Germany) was used for continuous CO$_2$ monitoring. To perform CO$_2$ measurement the CO$_2$ sensor was placed on the “breath path” between a rat and the e-Nose (Figure 1).

In addition, exhaled air was captured from 3 rats to examine whether it included specific breath compounds that might potentially be useful for diagnosis and prediction of ALF. First, breath was captured from 3 healthy rats. Second, breath was captured from the same 2 rats (one rat died during PCS procedure) once in the PCS period (‘PCS’). Third, breath from the same 2 rats was captured two times in the LIS period, once at 2$^{nd}$ grade of HE and once at 4$^{th}$ grade of HE. GC-MS samples were obtained directly after the sample collection by the e-Nose while the rat remained in the tube for another 10 minutes. The exhaled breath was led through absorption tubes filled with Tenax GR® (Supelco, Zwijndrecht, The Netherlands) placed in the “breath path” so the breath could pass through the sorbent to capture VOCs (Figure 1). Additionally, in a separate situation, air flowing through the empty system (without a rat) was sampled to analyze the VOC's coming out of tubing and connectors (‘blank’ measurement). Sorbent tubes for GC-MS analysis were sent to Philips (Eindhoven), and upon arrival they were stored at 6-8°C.

**Design**

Rats were divided into following subgroups:

1. In the first days (3-5 days) the exhaled breath of healthy rats was measured twice a day. These measurements constitute the “healthy group” (of measurements).

2. In the next 3 days, after the PCS procedure, the exhaled breath of PCS rats was measured twice a day. Those measurements constitute the “PCS group”.

3. In the next day the exhaled breath of rats, after the LIS procedure, was measured every hour till the rat was sacrificed. These measurements constitute the “LIS group”.

To capture a potential confounding effect of PCS and LIS operations, two sham operations were performed in 3 rats, one instead of PCS operation and one instead of LIS operation. Measurements in these rats constitute the “sham1” and “sham2 groups”, respectively. “Sham1” rats were subjected to anaesthesia once and “sham2” rats two times.

Measurements from the “healthy”, “PCS” and “sham groups” formed one combined group called hereafter “control”.

Although the rats were their own controls we subcategorised them into above mentioned subgroups to assure clarity in the study.
Blood analysis
Blood samples were directly used for determination of glucose (Ascensia ELITE, Bayer Corporation, USA) and ammonia (Ammonia Checker II AA-4120, Kyoto Daiichi Kagaku Co Ltd, Japan) levels. Plasma ASAT and ALAT were analysed spectrophotometrically according to standard clinical chemistry.

Analysis of e-Nose data
We performed PCA analyses on the data of the 8 sensors of e-Nose with and without the data obtained from the 9th external CO₂ sensor. PCA reduces the dimensionality (number of sensors in our case) of the data by identifying principal components. The first principal component is the linear combination of the sensor values representing the maximum variance of the data. The second principal component is orthogonal to the first and captures the maximum variance remaining after removing the first component, etc. One usually selects a small number of the principal components obtained in this manner for further analysis. For each rat and for each sequence of measurements the differences in the sensors’ responses were calculated according to the equation:

\[ \Delta_{ij} (\text{delta}) = \max(X_{ij}) - \min(X_{ij}), \]

where \(X_{ij}\) is \(i^{th}\) response of \(j^{th}\) sensor. A delta value is hence the difference between the maximum and minimum resistance values for each sensor. These deltas were taken for further analysis. The median values of deltas in each group (for control and for LIS) were calculated for each rat and formed the starting point for PCA analysis, for example if a rat had 4 measurements in the healthy and PCS periods then the median of these deltas was obtained. To compare the differences between measurement stages the paired t-test was used.

PCA analyses were additionally performed for short duration of ALF, i.e. till 1, 2, 3 and 4 hours of LIS period duration.

For classifying a given (new) set of sensor measurements (in effect their deltas), i.e. predicting the group to which the given new set of sensor measurements belongs, we used the methods defined in Table 2.

<table>
<thead>
<tr>
<th>Table 2. Classification methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>PC1 threshold</td>
</tr>
<tr>
<td>Average distance</td>
</tr>
<tr>
<td>3 neighbours</td>
</tr>
<tr>
<td>5 neighbours</td>
</tr>
</tbody>
</table>
The idea is that the PC1 and PC2 values of the set of measurements are first calculated and then a decision is made on the group (control or LIS) to which this set belongs. We selected four ways to make this decision (each based on a different criterion), as described in Table 2. The analysis was performed in the statistical environment R version 2.15.1.

