Diagnosis of pulmonary injury and infection by exhaled breath analysis
Bos, L.D.J.

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Chapter 5.

Alterations of Exhaled Breath Metabolite–mixtures in Two Rat Models of Lipopolysaccharide–induced Lung Injury

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Abstract

Exhaled breath contains information on systemic and pulmonary metabolism, which may provide a monitoring tool for the development of lung injury. We aimed to determine the effect of an intravenous and an intra–tracheal lipopolysaccharide (LPS) challenge on the exhaled mixture of volatile metabolites and to assess the similarities between these two models.

Male adult Sprague–Dawley rats were anesthetized, tracheotomized and ventilated for six hours. Lung injury was induced by intravenous or intra–tracheal administration of LPS. Exhaled breath was monitored continuously using an electronic nose (eNose) and hourly using gas–chromatography and mass–spectrometry (GC–MS).

GC–MS analysis identified 34 and 14 potential biological markers for lung injury in the intravenous LPS model and the intra–tracheal LPS model, respectively. These volatile biomarkers could be used to discriminate between LPS challenged rats and control animals within 1 hour after LPS administration. eNose analysis resulted in a good separation 3 hours after the LPS challenge. Hexanal, 6,10–dimethyl–5,9–undecadien–2–one and pentadecane concentrations decreased after both intra–tracheal and intravenous LPS administration. Nonanoic acid was found in a higher concentration in exhaled breath after LPS inoculation into the trachea but in a lower concentration after intravenous infusion.

LPS–induced lung injury rapidly changes exhaled breath metabolite mixtures in two animal models of lung injury. Changes partly overlap between an intravenous and an intra–tracheal LPS challenge. This warrants testing the diagnostic accuracy of exhaled breath analysis for ARDS in clinical trials, possibly focusing on biological markers described in this study.
Introduction

The acute respiratory distress syndrome (ARDS) results either from an indirect insult (e.g., systemic inflammation with sepsis or pancreatitis), or from a direct pulmonary challenge (e.g., pneumonia, aspiration or inhalation trauma). Whatever its cause, ARDS is characterized by pulmonary inflammation, alveolar protein leakage and disrupted repair mechanisms [1]. The diagnosis of ARDS is based on clinical symptoms and radiological findings potentially hampering and delaying its recognition [2-5]. Biological markers may have the potential to improve and accelerate the diagnostic process of ARDS, but systemic biological markers (i.e., markers within blood) studied so far exhibit only limited diagnostic value [6, 7]. Therefore, recent efforts have focused on sampling local biological markers (i.e., markers within the pulmonary compartment) (7–9). This has demonstrated that multiple biological markers reflect ARDS better than one single biological marker [8, 9].

Extensive metabolic profiling (metabolomics) represents a comprehensive tool for biomarker discovery in complex metabolic mixtures [10]. Metabolomics of blood and broncho–alveolar lavage (BAL)–fluid shows changes in metabolism during pulmonary inflammation and injury [11-15]. Some of the produced metabolites are volatile and therefore detectable in the exhaled breath [16]. So–called volatile organic compounds (VOCs) can be separated, identified and quantified using gas–chromatography and mass–spectrometry (GC–MS), which is considered the gold standard for (volatile) metabolite discovery [17]. VOCs can also be rapidly detected on–site using sensor–based devices called electronic noses (eNose). This method does not allow for metabolite discovery, but provides probabilistic evidence for the presence or absence of disease by pattern recognition [18]. In other inflammatory diseases of the lung (e.g., asthma and chronic obstructive pulmonary disease) exhaled breath analysis has been examined for diagnostic purposes [19, 20]. Subsequently, several metabolites have been linked to cellular inflammation [21-24]. This suggests that volatile metabolic changes could also reflect inflammatory responses and injury with development of ARDS.

Ideally, potential biomarkers are investigated in high–risk patient groups using gold standard reference tests. However this is hampered by an
imperfect clinical reference standard, a high prevalence of co-morbidities and a veritable pharmacopeia of drugs in patients with ARDS [2]. Therefore, animal models of lung injury may allow for a first selection of potential early volatile biological markers of ARDS. Both systemic and local administration of lipopolysaccharide (LPS) lead to accumulation of inflammatory cells in the lung and disrupted barrier mechanisms similar to those found in the early phases of human ARDS and correspond to an indirect (e.g. sepsis) and direct (e.g. pneumonia) insults, respectively [25, 26].

