Non-invasive diagnostic of head and neck cancer:
feasibility of a portable electronic nose

Martinus Gerardus Eimbertus van de Goor

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CHAPTER 1

General introduction and Outline of this thesis

The human nose and the electronic nose

Humans have five basic senses: sight, hearing, taste, smell, and touch. For centuries, medical professionals have used their senses to diagnose and treat patients. Although vision and touch take primacy – especially when examining patients – as practitioners we should not underestimate the power of our olfactory sense for the diagnosis of pathological processes in the human body.

As early as 2000 BCE Greek doctors diagnosed infectious diseases such as tuberculosis by means of scent. Heating a person's sputum released a smell specific to patients with tuberculosis that the doctor recognized in the fumes. Other examples include the odor produced by gas gangrene of the limbs and the fruity smell of ketones in the exhaled breath of patients with diabetic ketoacidosis.[1] The relationship between disease and aroma producing organisms was already reported by Omlanski in 1923 were he described the distinctive aroma produced by the pseudomonas bacteria.[2] The downside of using smell for diagnosis is that it takes years to develop this skill and it only works for a limited number of diseases. With the rise of modern laboratory and radiologic diagnostic techniques, the art of olfactory diagnosis has almost disappeared. However, this method might be suitable for identifying diseases in the laboratory or even at the bedside, given the theoretical simplicity and ease of olfactory diagnostics.

Laboratory-based identification can use volatile organic compounds (VOCs), gaseous compounds produced during physiological and pathological processes in the human body. These compounds are found in feces, headspace of micro-organism cultures, blood, urine, and exhaled breath.[3] The VOCs produced by the human body can be considered as an individual's odor-fingerprint. Disease is often associated with an altered metabolism, resulting in a modified VOC output with a distinctive fingerprint.[4] Several techniques have been used to assess these VOCs. One combines gas chromatography with mass spectrometry (GC-MS) for separation and identification of specific VOCs instead of unique composite breath signals. This technique is complex and relatively expensive. Furthermore, it is doubtful whether GC-MS is reliable enough to discriminate between cancer patients and healthy controls. Schallschimdt et al. concluded in an observational study on VOC patterns that GC-MS is not reliable enough to discriminate between cancer patients and healthy controls.[5] Wang et al. described a confounding effect of benign pulmonary diseases in selecting individual VOCs when using GC-MS for detection of lung cancer.[6]

Another technique, using an electronic nose (e-nose), is based on pattern recognition rather than individual molecular identification such as with GM-MS.[7] VOC patterns identified with e-nose technology have been found in feces, urine, headspace of micro-organism cultures, and exhaled breath. These patterns have been associated with respiratory,[8] urogenital,[9] and neurological disease,[10] as well as with malignancies of lung,[11-14] colorectal,[15,16] and head and neck origin.[16-20]

When using odor as a diagnostic instrument in an outpatient or bedside setting, a portable handheld instrument is warranted. Advances in sensor technology, electronics, biochemistry, and artificial intelligence made it possible to develop devices capable of measuring and characterizing VOC patterns. E-noses were engineered to mimic the mammalian olfactory system within an instrument designed to obtain repeatable measurements, allowing the identification and classification of volatile organic compound patterns.

Human nose

Before explaining the principle of the e-nose, it is best to briefly review the physiology of the human olfactory system. Olfaction is a chemical reaction that produces the sense of smell. To stimulate the olfactory system, an odor must activate receptors in the olfactory epithelium below the cribriform plate (Figure 1).[21]

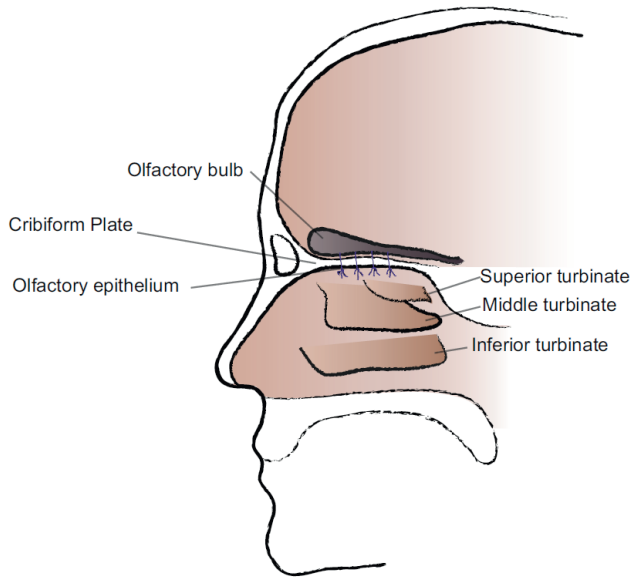

Figure 1. Lateral view of the human nasal cavity. The dorsal area of the nasal cavity is lined with olfactory epithelium. Axons from olfactory sensory neurons in the epithelium coalesce to form the olfactory nerve, which projects through the bony cribriform plate to the olfactory bulb.
Reprinted from ***Conn's Translational Neuroscience****, 2017 C. Trimmer, J.D. Mainland, The Olfactory System, Page 366., Copyright (2017), with permission from Elsevier. DOI: 10.1016/B978-0-12-802381-5.00029-4*

