Internationales Symposium
Lung disease – what can be learned from physiology?

Exhaled breath analysis –
techniques and current clinical impact

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Background

Lungs: large surface (100 m2)

Components of exhaled air:
- \( N_2, O_2, CO_2 \)
- inert gases
- water vapour
- thousands of volatile and non-volatile organic components (VOC)

  - Isoprene (12-580 ppb)
  - Ethanol (13-1000 ppb)
  - Methanol (160-2000 ppb)
  - Acetone (1.2-1880 ppb)
  - ....

  - isoprostanes, cytokines, leukotrienes, hydrogen peroxide
## Background

<table>
<thead>
<tr>
<th>purpose (examples)</th>
<th>target organ</th>
<th>conventional method</th>
<th>replacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (forensic)</td>
<td>blood</td>
<td>venous blood sampling</td>
<td>exhaled breath</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>stomach</td>
<td>gastric biopsy</td>
<td>exhaled breath (C\textsuperscript{13})</td>
</tr>
<tr>
<td>CO-Hb</td>
<td>lungs</td>
<td>venous blood sampling</td>
<td>exhaled breath</td>
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</tbody>
</table>
Ideal biomarker

Sensitive and specific for the diagnosis of the disease
Reflect or be a very clear surrogate of the pathophysiologic mechanism
Be stable and only vary within events known to relate to disease progression
Predict early-stage disease development
Predict disease progression
Be responsive to interventions known to be effective
Sources of exhaled Nitric Oxide

Three different isoenzymes:

**iNOS (NOS II)** expressed exclusively in the respiratory epithelium including the squamous epithelium of the parnyngo-oral tract

**endothelial NOS (NOS III)** no detectable eNOS mRNA in lower airway epithelial cells. Expressed in endothelial cells in the respiratory tract, involved in the control of pulmonary/bronchial circulation

**neuronal NOS (NOS I)** expressed in postganglionic parasympathetic neurons in the airways. inhibitory nonadrenergic noncholinergic neurotransmitter mediating bronchodilatation upon vagal stimulation.

High NO concentrations in the nasal airways in contrast to much lower levels in the lower airways (more dense iNOS expression in the epithelium of the nasal airways)

Formed non-enzymatically from nitrite (gastric ventricle, oral cavity, facultative anaerobic bacteria reduce nitrate to nitrite, vegetables (lettuce, spinach))
Relative contribution of different airway NO sources

<table>
<thead>
<tr>
<th>Anatomical location</th>
<th>Biochemical source</th>
<th>Contribution to exhaled NO ppb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharyngo-oral tract</td>
<td>Salivary nitrite</td>
<td>4 (20)</td>
</tr>
<tr>
<td></td>
<td>Epithelial iNOS</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Hypopharynx, larynx, upper trachea</td>
<td>Salivary nitrite/epithelial iNOS?</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Lower trachea, bronchi</td>
<td>Epithelial iNOS</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Bronchioli</td>
<td>Epithelial iNOS</td>
<td>1–2 (5–10)</td>
</tr>
<tr>
<td>Alveoli</td>
<td>Endothelial NOS</td>
<td>0.5 (2.5)</td>
</tr>
</tbody>
</table>

The exhaled NO concentration at a standard flow rate of 50 mL·s⁻¹ is set to 20 parts per billion (ppb) in this subject. iNOS: inducible NO synthase; NOS: NO synthase.
FeNO in Asthma

Not a marker specifically of eosinophilic airway inflammation!
FeNO broader marker of Th2-mediated allergic inflammation (includes airway eosinophilia rather than eosinophilic inflammation only)
Mepolizumab (anti-IL-5)  
FeNO idem  
↓ eosinophils (sputum/blood) reduced

Lebrikizumab (anti-IL-13)  
↓ FeNO  
eosinophils idem
### Factors affecting exhaled NO