We hypothesized that LPS–induced lung injury changes the mixture of exhaled metabolites, and that these changes overlap between systemic or local administration of LPS. To test these two hypotheses, we challenged rats with intravenous or intra–tracheal LPS in a model of ventilation [27].

**Materials and methods**

**Animals**

Adult specific pathogen–free Sprague–Dawley rats weighing ~ 400 grams (Harlan, the Hague, The Netherlands) were used in all experiments. The Animal Care And Use Committee of the Academic Medical Center at the University of Amsterdam, Amsterdam, The Netherlands, approved the study. All animal procedures were carried out in compliance with the Institutional Standards for Human Care and Use of Laboratory Animals.

**Study design and experimental groups**

This was a parallel study with 4 animal groups. Lung injury was induced by intravenous (IV) infusion or intra–tracheal (IT) instillation of LPS (7.5 mg/kg in 2 ml normal saline, or 1.0 mg/kg in 200 µl, respectively) (from *Escherichia coli*, L4131, Sigma Aldrich, Steinheim, Germany). Two control groups received an equal amount of sterile normal saline IV or IT. Each group consisted of 12 rats; per day, 2 rats were randomly assigned to one of the four groups and challenged.

**Experimental protocol**

Ventilation was performed as described before [27]. In short, anaesthesia was induced using a mixture of ketamine (Eurovet Animal Health B.V.,
Bladel, The Netherlands), dexmedetomidine (Pfizer Animal Health B.V., Capelle a/d IJssel, the Netherlands) and atropine (Pharmachemie, Haarlem, The Netherlands) and maintained with ketamine. The ventilation pattern could influence exhaled breath VOCs [28, 29]. Therefore, rats were tracheotomized and subsequently ventilated using a Servo 300 ventilator (Siemens, Upplands Väsby, Sweden) with tidal volumes of 6 ml/kg with positive end-expiratory pressure (PEEP) at 5 cmH\(_2\)O and a respiratory rate of 40/min or tidal volumes of 12 ml/kg with PEEP at 0 cmH\(_2\)O and a respiratory rate of 20/min. These two ventilation protocols represent the two extremes of clinically applied settings [30] and were used to investigate the robustness of the diagnostic algorithm with different ventilator settings. Tidal volumes were monitored using a pneumotachometer (HSE; Harvard Apparatus, Manheim, Germany) and adjusted if necessary. The lungs were recruited hourly and respiratory rate was adjusted based on hourly blood gas analysis (Rapidlab, Siemens, Erlangen, Germany) to keep normo-pH.

LPS was administered 30 minutes after the start of mechanical ventilation. Blood pressure and temperature were recorded continuously (Braun, Melsungen, Germany connected to Siemens SC900; Siemens Medical Systems Group, Danvers, Mass). If the mean arterial pressure fell below 65 cmHg, nor-adrenaline was started. Temperature was maintained between 36.5\(^\circ\)C and 37.0\(^\circ\)C using a warming pad (ama-digit ad 15th; Amarell, Kreuzwertheim, Germany). Urine was collected at baseline, and after 3 and 6 hours.

After 6 hours the rats were sacrificed. Lung wet-to-dry (WD) ratios were determined as described before [27], using the right lower lobe of the lung. To obtain BAL-fluid, 3 x 2 ml of normal saline was instilled in and directly withdrawn from the contra-lateral lung, and kept at 4\(^\circ\)C until further processing.

**Measurements**

The number of cells in BAL-fluid was counted (Z2 Coulter Particle Counter; Beckman Coulter Corporation, Hialeah, Fla) and cell-differentiation with Giemsa Stain (Dade Behring AG, Dudingen, Switzerland) was performed. Total protein levels (Oz Biosciences, Marseille, France) and levels of tumor
necrosis factor (TNF–α), interleukin (IL)–6, cytokine induced neutrophil chemoattractant (CINC)–3 and IL–10, (ELISA; R&D systems, Abingdon, United Kingdom) were measured in BAL–fluid.