These olfactory receptors are expressed in the cilia of the olfactory sensory neurons (OSNs), which are surrounded by a thin layer of mucus. The cilia are whip-like extensions of the OSNs. Each OSN has 8 to 20 cilia (Figure 2). To be perceived, an odor must be absorbed by the mucous layer to reach the olfactory receptors.[21]

Each aroma triggers a specific selection of olfactory receptors. The brain combines the signals received and determines what combination of molecules is detected and which odor "belongs" to this combination. The brain needs to be trained to classify a combination of molecules as an odor.

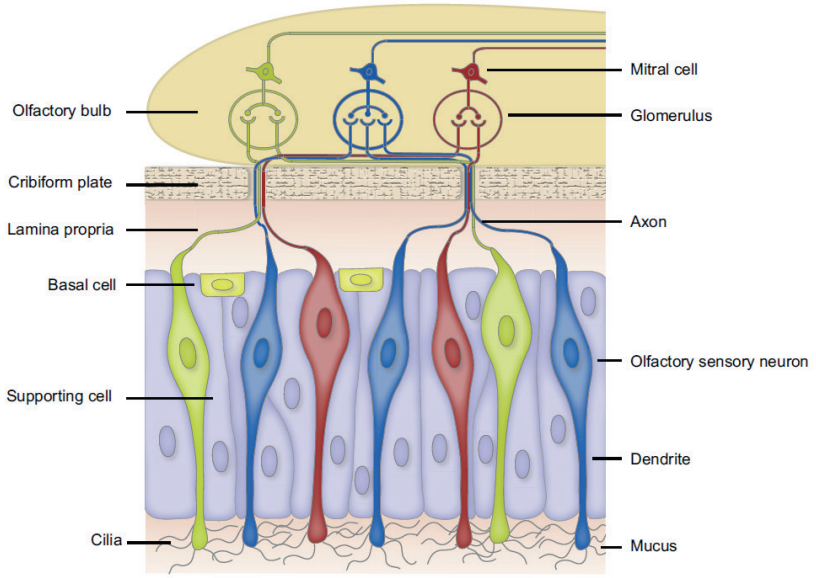

Figure 2. Schematic of the main olfactory epithelium showing the major cell types. Odorants are detected by the olfactory sensory neurons (OSNs). The OSNs are bipolar neurons, with their cell body found in the basal portion. A dendrite extends from the apical pole of the neuron to the epithelial surface. At the surface, the dendrite forms the olfactory knob, from which 8-20 cilia extend into the mucous layer. A single axon extends from the basal pole of the OSN, where it merges with the axons of other OSNs, passing through the cribriform plate before joining other bundles (termed olfactory fila). These fila comprise the olfactory nerve, which synapses on mitral/tufted cells in the main olfactory bulb (MOB).
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The effect is shown in figure 3 as a response matrix for different odorants and different receptors. Volatile molecules activate specific populations of OSNs, which vary in relation to both odor identity and odor concentration. This pattern of activation determines the information sent to the brain for further processing. The unique ensemble of activated receptors allows the brain to identify a very large set of (mixed) odorants.[21,22]

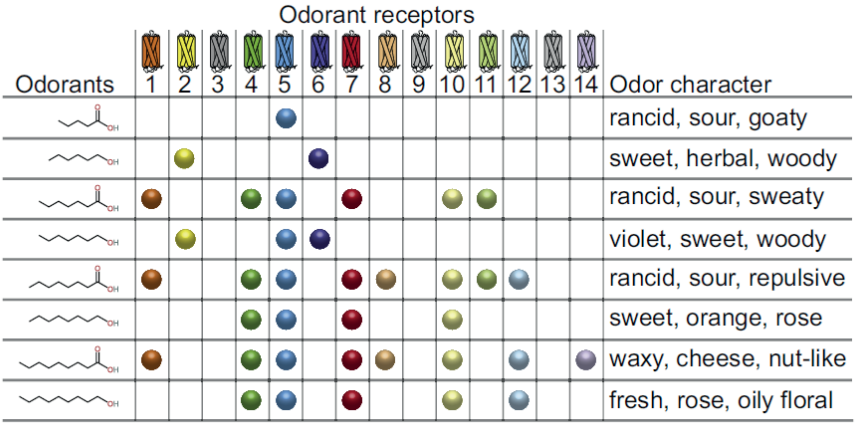

Figure 3. Different odorants are recognized by unique but overlapping ensembles of odorant receptors. These ensembles are translated into diverse odorant perceptions.[21]
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The electronic nose