**Validation**

In order to have an unbiased estimate of classification performance we used the leave-one-out validation design. It allows classifying the rat’s group for a specific measurement based of all the other measurements. This means that in each step one rat was excluded from the analysis, and classified by the PCA function.

We assessed the accuracy of this classification by methods described in Table 2. In each method points were scored as follows: for each correct prediction of a rat being both in LIS and control group 1 point was given; for correct prediction of a rat being in only one of the groups (e.g. control or LIS) but wrongly predicted when a rat was in the other group 0.5 point was given; for wrong prediction of a rat in both groups (control and LIS) 0 points were given. For rats which were only in control and never in LIS group points were scored as follows: 1 point for correct prediction and 0 points for wrong prediction. For correct prediction of all rats in both groups 14 points could be maximally scored.

Statistical significance was calculated with two-tailed binomial test.

Statistical measures of the performance were expressed in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and classification accuracy.

**Breath analysis by GC-MS**

To investigate specific breath compounds of ALF we also performed GC-MS analysis of exhaled air from 3 rats, as described. Sorbent tubes were thermically desorbed at 250°C (TDSA, Gerstel, Mülheim an der Ruhr, Germany). Solvent venting mode was used to transfer the sample without loss to the packed liner (filled with Tenax TA), held at 0 °C which was subsequently heated to 300°C in splitless mode. A cold trap (CTS2, Gerstel, Mülheim an der Ruhr, Germany) was used to minimize band broadening (initial temp –150°C, after 2 min heated to 250°C). A gas chromatograph (6890N GC, Agilent, Santa Clara, CA, USA) equipped with a VF1-MS column (30m*0.25mm, film thickness 1 μm, 100% dimethyl polysiloxane, Varian Chrompack, Middelburg, The Netherlands) was used with the following temperature program: 40 °C isothermal during 5 minutes, raised with 10 °C/minute until 310 °C, held isothermal for 5 minutes. Helium was used as a carrier gas with a column flow of 1ml/minute. A quadrupole mass spectrometer (5975 MSD, Agilent, Santa Clara, CA, USA) was used in electron ionization mode at 70eV, with a scan range of m/z 29-450 Da. Gaseous
calibration standards (10 ppmv acetone-D8, hexane-D16, toluene-D8 and xylene-D14 in nitrogen, Air Products, Amsterdam, The Netherlands) were made by use of a home built dilution system and loaded on adsorption tubes to check the sensitivity of the system. Agilent Chemstation (Agilent, Santa Clara, CA) was used for collection and integration of the data. Peak identification was done using a NIST mass spectral database (NIST version 2.0, National Institute for Standards and Technology, USA).

Because of the small sample size of the data taken to GC-MS analysis (3 healthy and 2 PCS/LIS rats) statistical significance calculation was not attempted.

**Results**

Mean survival time of ALF rats was 8 hours and median 7.5 hours (range 5-10 hours). Grade of HE assessed during LIS period is presented in Supplement Figure 2.

**Biochemical analysis**

Blood glucose levels were maintained between 5 and 10 mM in all rats by intraperitoneal injection of 3-5mL of 5% glucose. Progress of ALF was observed by rising trend of ammonia level. Means ± sd values of ammonia at the start of LIS, after two hours and at the end were 65±20, 599±290 and 1191±546µM, respectively. High levels of ASAT and ALAT measured in blood samples taken at the end of life confirmed presence of ALF. Means ± sd values were 20331±7948 and 15421±5714 U/L, respectively.

**Analysis of e-Nose data**

Figure 2 shows the median (with interquartile range) changes in electrical resistance on each of the 8 e-Nose sensors and external CO2 sensor.