**Exhaled breath analysis**

Measurements with eNose were performed in half the experimented animals. For this, directly after the initiation of ventilation, an eNose (Comon Invent, Delft, The Netherlands) was connected, via a T–piece, to the expiratory limb of the ventilator tubing. The eNose contains seven metal–oxide sensors, which reversibly bind a broad spectrum of VOCs. VOC–binding results in a change in electrical resistance, measured by altered electrical current, which was recorded continuously for 6 hours and stored every 10 seconds.

GC–MS analysis was performed in all experimented animals. For this, a new stainless steel tube containing Tenax GR (Tenax™ GR 60/80, Interscience, Breda, The Netherlands) was connected to the expiratory limb of the ventilatory circuit every hour. VOCs were absorbed onto this tube every hour for 30 minutes with a flow of 35 ml/minute. The Tenax tubes were transported to a thermal desorption unit (Markes TD100 Cincinnati, Ohio, USA) and heated to 280°C for 15 minutes with a flow of 30 ml/min. The VOCs were captured on a cold trap at 10°C, which was rapidly heated to 300°C for one minute, after which the molecules were split less injected through a transfer line at 180°C onto a Inertcap 5MS/ Sil gas–chromatography column (30 m, ID 0.25 mm, film thickness 1 μm, 1,4–bis(dimethylsil oxy)phenylene dimethyl polysiloxane, Restek, Breda, The Netherlands) at 1.2 ml/min. The oven temperature was isothermal at 40°C for 5 minutes, then increased to 280 at 10°C/min and kept isothermal at 280°C for 5 minutes. Molecules were ionized using electron ionization (70 eV) and the fragment ions were detected using a quadrupole mass–spectrometer (GCMS–GP2010, Shimadzu, Den Bosch, the Netherlands) with a scan range of 37–300 Da. Ion–fragment peaks were used for statistical analysis. The predictive fragment–ions were manually checked in the raw chromatograms and the corresponding metabolites were tentatively identified based on NIST–library matching. Metabolites were considered identified if the first five hits in the library were the same compound and all matching factors were above 90%. In the case of multiple
likely library hits, a chemical standard (Sigma–Aldrich, Zwijndrecht, The Netherlands) was injected for identification. When these two procedures did not result in identification, the compound was called unidentified.

**Statistical Analysis**

Primarily, the groups challenged with LPS were compared with their corresponding controls. Secondarily, intravenous and intra–tracheal LPS-induced lung injury groups were compared using a Venn diagram [31]. Time dependent changes between groups in continuous variables were analyzed using linear mixed models. Differences in end points that were measured once were analyzed using the Mann–Whitney U test. P–values below 0.05 were considered significant. All analysis were performed in R–studio (V 0.97) using the statistical data environment R (V2.15) [32].

GC–MS analysis, de–noising, peak–detection and alignment were performed using the Xcms–package [33] (Scripps center for metabolomics, La Jolla, CA, USA) and resulted in an ion–fragment peak table as input for statistical analysis. eNose analysis resulted in seven sensor signals per 10 seconds, per animal. Fragmented–ion peaks and sensor signals were expressed as a percentage change of baseline measurement (first thirty minutes of mechanical ventilation, before LPS administration). Fragment–ion peaks were checked for outliers by principal component analysis. Outliers were defined as a chromatogram with many infinite values (compared to baseline) as detected by PCA. When no valid baseline chromatogram was available, all data from that animal was neglected.

Fragment–ion peaks that changed significantly after LPS administration were detected using six steps (figure 1). First, a linear mixed effect model was fitted using the peak as dependent variable with time after start of mechanical ventilation as independent variable and with a random intercept per animal. Time was modeled with a basic spline to allow for non–linear fluctuations in metabolites (e.g. increase in the first three hours after LPS and decrease thereafter). Secondly, this procedure was repeated with the addition of the LPS group (yes/no) as a second independent variable. Thirdly, the improvement of the model with the addition of LPS allocation was expressed as the difference in Akaike information criterion [34] (AIC) between the two models.
Figure 1: Selection of statistically significant ion-fragments.
Fourthly, step two and three were 1000 times repeated but with a random group allocation instead of LPS allocation to simulate chance findings. Fifthly, delta AICs were log–transformed and a normal distribution was fitted through the randomly generated values. Finally, the false–discovery rate (FDR) per AIC cut–off value was calculated and peaks with a FDR of below 5% were selected.