E-noses are biomedical instruments that are inspired by the human olfactory system and simulate odor discrimination by combining an array of sensors interacting with VOCs in the air. The instrument combines a signal transduction mechanism with a pattern recognition algorithm to create a model. As the processing capacity of computers has continuously improved, it is possible to apply pattern recognition techniques in real-time diagnostics. Pattern recognition is a process that aims to find statistical relationships in datasets.[23] In healthcare pattern recognition, it is mostly used for classification. The general idea is to develop a broadly responsive sensor system and use pattern recognition systems to match "unknown response" patterns to previously "observed response" patterns, thereby identifying the specific odors present within complex mixtures. In fact, this is analogous to the way humans smell complex mixtures of unknown substances containing a characteristic odor.[24]

The principles underlying this approach are illustrated in figure 4 and are compared to the routing of the human sense of smell.

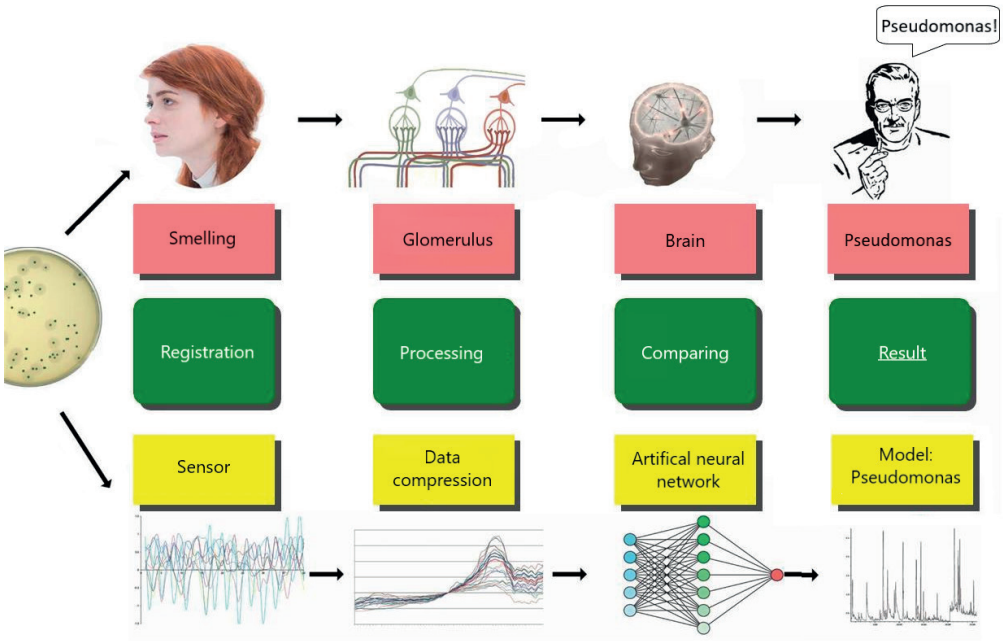

Figure 4. Comparison of the steps required to detect an odor using either a biological nose or an e-nose. Reprinted and adjusted from ***Transferable Odor Differentiation Models for Infectious Disease Diagnostics****, 2014, M. Bruins, The Electronic nose, Page 16, with permission from M. Bruins ISBN 978-94-6108-600-6*

In recent years numerous studies have reported the use of VOC pattern analysis to detect malignancies and have demonstrated fairly high diagnostic accuracy.[8,9,11-14] One major limitation of VOC pattern analysis is the reproducibility of apparently similar sensors used in different e-nose devices.[22] There seems to be an inter-sensor difference, mainly due to small differences in the sensing layer and the absence of temperature control.[22] Reproducibility would be improved if VOC patterns could be compared without building a new model for each e-nose, thereby possibly saving scarce resources such as personnel and money. Another impediment to reproducibility could lie in the statistical or mathematical methods used to detect VOC patterns in exhaled breath.[25] Advances in machine learning and vector analysis by means of an artificial neural network (ANN) could improve pattern recognition.[26]

Therefore, in this thesis we used the Aeonose™ (the eNose Company, Zutphen, the Netherlands). It is a handheld, portable, and easy-to-use device, which made it possible to use it in an outpatient clinical setting.