<table>
<thead>
<tr>
<th>Increase</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airway infection</td>
<td>Smoking</td>
</tr>
<tr>
<td>Allergic rhinitis / IgE sensitisation</td>
<td>Exercise</td>
</tr>
<tr>
<td>Nitrate-rich diet (spinach, lettuce) *</td>
<td>Spirometric maneuvers</td>
</tr>
<tr>
<td>Height ** / age in children (&lt; 12 years)</td>
<td>Alcohol consumption</td>
</tr>
<tr>
<td>Bronchodilator</td>
<td>Bronchoconstriction</td>
</tr>
<tr>
<td>Diurnal variation (+ 15% afternoon)</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td></td>
<td>Ciliary dyskinesia</td>
</tr>
<tr>
<td></td>
<td>Pulmonary hypertension</td>
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</tbody>
</table>

* 200 g spinach FeNO + 150%

** Increase from 120 to 180 cm associated with a doubling of FeNO.

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### Effect size of different patient-related factors on FeNO

<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex %</td>
<td>21 (7–39)</td>
<td>15 (1–31)</td>
<td>8 (-6–23)</td>
<td>17 (7–29)</td>
<td>25*</td>
</tr>
<tr>
<td>Height (per 10 cm increase) %</td>
<td>14 (1–41)</td>
<td>10 (3–18)</td>
<td>62 (45–86)</td>
<td>11 (5–17)</td>
<td>Not significant</td>
</tr>
</tbody>
</table>
| Previous smoking %   | -13 (-22– -3) | -9 (-18–1)        | Not significant
| IgE sens. %          | 15 (0–32)     | 27 (15–40)        | 62 (45–86) | 50 (37–63)§   | 41*           |
| Asthma %             | 17 (5–32)     | 46 (26–69)        | 41 (5–86)  | Not studied separately§ | 26%*          |

Practical aspects of measuring exhaled NO

Chemiluminescence method
flow rate of 50 ml/s

Electrochemical sensors
**FeNO and its clinical impact: ICS responsiveness**

**ATS Guidelines 2011**

*We recommend the use of FeNO in determining the likelihood of steroid responsiveness in individuals with chronic respiratory symptoms possibly due to airway inflammation (strong recommendation, low quality of evidence).*

Use of cut points rather than reference values

<table>
<thead>
<tr>
<th>FeNO (ppb)</th>
<th>Response to Inhaled Corticosteroids</th>
<th>Consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 25</td>
<td>Unlikely</td>
<td>Noneosinophilic asthma</td>
</tr>
<tr>
<td>&lt; 20, children under 12 years</td>
<td></td>
<td>VCD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rhinosinusitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GERD</td>
</tr>
<tr>
<td>25-50</td>
<td>Possible, Cautious Interpretation</td>
<td>Anxiety</td>
</tr>
<tr>
<td>20-35, children under 12 years</td>
<td></td>
<td>Cardiac disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High levels of allergen exposure</td>
</tr>
<tr>
<td>&gt; 50&lt;sup&gt;a&lt;/sup&gt; 39</td>
<td>Likely</td>
<td>Atopic asthma</td>
</tr>
<tr>
<td>&gt; 35&lt;sup&gt;a&lt;/sup&gt;, children under 12 years</td>
<td></td>
<td>High levels of allergen exposure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COPD with mixed inflammatory phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eosinophilic bronchitis</td>
</tr>
</tbody>
</table>

*New reference values (National Health and Nutrition Examination Survey)*

*FeNO Interpretation in patients with asthma-like symptoms not treated with ICS*

FeNO and its clinical impact: Asthma Management

Conflicting results on whether FeNO-guided management results in reduced exacerbation rates

**Cochrane review 2016** (n=1700, age 28-54 years)

- Asthma exacerbations significantly lower in the FeNO group compared to the control group (OR 0.60, 95% CI 0.43 to 0.84), number needed to treat to benefit over 52 weeks was 12 (95% CI 8 to 32).