A logistic regression model was trained using the previously selected fragment–ion peaks. Sparse–partial least square (SPLS) logistic regression was used as an alternative to “normal” regression techniques in this study, because SPLS can limit false discovery in situations were a large number of independent variables are investigated in low numbers of individuals [35]. The mean and 95% confidence–interval (95%–CI) of the predicted probability of group membership over time was displayed in a figure.

A SPLS logistic regression model was also trained on the mean change in electrical resistance per sensor after 330 to 360 minutes of mechanical ventilation. This logistic regression model was there–after applied to all other sensor data.

**Results**

*Animals*

Of 55 included animals 8 rats died during instrumentation (figure 2). Forty–seven rats completed the experiment. Exhaled breath was continuously analyzed by eNose from 6 animals per group. Exhaled breath for GC–MS analysis was hourly obtained from all 47 rats. However, since baseline measurements were unsuccessful in four animals due to technical problems with GC-MS, GC–MS data from these animals were excluded from further analysis.

*Extra–pulmonary manifestations of LPS administration*

Intravenous administration of LPS resulted in a drop in mean arterial blood pressure (p < 0.001, figure 3A), but the mean arterial blood pressure remained above 65 mmHg at all time points in all rats. These rats also had a lower pH throughout the experiment (p = 0.003, figure 3C), with the largest difference after 2 hours of start of ventilation. Urine output also
declined \( (p = 0.007, \text{figure } 3F) \). Systemic blood pressure, pH and urine output were not affected by intra–tracheal administration of LPS (figure 3B, D, and F).

**Figure 2:** Flowchart

![Flowchart](image)

**Pulmonary manifestations of LPS administration**

Rats challenged with intravenous LPS had lower numbers of neutrophil cells in BAL–fluid \( (p < 0.001 \text{ vs. control}) \), while rats challenged with intra–tracheal LPS had higher numbers of neutrophil cells in BAL fluid \( (p < 0.001 \text{ vs. control}) \) (figure 4A, and B). All LPS–challenged rats showed signs of pulmonary inflammation, with higher levels of TNF–alpha \( (\text{IV } p = 0.045 \text{ vs. control}; \text{ IT } p < 0.001 \text{ vs. control}) \), IL–6 \( (\text{IV } p < 0.001 \text{ vs. control}; \text{ IT } p < 0.001 \text{ vs. control}) \) and CINC–3 in BAL fluids \( (\text{IV } p < 0.001 \text{ vs. control}; \text{ IT } p < 0.001 \text{ vs. control}) \) (figure 4C, and D).

**Alterations in exhaled metabolites analyzed by GC–MS**

372 and 355 mass–spectrometer generated fragmented ions of VOCs were present in all air samples from intravenous or intra–tracheal challenged rats, respectively. In the intravenous model, 120 ion–fragments were found to be significantly altered after LPS administration. In the intra–tracheal model, 31 ion–fragments were significantly altered after LPS inoculation.
**Figure 3:** Physiological changes after LPS infusion

Squares represent the median of the LPS group, round of the control group. The spread shows the minimum and maximum value per time point. MAP = mean arterial pressure.
Figure 4: Immune response after LPS administration

Discrimination between rats challenged with intravenous LPS and control animals was based on 30 ion–fragments. The logistic regression function diverged between the two groups 30 minutes after LPS administration or 60 minutes after the start of mechanical ventilation (figure 5A). The area under the receiver–operating characteristic curve (AUC–ROC) was 0.84 [95% CI: 0.66–1.0] 30 minutes after LPS administration and increased to 0.94 [95%–CI: 0.83–1.0] during the next hour. The AUC–ROC remained similar for the rest of the experiment.

