Breathomics in pulmonary disease
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General introduction and
Aims of the thesis
**DIAGNOSTICS IN OBSTRUCTIVE LUNG DISEASES**

**Asthma**
Asthma is a chronic inflammatory disease of the airways, and is associated with variable airways obstruction, airway hyperresponsiveness and symptoms of chronic cough, dyspnea, wheeze, chest tightness and sputum production. The disease affects people of all ages, and its spectrum ranges from mild intermittent to severely disabling when uncontrolled. Due to the heterogeneity of the disease and because symptoms can be non-specific, diagnosis can be challenging. Asthma is often not recognized, or misdiagnosed as wheezy bronchitis, COPD, hyperventilation syndrome or breathlessness of old age [1]. Besides this, there is no single definition of the disease. Nonetheless, correct and early diagnosis of asthma significantly reduces the socioeconomic burden of this disease and increases the patient’s quality of life.

Asthma is usually diagnosed on the basis of symptoms, the predominant feature being intermittent shortness of breath, especially at night, often accompanied by cough [1]. The main physiological feature is episodic airway obstruction which is characterized by expiratory airflow limitation, often responsive to bronchodilators. The finding of airway hyperresponsiveness to inhaled methacholine or histamine also favors the diagnosis of asthma.

**COPD**
Chronic Obstructive Pulmonary Disease (COPD) is a disease that is associated with an abnormal inflammatory response to noxious particles or gases, of which cigarette smoke is the most important. Besides cigarette smoke, COPD can also be caused by occupational exposure to dust or fumes, and indoor biomass fuel cooking in developing countries [2,3].

As such, this disease is largely preventable, yet it is one of the major causes of chronic morbidity and mortality [4,5]. Underdiagnosis and misdiagnosis are frequent, especially in case of early or mild disease. Early and correct diagnosis of COPD is necessary to help the patient in quitting smoking to prevent further damage, and to start treatment timely.

COPD is characterized by airway obstruction that is not fully reversible and usually progressive over the years [6]. COPD is currently diagnosed when the ratio of expiratory volume in 1 second (FEV₁) to the forced vital capacity volume is smaller than 0.70 after bronchodilation [7]. This is irrespective of the presence of symptoms of cough, sputum production or dyspnea and the cumulative smoking history, although these factors certainly contribute to the diagnosis. However, diagnostics for COPD are far from ideal, leaving room for improvement.

**DETERMINING THE ACCURACY OF DIAGNOSTIC TESTS**
Diagnostic tests are an essential part of modern medicine, including in respiratory medicine. The ultimate goal of a diagnostic process is to optimize the outcome or prognosis for the patient by giving the clinician directions for a clinical management strategy. An evidence-based evaluation of a diagnostic test after its technological validation requires the analysis of the
association between the presence of the target disease and the test result using clinical epidemiological research data [8].

The ideal diagnostic test should be both sensitive (the percentage of sick people who are correctly identified as having the condition is high) and specific (the percentage of healthy people who are correctly identified as not having the condition is high). The overall percentage of subjects correctly classified as being sick or healthy is called the test accuracy. Studies to assess diagnostic accuracy have a common basic structure [9,10]. In phase I, the test result distribution in patients with the target disease is compared to the test result distribution in healthy controls. This is usually performed in a cross-sectional case-referent study and provides an internal validity of the test. In phase II, a newly recruited somewhat broader spectrum of patients with the target disease is compared to healthy controls or to patients with differential diagnoses that should be discriminated from the target disease. This phase is also performed in a cross-sectional case-referent study, using the diagnostic model that was created in phase I. This phase provides the external validity of the test. In phase III, a new group of patients with clinical and pathological variety of the target disease and/or comorbidities is prospectively recruited and compared to the diagnostic model. This phase provides an external validity for clinical or difficult-to-diagnose groups of patients. Finally, in phase IV, the test performance is prospectively evaluated in large series of subjects with an ‘intention-to-diagnose’, i.e. subjects who are suspected of the target disorder [8].

The results of the new test are compared to the results of the reference test: the gold standard test for a specific condition [11]. The STARD (STAndards for the Reporting of Diagnostic accuracy studies) initiative has has been instigated to improve the completeness and accuracy of studies of diagnostic accuracy. The STARD statement consists of a 25 item checklist that can be followed when designing and reporting diagnostic studies [11]. This checklist can be found at www.stard-statement.org.

**EXHALED BREATH ANALYSIS**

Using exhaled breath as a diagnostic test is an old concept. Already in ancient times it was recognized that breath can provide information about health and disease. For example, a fishy smell of breath could indicate liver disease, the sweet acetonic smell of breath is related to uncontrolled diabetes, a urine-like odor to kidney disease, the smell of grapes to *Pseudomonas* infections and the smell of baked bread to typhoid fever [12].

Exhaled breath is a mixture of nitrogen, oxygen, carbon dioxide, water, and inert gases such as nitric oxide and carbon monoxide. In the 1970's it has been shown that exhaled breath also contains a complex mixture of volatile organic compounds (VOCs) in very low concentrations (see Figure 1) [13-15]. VOCs originate from both systemic and local metabolic processes, which can be associated with normal physiology or inflammatory or oxidative activity (see Figure 2).