- No difference between the groups for exacerbations requiring hospitalisation (OR 0.14, 95% CI 0.01 to 2.67) or rescue oral corticosteroids (OR 0.86, 95% CI 0.50 to 1.48)

- No significant difference between groups for any of the secondary outcomes (FEV1, FeNO levels, symptoms scores, or inhaled corticosteroid doses at final visit)

**Authors’ conclusions**

... showed that tailoring asthma medications based on FeNO levels (compared with primarily on clinical symptoms) decreased the frequency of asthma exacerbations but did not impact on day-to-day clinical symptoms, end-of-study FeNO levels, or inhaled corticosteroid dose. Thus, the universal use of FeNO to help guide therapy in adults with asthma cannot be advocated. As the main benefit shown in the studies in this review was a reduction in asthma exacerbations, the intervention may be most useful in adults who have frequent exacerbations. Further RCTs encompassing different asthma severity, ethnic groups in less affluent settings, and taking into account different FeNO cutoffs are required.

Petsky H et al. Exhaled nitric oxide levels to guide treatment for adults with asthma. The Cochrane Library September 2016
FeNO testing is a noninvasive point-of-care method / indicator of Th2-mediated allergic airway inflammation.

FeNO testing can assist clinicians in identifying patients with airway inflammation responsive to ICS.

Incorporating FeNO testing into the management of patients with asthma may result in reductions in exacerbation rates.

FeNO tests may have limited utility in patients with asthma phenotypes not characterized by allergic airway inflammation. Increases in FeNO levels are also seen in atopic patients without asthma.
Exhaled breath condensate (EBC)

99% water vapor, small fraction of respiratory airway lining fluid droplets
Simple, safe, non-invasiv, cheap
Formed by cooling exhaled breath of patients in a condenser system
Exhaled breath condensate (EBC)

Nonvolatile biomarkers from the epithelial lining fluid of the airway epithelium
Extremely low concentrations (ultrasensitive techniques required for analysis)
pH, H$_2$O$_2$, 8-isoprostane, eicosanoids, NO, interleukins, metabolomics, proteomics, genomics …

Asthma, COPD, bronchiectasis and cystic fibrosis: elevated levels of inflammatory and oxidative stress biomarkers (hydrogen peroxide, leukotrienes, isoprostanes, hydrogen ions, prostaglandins, and nitrogen oxides).

Significant difference in EBC matrix metalloproteinase 9 (MMP-9) concentrations between NSCLC and control subjects with transudative pleural effusion. Positive correlations between MMP-9 concentration and pack years smoking history (r=0.8, P<0.0001) and stage of lung cancer (r=0.6, P<0.01). No correlation with the histopathological type of lung cancer
Volatile organic compounds

Oscar the cat (NEJM 2007:357:328-329)
Steere House Nursing and Rehabilitation
Volatile organic compounds (VOC)

- Gaseous organic molecules, emitted from the fluid phase (highly volatile)
- Released from skin, with feces, urine, and breath
- Derived from many metabolic pathways.
- Rate at which VOCs are exhaled is the net effect of several interacting (bio)chemical processes; intracellular and extracellular degradation; solubility of the compound in extracellular fluid, fat, and blood; the affinity with extracellular matrix and carrier proteins; the concentration gradient with the alveolar and bronchial air; the vapor pressure; and alveolar ventilation.
Only 1% of the exhaled breath composition is likely to contain disease specific compounds.

Other 99% only certain differences in compound abundance suggest disease-related information.