Discrimination between rats challenged with intra–tracheal LPS animals and control animals was based on 13 ion–fragments. Discrimination was good (ROC–AUC: 0.94 [95%–CI: 0.84–1.0]) 30 minutes after LPS inoculation and remained good until the end of the experiment (figure 5B).
Alterations in exhaled breath profile analyzed by electronic nose

Continuous electronic nose analysis of the exhaled breath resulted in good discrimination between animals that were challenged with LPS intravenously and control animals after approximately 3 hours (ROC-AUC: 0.85 [95%–CI: 0.78–0.97]) (figure 6A). After intra-tracheal LPS administration, good discrimination was reached within 90 minutes (ROC-AUC: 0.82 [95%–CI: 0.52–0.93]) (figure 6B). Notably, the control group deviated from the baseline measurements instead of the LPS group.

Metabolite identification and model comparison

The 120 significant ion-fragments found in the intravenous LPS model corresponded to 35 metabolites, of which 21 could be tentatively identified (table 1). The intra-tracheal LPS challenge resulted in 31 significantly altered ion fragments that corresponded to 14 metabolites, of which 9 could be identified (table 2). 5 metabolites changed significantly after the challenge with LPS in both models, as shown in the Venn diagram (figure 7). Hexanal, 6,10-dimethyl-5,9-undecadien-2-one, pentadecane and an unidentified compound were found in lower concentrations after both pulmonary and intravenous LPS administration. In contrast, nonanoic acid was found in a higher concentration in the exhaled breath after LPS inoculation into the trachea but in a lower concentration after intravenous infusion.

Dependence upon ventilation protocol

The logistic regression algorithm was not dependent on the ventilation protocol that was used (p = 0.97 / p = 0.60, for intravenous or intra-tracheal LPS administration, respectively).
**Figure 5:** Predicted probability of LPS administration by GC-MS

Logistic regression function based on GC–MS detected ion–fragments. A – The blue line indicates the mean logistic function for intravenous LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval. B – The yellow line indicates the mean logistic function for intra–tracheal LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval.

**Figure 6:** Predicted probability of LPS administration by eNose

Logistic regression function based on electronic nose analysis. A – The blue line indicates the mean logistic function for intravenous LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval. B – The yellow line indicates the mean logistic function for intra–tracheal LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval.
Table 1: Significantly altered metabolites in the intravenous LPS model

<table>
<thead>
<tr>
<th>Name</th>
<th>M/Z</th>
<th>RT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-Butanedione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Butanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluorobenzene (unknown isomer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>163</td>
<td>310</td>
</tr>
<tr>
<td>Butanal, 3-methyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>38/49/79</td>
<td>367</td>
</tr>
<tr>
<td>Unknown</td>
<td>181</td>
<td>374</td>
</tr>
<tr>
<td>Hexanal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>82</td>
<td>683</td>
</tr>
<tr>
<td>3-Heptanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclohexanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>79/99/122</td>
<td>898</td>
</tr>
<tr>
<td>Hexanal, 2-ethyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>40/43/53/55/65/93</td>
<td>1006</td>
</tr>
<tr>
<td>Decane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octanal</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>101</td>
<td>940</td>
</tr>
<tr>
<td>1-Hexanol, 2-ethyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Limonene</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>50/51/62/89</td>
<td>1068</td>
</tr>
<tr>
<td>Benzenemethanol, dimethyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Hexene, 3,3,5-trimethyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>73/101/191/244/249</td>
<td>1169</td>
</tr>
<tr>
<td>Nonanoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>145</td>
<td>1180</td>
</tr>
<tr>
<td>n-Decanoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>102/107/118</td>
<td>1206</td>
</tr>
<tr>
<td>Unknown</td>
<td>73</td>
<td>1241</td>
</tr>
<tr>
<td>5,9-Undecadien-2-one, 6,10-dimethyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Dodecanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentadecane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol, 3,5-bis(1,1-dimethylethyl)-</td>
<td></td>
<td></td>
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</tbody>
</table>

*RT: Retention time in seconds. M/Z: Mass over charge; the mass of the ion fragment(s)*
Table 2: Significantly altered metabolites in the intra–tracheal LPS model

<table>
<thead>
<tr>
<th>Name</th>
<th>M/Z</th>
<th>RT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4–Hexadiyne</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexanal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–Cyclopenten–1–one</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclopentanone, 2–methyl–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–Pentylfuran</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonanoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,9–Undecadien–2–one, 6,10–dimethyl–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentadecane</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RT: Retention time in seconds. M/Z: Mass over charge; the mass of the ion fragment(s).