PCA analysis of the data from 8 sensors of the e-Nose for “healthy” versus “PCS” rats showed some overlap of these two groups (Supplement Figure 3; left plot), and for “healthy” versus “LIS” showed clear separation (Supplement Figure 3; right plot). The statistical comparison of the sensor responses in “healthy”, “PCS”, ”sham1” and “sham2” groups revealed no significant differences. For this reason we combined those 4 initial groups into one “control” group and conducted further analysis comparing control and LIS groups.
PCA analysis of the data from 8 sensors of the e-Nose showed that the first three PCs explained 73.7, 21.5 and 4.2% of the data, respectively. Figure 3 shows the 14 control measurements (squares) and 9 LIS measurements (circles) of the 14 rats in the PC1-PC2 plain. Indicator of rats’ identities (from 4 to 17) is depicted above each point. Also shown is the threshold on PC1 (for the first classification method in Table 2) that minimizes classification error. Notably, the discrimination could be easily achieved by PC1 threshold. There is only one measurement, the LIS measurement of rat “14”, which is incorrectly classified (its other control measurement is correctly classified) and hence the threshold-based classification method scores 13.5/14 (96.4%) on the whole dataset.
Figure 3. Principal component analysis plot of ∆ responses of 8 sensors in LIS and control rats; each point (square or circle) is connected with centre of own cluster; rats' indicators (from 4 to 17) are depicted.

The results of additional analyses for short duration of ALF: till 1, 2, 3 and 4 hours of LIS duration are presented on Figure 4 a,b,c,d, respectively. Classification based on PC1-threshold method was possible from 3 hours of LIS duration and scores were 13.5/14 (96.4%) for both 3 and 4 hours.

The results of leave-one-out validation (Supplement Figure 4) for the various methods are summarized in Table 3, which shows the classification accuracy. The results are shown separately for the case when the LIS period is taken as a whole and when this period is segmented per hour.
**Figure 4.** Principal component analysis plot for short duration of ALF

**Table 3.** Assessment of prediction based on the leave-one-out validation; data from e-Nose 8 sensors

<table>
<thead>
<tr>
<th>Duration of LIS</th>
<th>Method</th>
<th>PC1 threshold</th>
<th>Average distance</th>
<th>3 neighbors</th>
<th>5 neighbors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total LIS period</td>
<td>13.5 *** (0.96)</td>
<td>10.5 (0.75)</td>
<td>12.5 ** (0.89)</td>
<td>12.5 ** (0.89)</td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td>-</td>
<td>10 (0.71)</td>
<td>8.5 (0.61)</td>
<td>10.5 (0.75)</td>
<td></td>
</tr>
<tr>
<td>2 hours</td>
<td>-</td>
<td>10 (0.71)</td>
<td>10.5 (0.75)</td>
<td>9.5 (0.68)</td>
<td></td>
</tr>
<tr>
<td>3 hours</td>
<td>13.5 *** (0.96)</td>
<td>10.5 (0.75)</td>
<td>12 * (0.86)</td>
<td>12.5 ** (0.89)</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>13.5 *** (0.96)</td>
<td>10.5 (0.75)</td>
<td>13 ** (0.93)</td>
<td>12.5 ** (0.89)</td>
<td></td>
</tr>
</tbody>
</table>

Probability of success in brackets (); * <0.05, **<0.01, *** <0.001

Sensitivity, specificity, PPV, NPV and classification accuracy according to the PC1 threshold classification method (first method in Table 2) were 0.89, 1, 1, 0.95, 0.96,
respectively. These performance measures based on average distance (2nd method in Table 2) were: 0.89, 0.68, 0.57, 0.93, 0.75, and for both methods “3 neighbours” and “5 neighbours” (3rd and 4th methods in Table 2) were as follows: 0.67, 1, 1, 0.86, 0.89.

Additionally, we inspected the classification accuracy when considering any combination of only two e-Nose sensors. The best leave-one-out estimate of a sensor pair was achieved for combination with sensor 6, 7 or 8 (Supplement Table 1). Notably, those sensors were among others sensitive to carbon monoxide (CO).

In the next step, PCA analysis was performed with the data from 9 sensors: 8 sensors of e-Nose and external CO$_2$ sensor. Figure 5 presents CO$_2$ concentrations in exhaled air of each rat.

**Figure 5.** Mean CO$_2$ concentrations in exhaled air of each rat; rats’ indicators (from 4 to 17) are depicted

Figure 6 shows the 14 control measurements (squares) and 9 LIS measurements (circles) of the 14 rats in the PC1-PC2 plain with the PC1 threshold. One measurement of rat “14” is incorrectly classified in LIS period and the threshold-based classification method scores 13.5/14 (96.4%).
Figure 6. Principal component analysis plot of $\Delta$ responses of 9 sensors in LIS and control rats; each point (square or circle) is connected with centre of own cluster; rats' indicators (from 4 to 17) are depicted.