Comorbidities?
# VOCs detected in lung diseases

<table>
<thead>
<tr>
<th>Author</th>
<th>Disease</th>
<th>Significant VOCs identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phillips 2003</td>
<td>Lung Cancer</td>
<td>butane, 3-methyl tridecane, 7-methyl tridecane, 4-methyl octane, 3-methyl hexane, heptane, 2-methyl hexane, pentane, 5-methyl decane</td>
</tr>
<tr>
<td>Machado 2005</td>
<td>Lung Cancer (NSCLC)</td>
<td>isobutane, methanol, ethanol, acetone, pentane, isoprene, isopropanol, dimethylsulfide, carbon disulfide, benzene, toluene</td>
</tr>
<tr>
<td>Poli 2005</td>
<td>Lung Cancer</td>
<td>2-methyl pentane, pentane, ethyl benzene, xylenes (total), trimethyl benzene, toluene, benzene, decane, octane, penta methyl heptane</td>
</tr>
<tr>
<td>Barker 2006</td>
<td>Cystic Fibrosis</td>
<td>ethanol, propane, pentane&lt;sup&gt;‡&lt;/sup&gt;, methanol&lt;sup&gt;‡&lt;/sup&gt;, ethanol, 2-propanol&lt;sup&gt;‡&lt;/sup&gt;, acetone, isoprene&lt;sup&gt;‡&lt;/sup&gt;, benzene, toluene, dimethyl sulfide&lt;sup&gt;‡&lt;/sup&gt;, limonene</td>
</tr>
<tr>
<td>Dragonieri 2007</td>
<td>Asthma</td>
<td>4-methyl octane, 2,4-dimethyl heptane, isopropanol, toluene, isoprene, alkane, acetic acid, acetone, 2,6,11-trimethyl dodecane, 3,7-dimethyl undecane, 2,3-dimethyl heptane</td>
</tr>
<tr>
<td>Chen 2007</td>
<td>Lung cancer</td>
<td>styrene, decane, isoprene, benzene, undecane, 1-hexene, hexanol, propyl benzene, 1,2,4-trimethyl benzene, heptanal, methyl cyclopentane</td>
</tr>
<tr>
<td>Peng 2010</td>
<td>Lung, breast, colon, prostate cancer</td>
<td>16 compounds identified that varied in abundance between healthy groups and cancer groups: 1-methyl-4-(1-methyl)benzene, toluene, dodecane, 3,3-dimethyl pentane, 2,3,4-trimethyl hexane, 1,1′-(1-butylidene) bis benzene, 1,3-dimethyl benzene, 1-iodo nonane, 1,1′-dimethylthethyl thio) acetic acid, 4-(4-propylcyclohexyl)-4'-cyanof[1,1′-biphenyl][4-yl ester benzoic acid, 2-amino-5-isopropyl-8-methyl-1-azulenecarbonitrile, 5-(2-methylpropyl) nonane, 2,3,4-trimethyl decane, 6-ethyl-3-octyl ester 2 trifluoromethyl benzoic acid, p-xylene, and 2,2-dimethyldcane</td>
</tr>
<tr>
<td>Fuchs 2010</td>
<td>Lung cancer</td>
<td>Aldehydes-butanal, formaldehyde, acetaldheyde, pentanal, hexanal, octanal, nonanal</td>
</tr>
<tr>
<td>Wang 2012</td>
<td>Lung cancer</td>
<td>Adenocarcinoma-2,4,6-trimethyloctane, 2-methyldecane, 2-tridecanone, 2-pentadecane, 8-methyl heptadecane, 2-heptadecane, nonadecane, eicosane, squamous-methanoic acid, 2-nonanone, 2-pentadecane, nonadecane, eicosane, SCC-2-decanone, 2-hendecane, 2-methylphthaline, 2-tridecanone, 2-pentadecane, 2,6-dimethylphthaline, 1-heptadecanol, 2-heptadecane, nonadecane, eicosane</td>
</tr>
</tbody>
</table>

Sources of Exhaled Volatile Organic Compounds (VOC)

Concept of Analysis of VOC
# Concept of analysis of VOC

## Chemical Analytical Techniques

**Gas chromatography (GC) coupled to mass spectrometry (MS)**

- Identification of individual chemical compounds.
- Clinical implementation: complex (highly trained personnel, laborious analysis)
- Costly application

Recent developments:
- Real-time quantitative analysis of VOCs, technically less demanding. Miniaturization of devices may allow low-cost, on-site detection of specific compounds