Figure 7: Venn diagram

Venn diagram for the two lung injury models, intravenous LPS (left) and intra–tracheal LPS (right). Were the circles overlap, the number of shared differences is indicated.
Discussion

This animal study shows that LPS induces a metabolic response that can be measured in exhaled breath. Longitudinal analysis revealed that the volatile metabolic changes in the exhaled air can be used to discriminate between rats challenged with LPS and control animals within one hour after LPS administration. This could be reproduced with a bedside electronic nose, although it took longer to reach good discrimination. Finally, this study provides evidence that changes in exhaled breath metabolites partly overlap between intravenous and intra-tracheal LPS administration. These data suggest that exhaled volatile metabolites may be used to monitor the development of ARDS.

To our knowledge, we performed the first experiment with longitudinal exhaled breath measurements in an animal model of acute lung injury, allowing for time-dependent analyses. The present data extend those by Gauman et al. [36], who observed a similar discriminatory power at a single time point, 24 hours after intra-peritoneal LPS-administration, and show that changes in exhaled VOCs already occur shortly after LPS instillation. However, the discriminating metabolites are different in both studies, which may be due to afore mentioned differences in study design.

Two complementary analytical techniques were used for the analysis of exhaled breath. GC-MS allowed for separation and identification of individual VOCs while eNose technology relies on pattern recognition. Arguably, pattern recognition is sufficient for clinical practice. This is the first study to perform continuous exhaled breath analysis during ventilation using eNose technology. The observed discrimination by eNose analysis in the intravenous LPS model mimics that of the GC-MS measurements, albeit later. However, after the intra-tracheal LPS challenge, the control group deviated from the baseline measurement instead of the challenged group. This suggests that another signal dominates or that the sensors in this eNose are not sensitive to the significantly altered volatile metabolites found by GC-MS but to unknown other compounds that apparently change after intra-tracheal saline administration.

The results of both challenge models were combined into a Venn diagram, which revealed that five metabolites were altered in both models. This
method has been proposed for meta-analysis in unbiased metabolomics research and limits false-discovery through external validation [31]. Four of the five shared metabolites were found in lower concentration after challenge with LPS in both models. Hexanal, is a breakdown product of oxidized linoleic acid and therefore a marker of oxidative stress [37]. Hexanal concentrations in exhaled air were previously found to be increased in COPD and lung cancer patients [38, 39]. Exhaled air concentrations of hexanal was found to be lower in smoking subjects, when compared to controls and lung cancer patients [40]. Apparently, exhaled hexanal is differentially associated with oxidative stress in health and disease, which may be related to differences in local oxidative stress and/or activation of alternative pathways. In line with these findings, pentadecane (a classic marker of oxidative stress) was also found in lower concentrations after LPS administration in our experiments. 6,10-dimethyl-5,9-undecadien-2-one is a product of acetone termination of geranyl phosphate, an intermediate in the sterol pathway [41]. We speculate that the lower concentration of this metabolite in exhaled air with development of lung injury could be explained in two ways. First, the sterol pathway is down-regulated after LPS-administration. Second, the specific shunt pathway through 6,10-dimethyl-5,9-undecadien-2-one could be reduced in order to increase cholesterol and steroid hormone synthesis. The latter is supported by studies showing that cholesterol and steroid hormone concentrations increase after endotoxin infusion (33, 34). The fourth shared marker was not identified. To summarize, previously described exhaled markers of oxidative stress were found in lower concentration after LPS administration. Possibly, however unlikely, oxidative stress is decreased after LPS administration. Alternatively, oxidative stress may cause activation of alternative pathways under different circumstances. This is very much in line with exhaled NO, which is a marker of oxidative stress when exhaled in either higher or lower concentrations [42].