The results of PCA analyses of 9 sensors for short duration of ALF, till 1, 2, 3 and 4 hours of LIS duration are presented in Supplement Figure 5 a,b,c,d, respectively. Classification based on PC1-threshold method was possible from 2 hours of LIS duration and scores were 13.5/14 (96.4%) for 2, 3 and 4 hours.

The results of leave-one-out validation (Supplement Figure 6) for the various methods are summarized in Table 4. The results are shown separately for the case when the LIS period is taken as a whole and when this period is segmented per hour.

Table 4. Assessment of prediction based on the leave-one-out validation; data from e-Nose 8 sensors + external CO2 sensor

<table>
<thead>
<tr>
<th>Duration of LIS</th>
<th>Method</th>
<th>PC1 threshold</th>
<th>Average distance</th>
<th>3 neighbors</th>
<th>5 neighbors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total LIS period</td>
<td></td>
<td>13.5 *** (0.96)</td>
<td>11.5 (0.83)</td>
<td>13 *** (0.93)</td>
<td>13 ** (0.93)</td>
</tr>
<tr>
<td>1 hour</td>
<td></td>
<td>-</td>
<td>11 (0.79)</td>
<td>10 (0.71)</td>
<td>11 (0.79)</td>
</tr>
<tr>
<td>2 hours</td>
<td></td>
<td>13.5 *** (0.96)</td>
<td>11 (0.79)</td>
<td>11.5 * (0.83)</td>
<td>10 (0.71)</td>
</tr>
<tr>
<td>3 hours</td>
<td></td>
<td>13.5 *** (0.96)</td>
<td>11 (0.79)</td>
<td>12.5 ** (0.89)</td>
<td>11.5 * (0.83)</td>
</tr>
<tr>
<td>4 hours</td>
<td></td>
<td>13.5 *** (0.96)</td>
<td>12 (0.86)</td>
<td>13.5 *** (0.96)</td>
<td>13.5 *** (0.96)</td>
</tr>
</tbody>
</table>

probability of success in brackets (); * <0.05, **<0.01, ***<0.001
Sensitivity, specificity, PPV, NPV, and classification accuracy according to the PC1 threshold classification method (first method in Table 2) were 0.89, 1, 1, 0.95, 0.96, respectively. These performance measures based on average distance (2\textsuperscript{nd} method in Table 2) were: 0.89, 0.79, 0.67, 0.94, 0.82; for “3 neighbours” (3\textsuperscript{rd} method in Table 2) were: 0.67, 1, 1, 0.86, 0.89 and for “5 neighbours” (4\textsuperscript{th} method in Table 2) were: 0.78, 1, 1, 0.90, 0.93.

**Breath analysis using GC-MS**

Analysis of the exhaled breath samples by GC-MS showed above two hundreds compounds per sample. The relative changes between groups were counted for each compound. Of those, only compounds with the largest difference (at least 50%) were selected. This accounted for 9 compounds (Figure 7).

The results of the analysis show a rising pattern for 2-butanol, MEK (2-butanone), 2-pentanone, and 1-propanol across healthy – PCS – LIS stages. A decreasing pattern from PCS to LIS stage is shown for dimethyl sulphide (DMS), hexane (n-C\textsubscript{6}) and pentane.

**Figure 7.** Volatile organic compounds detected by the GC-MS analysis
Discussion

To our knowledge the present study is the first that investigates breath prints by e-Nose technology as a non-invasive test for ALF.

We report the results of the breath analysis of rats developing ALF due to surgically induced complete liver ischemia in a relatively short period. This model has two advantages: one - to study possible changes in breath prints due to PCS, second - to study progression of ALF in one and the same rat, which is its own control. Using the rat-model rather than human breath allows one better control of the experimental conditions.