## Pattern Recognition-Based Sensors

**Electronic noses (eNoses)**

- VOCs competitively interact with cross-reactive sensors, allowing multiple VOCs to interact with the same sensor based on their affinity for both the sensor and its substrate.
- Does not identify individual compounds, provides probabilistic recognition, which forms the basis of assessing diagnostic accuracy.
- Relatively cheap, easy to use, provides on-site results holding promise for its use as a point-of-care tool if properly validated and standardized
Exhaled breath VOC analysers

Gas chromatography and mass spectrometry (GC-MS)

Portable/inexpensive devices
- Ion mobility spectrometry (IMS)
- Electronic noses: Cyranose 320
- Colorimetry
- Gold particle nanosensor
- Canine
Electronic nose (Cyranose 320)

Handheld, portable
32 nanocomposite array sensors
Vapor passes over each sensor, induces a swelling and change in resistance
Secondary Electrospray Ionization Source (SESI)

Secondary Electrospray Ionization Source

Orifice plate
Heated nitrogen gas
Curtain plate
Vapor sample
Flow restrictor
SESI mixing chamber
Top View

nano Electrospray (water + 0.1 % formic acid)

courtesy of Lukas Bregy, ETH Zurich
VOC in COPD: 2-hydroxyisobutyric acid
VOC in COPD

N=22 COPD (mean FEV$_1$ 59±20%, 32±19 py, age 59±7 yrs)
N=14 controls (mean age 58±8 years, FEV$_1$ 103±11% pred, 24±13 py, age 58±8 yrs)

<table>
<thead>
<tr>
<th>Condition</th>
<th>COPD</th>
<th>No COPD</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD</td>
<td>19</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>No COPD</td>
<td>1</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>total</td>
<td>20</td>
<td>16</td>
<td>36</td>
</tr>
</tbody>
</table>

accuracy 89% (CI 74% - 97%), sensitivity 93%, specificity 87%, PPV 81%, NPV 95%

Bregy L, Nussbaumer-Ochsner Y, submitted
N=26 OSA patients (Δ ODI 30/h (95% CI 20/h, 40/h))
sensitivity of 92.9% and a specificity of 84.6%

Schwarz EI et al. Effects of CPAP therapy withdrawal on exhaled breath pattern in OSA. Thorax 2016;71:110-7
Real-time breath analysis by SESI-MS allows molecular profiling of exhaled breath, can distinguish patients with COPD / OSA from matched healthy controls and provides insights into the disease pathogenesis.

Exact origin of most of these VOCs is unknown:

- **COPD**: metabolites from oxidative stress processes like fatty acids, aldehydes and amino acids resulting from lung muscle degradation.

- **OSA**: mainly aldehydes (cell membrane lipid peroxidation leads to generation of various aldehydes, e.g. isoprene (cholesterol synthesis) – sleep regulation, increased in stressful conditions such as exercise, myocardial infarction and increased cardiac output (sympathetic activity?)

- **Lung fibrosis**: different aminoacids (one of them proline).

Limitations: no recommended guidelines in sampling

Many variations on statistical analysis

No comparison between equipment

Confounders
Where the future goes

BreathCloud database
BreathCloud is an online reference database of exhaled biomarker profiles linked to a computer programme by a corresponding application to enable point-of-care personalized medicine. This database provides immediate diagnostic answers for the individual patient. For each patient BreathCloud will automatically select the most optimal model, based on patient characteristics and differential diagnosis, in order to create a final report. The breath analysis outcomes of each patient will eventually - after diagnosis, established according gold standard methods, be added to the expanding database and will automatically make subsequent diagnoses more accurate.

SpiroNose - BreathCloud
The combination
Diagnostic tests are an essential part of modern medicine. The ultimate goal of diagnostics and monitoring is to optimize the outcome or prognosis for the patient by giving the clinician directions for a clinical management strategy. Even though physiological and cell-based procedures, such as spirometry, blood and induced sputum are often routinely available in respiratory medicine, molecular diagnostics are not widely applicable at point of care.