A well-known breath test is the ethanol and acetaldehyde test that is used by the police to check for alcohol consumption. There are a few VOCs present in high concentrations (parts per
Introduction

Chapter 1

**Figure 1** The composition of exhaled breath.

- **O₂**: 17%
- **CO₂**: 4%
- **Ar**: 1%
- **VOCs**: <0.001%
- **N₂**: 78%
- **NO, CO**: <0.1%

**Figure 2** The origin of exhaled volatile organic compounds (VOCs).

- **Oxidative stress**
  - ↑ Reactive oxygen species from mitochondria
    - Lipid peroxidation of poly-unsaturated fatty acids in cell membranes
    - Oxidation of biologically important molecules (DNA, proteins) in cytoplasm
  - Non-volatiles
    - Volatiles (VOCs)
      - alkanes
      - methyl alkanes
      - aldehydes
      - etc.
    - Metabolism via cytochrome p450 or enzymes
    - Excreted in breath

- **Airway inflammation**
  - Production of inflammation-specific VOCs
billion range; ppb) in exhaled breath, such as ammonia, acetone, isoprene, methanol, ethanol, acetaldehyde and alkanes of varying chain lengths. However, most VOCs in breath have concentrations in the picomolar (parts per trillion; ppt) range. Most (pulmonary) diseases are not characterized by the increase of a single component, so the challenge is to capture the profile of VOCs in exhaled breath in order to detect diseases.

The great advantage of exhaled breath testing is the non-invasiveness for patients. Patients are asked to either blow in a device or a balloon, and for small children and sedated ICU-patients, breath collection methods that require no cooperation of the patient have been developed. Despite the advantages of exhaled breath testing, its use in clinical practice is still limited. The main reasons are a lack of thoroughly validated techniques in different disease states and of normalization and standardization methods.

**METHODS OF EXHALED BREATH ANALYSIS: BREATHOMICS**

There are several methods that can be applied for exhaled breath analysis. The gold-standard test is considered to be gas chromatography coupled by mass spectrometry (GC-MS). Using this laborious and expensive technique, both the composition and the concentrations of components of a gas mixture can be determined [16,17]. GC-MS allows broad spectrum specific identification of VOCs, aiding in understanding pathophysiological molecular pathways.

Electronic noses (eNoses) follow a different approach, by measuring the whole spectrum of VOCs without identification of the individual components [18]. The output of eNoses is a signature of the expired VOC-profile, which is called a ‘breathprint’ and can be analyzed by pattern recognition algorithms (see Figure 3).

![Figure 3](image_url) Methods of measuring exhaled VOCs.
Photography: Netherlands Asthma Foundation and Kees Tabak Fotografie.
eNoses come in different shapes using different types of VOC-sensors. Sensors can be made of organic polymers [19], metaloxide [20], ion mobility spectrometry [21], colorimetric sensors [22,23], optical sensors [24], quartz crystals [17,25-27], gold nanoparticles [28] etcetera. The suitability of the instrument for detection of specific disease states depends on the selection of sensors in relation to the gas mixture of interest and the choice of pattern recognition algorithms. Unlike GC-MS, eNoses do not contribute to the discovery of biomarkers that are specific for a disease, or targets for therapy. However, breathprints do contribute to molecular phenotyping of patients in an easy and relatively cheap way.

Several other methods of exhaled breath analysis exist, such as proton-transfer mass spectrometry (PTR-MS), gas chromatography time-of-flight (GC-TOF), selected-ion flow-tube mass spectrometry (SIFT-MS) and exhaled breath condensate (EBC). This thesis will focus on the applicability of exhaled breath molecular profiling techniques in pulmonary diseases.

OBJECTIVES OF EXHALED BREATH ANALYSIS IN MEDICINE
The objectives of exhaled breath analysis in medicine can be summarized as follows and will be discussed in this thesis.
1. To determine and improve diagnostic accuracy of exhaled breath testing for specific diseases.
2. To unravel the molecular pathways associated with (patho)physiology that contribute to the exhaled breath signal.
3. To identify disease subphenotypes in relation to clinical course and treatment response.

OUTLINE OF THIS THESIS
1. In chapter 2, we describe the current status of exhaled breath molecular profiling by electronic noses in medicine.
2. Following the STARD guidelines for determination of diagnostic accuracy of electronic noses in obstructive lung diseases, we assessed the internal and external validity in chapters 3 and 4.
3. In order to unravel what determines the exhaled signal that is specific for asthma and COPD, we investigated the relation of airway caliber and inflammatory markers in sputum to exhaled breathprints in chapters 5 and 6.
4. A cluster analysis on a community-derived population of COPD patients was performed in chapter 7 to assess the additive value of exhaled breath profiles in subphenotyping this heterogeneous disease.
5. In chapter 8, core methodological issues concerning breath testing, such as signal stability and inter-device compatibility are highlighted.
6. A sidestep to vascular pulmonary medicine was undertaken by assessing the diagnostic accuracy for acute pulmonary embolism in chapter 9.
7. In chapter 10, all findings are summarized and the implications for further implementation and research are discussed.
REFERENCES