Interestingly, nonanoic acid was found in a higher concentration in the exhaled breath of rats that received LPS in the trachea but in a lower concentration when LPS was infused intravenously. Nonanoic acid is a ligand to the Orphan G protein-coupled receptor GPR84 [43]. This receptor is mainly expressed by leukocytes and induces an inflammatory response.
(leukocyte influx and activation) when stimulated [43]. The present data are suggestive of a relationship between nonanoic acid concentration and the number of neutrophils in the broncho–alveolar lavage fluid, as these follow similar trends between the two models (lower after intravenous LPS and higher after intra–tracheal LPS) (figure 4A–B).

Our study knows several limitations. Some of the significant metabolites could not be identified because the mass–spectrum was not sufficiently informative. In the future, this could be overcome by applying high resolution mass–spectrometry or the inclusion of more external standards of known metabolites. Additionally, very low molecular weight volatile organic compounds (C1–C3) could not be monitored due to their limited retention on the absorbent tubes used in these experiments. Future studies aiming at these compounds should use a multi–sorbent VOC trap or new, rapid analytical techniques that do not require sample transportation (e.g. ion–mobility spectrometry [36, 44] or proton–transfer mass–spectrometry [45]).

A second weakness of this study is the limited number of animals investigated. Unfortunately, small numbers per group and a highly dimensional dataset result in increased chances of type I errors [46]. However, we limited false–discovery by repeating the statistical procedure with random group allocation and selection of only those peaks with a FDR of below 5%. Furthermore, true external validation was applied through the earlier described process of meta–analysis, thereby further reducing the chances on false–discovery for hexanal, pentadecane 6,10–dimethyl–5,9–undecadien–2–one and an unidentified fourth metabolite as biomarkers for lung injury. On the other hand, this stringent procedure inevitably promotes false negative results. Hence, our data do not exclude that other described markers can be representative for the phenotypic presentation of lung injury due to a pulmonary or a non–pulmonary challenge.

The mild nature of the used LPS–models could be considered a final limitation. Indeed, larger differences between the groups would probably have been found if a higher dose of LPS, injurious mechanical ventilation or a combination of both was used. However, since several physiological parameters (e.g., alveolar ventilation, cardiac output, ventilation/
perfusion mismatch) influence the concentration of VOCs in the exhaled breath, the investigated model should allow for maintaining the physiology within normal levels [47]. In our experiments, we were able to rapidly counter–act disturbances in homeostasis due to LPS, because the rats were instrumented and monitored continuously before the administration of LPS. This required multiple interventions: shock was prevented by fluid and noradrenalin infusion, acid–base imbalance by bicarbonate infusion and changes in respiratory rate and atelectasis by hourly recruitment maneuvers. These are all risk factors for ARDS [48]. Hence, by keeping LPS-induced injury relatively mild, we aimed to limit the confounding effects of gross physiological disturbances. However, the given dosage was sufficient to induce an inflammatory response comparable to that observed in humans. Finally, an early marker for lung injury is clinically required. Waiting for a longer period (e.g. 24 hours) after LPS administration, as in other models, was therefore not desirable.

The present data suggests that exhaled breath metabolites may be used to monitor lung injury. If these results can be translated to the clinical setting, this would provide intensive care physicians with a non-invasive diagnostic tool that allows for continuous monitoring.

To conclude, LPS–induced lung injury rapidly changes the mixture of exhaled metabolites in two animal models. Alterations in exhaled breath metabolites can be detected as soon as one hour after LPS administration. These results were partly reproduced by exhaled breath profiling with an electronic nose. Exhaled breath metabolite alterations partly overlap between rats challenged with LPS intravenously and intra–tracheally. We identified the following biomarkers for lung injury in general: hexanal, pentadecane 6,10–dimethyl–5,9–undecadien–2–one and an unidentified fourth metabolite. Furthermore, exhaled nonanoic acid could be a marker for alveolar neutrophil influx. We also described 30 potential volatile biomarkers for non–pulmonary lung injury and 9 potential volatile biomarkers for pulmonary lung injury. This implies that diagnostic accuracy of exhaled breath analysis for ARDS should now be further investigated in clinical trials, possibly focusing on the biomarkers described in this pre–clinical study.
References


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