Our study shows that the e-Nose can discriminate exhaled breath of control rats from rats developing ALF, indicating that the composition of exhaled VOCs is different in healthy and ALF subjects. The results of leave-one-out validation revealed e-Nose as a good discrimination tool. The e-Nose had high classification accuracy already within 3 hours after induction of complete liver ischemia. Different combinations of two e-Nose sensors show also good discrimination when one of the sensors was among others sensitive to CO. Simultaneous monitoring of CO$_2$ during the measurements, performed by an external sensor achieved high classification accuracy even earlier: already within 2 hours.

Breath analyses of patients with liver disease were performed in cirrhosis of various degrees and etiologies $^3$, $^{10}$, $^{33}$, $^{34}$, alcoholic and non-alcoholic fatty liver disease $^{35}$, alcoholic and non-alcoholic hepatitis $^{36}$. Breath analysis by GC–MS made it possible to discriminate patients with malodorous breath related to hepatic pathologies $^3$, $^4$, $^{33}$. Recently, breath analysis by proton transfer reaction time-of-flight mass spectrometry was able to distinguish cirrhotic patients from healthy subjects and to discriminate between different severity stages $^{34}$.

Interestingly, 2-butanone and 2-pentanone were reported to be increased in breath of cirrhotic patients $^3$, $^{33}$, $^{34}$, like in our study. Increased ketone formation is a consequence of increased insulin resistance and lipolysis when glycogen stores in the liver have run out and the body switches to fat metabolism for energy production $^5$. It has been reported that in cirrhotic patients 1-propanol decreased and DMS increased $^3$, $^{33}$, in contrast to our study. This is unexpected since DMS has been reported in breath of patients in hepatic coma $^4$. A possible explanation could be the fact that our Tenax trapping of DMS was insufficient for these VOCs. Phenol has been reported to decrease $^3$, $^{33}$ in breath of patients with cirrhosis. Our results do not allow firm conclusions about the effect of ALF on exhaled phenol because the empty system showed already the presence of phenol. Another animal study $^{16}$ on rats with renal failure showed presence of phenol and ethanol in exhaled air. Since ALF results often in multi organ failure like kidney failure, respiratory distress syndrome, etc $^{37}$, these
VOC's will contribute to the breath prints. In our model this was less evident, possibly due to the relatively short period in which ALF developed.

Another important observation from this study is that already in an early stage of ALF development (3-4 hours) when HE is usually not higher than grade 2, the breath prints of control rats became different from ALF rats, without a major change to the end stage. This indicates that the observed changes in the exhaled breath can be predictive for a fatal outcome.

Surprisingly the features of the ALF breath print could already be monitored by only 2 sensors of which one detects CO or CO\textsubscript{2}. This was probably due to the fact that mean CO\textsubscript{2} concentrations in exhaled air were significantly lower (p<0.0001) in ALF rats (42.3±3.2 ppm) compared to healthy subjects (61.1±4.6) (see Figure 5). A lower output of CO\textsubscript{2} might be caused by hyperventilation probably combined with less aerobic metabolism due to complete liver ischemia. As a consequence increased lactate production occurs and finally respiratory compensation of metabolic acidosis. However, clinically we did not observe manifest hyperventilation by the rats when they were outside the e-Nose circuit, but we may have missed that.

This study shows that e-Nose breath prints can adequately and rapidly distinguish between control and ALF rats. This technique combines the non-invasive measurement with real-time analysis of the broad spectrum of VOCs. Our results raise expectations for e-Nose as a tool for diagnosis of developing ALF in humans. Confirmation of these results in a clinical study will be an important step forward in the non-invasive diagnosis of ALF. In particular, it has the potential to recognize ALF early and to list the patient for transplantation timely\textsuperscript{38} and to speed up the therapeutic measures, by which the rate of recovery can be improved and consequently costs for the health care system can be saved.
Supplement Figure 1. Real rat-model constructed for the purpose of the study.

Supplement Figure 2. HE grading of rats during LIS period. The solid line is obtained by smoothing.
Supplement Figure 3. Results of principal component analysis between two groups: healthy vs. PCS (left), healthy vs. LIS (right)

Supplement Figure 5. Principal component analysis plot
Supplement Figure 4. Leave-one-out validation of (excluded rat reported above each plot; predicted rat plotted in black-edged circle; circle – rats in LIS group; square – rats in control group)
Supplement Figure 6. Leave-one-out validation (excluded rat reported above each plot; predicted rat plotted in black-edged circle; circle – rats in LIS group; square – rats in control group)
References