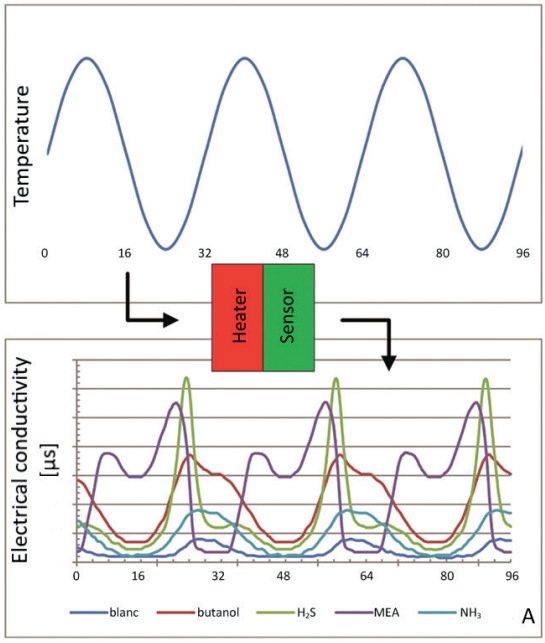

Figure 5 A.

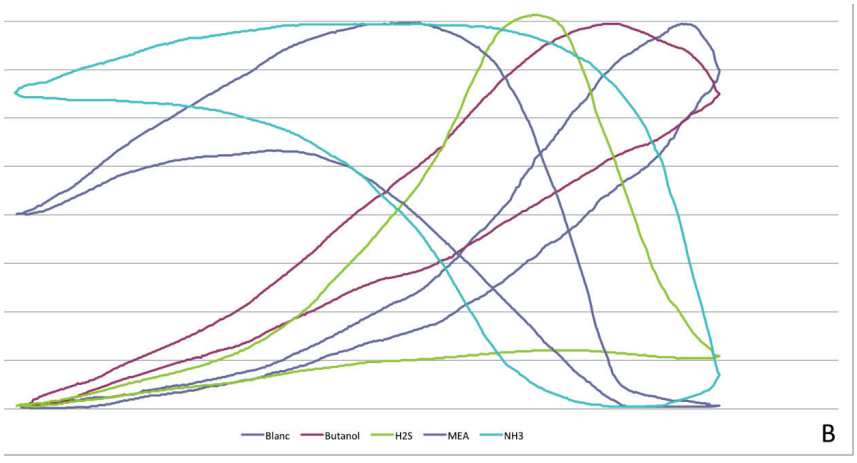

Figure 5 B

Figure 5. (A) Measuring principle of the Aeonose™: a continuous sinusoidal temperature cycle in which the heater is thermally cycled (top) and the conductivity of the sensor (bottom) is recorded as a function of momentary temperature. The temperature profile is applied to the heater while the response is recorded at the sensor. Substances can show temperature-dependent responses for the same chemical sensor type. (Blanc = clean air, H2S = Hydrogen sulphide, MEA = methylamine, NH3 = ammonia). (B) Thermal response loops resulting from the normalized conductivity of the sensor plotted as a function of the heater temperature during a full period. (Blanc =clean air, H2S = hydrogen sulphide, MEA = methylamine, NH3 = ammonia). Reprinted and adjusted from ***Transferable Odor Differentiation Models for Infectious Disease Diagnostics****, 2014, M. Bruins, The Electronic nose, Page 16, with permission from M. Bruins ISBN 978-94-6108-600-6*

Kort et al. 2017 give a descriptive explanation of the working mechanism of the Aeonose™. 'The Aeonose™ consists of three micro hotplate metal oxide sensors (MOS) that are rigid, mass producible, and offer the opportunity for transferring calibration models. This means that once a calibration model has been developed, it can easily be transferred to other Aeonose™ devices. In an Aeonose™ multiple metal oxides behave as semi-conductors at higher temperatures. The sensors vary in terms of metal-oxide type and catalyzing agent. Redox reactions occurring at the sensor surface result in changes in conductivity that can be measured and quantified, resulting in a unique breath signal. These redox reactions depend on the different types of metal oxides and catalysts, the reacting gas(es), and the temperatures created within the e-nose. A broad range of VOCs in exhaled breath will give different redox reactions. Redox reactions are temperature dependent, and this dependency can be determined as a function of time. Different VOCs show different responses at varying temperatures for the same chemical sensor type (figure 5A). The breath patterns are obtained by taking the response of a complete cycle and can be presented as a function of the temperature (figure 5B). In this way the temperature dependency of the redox reactions is acquired on a single sensor. The patterns obtained by thermal cycling do not only depend on the applied temperatures, but also on the dynamics of the temperature, because intermediate products created at the sensor surface have limited lifetimes.

The eNose Company uses a proprietary software package for data analysis called 'Aethena'. This package retrieves raw data from a database and takes care of data compression, data analysis, and data reporting to obtain the best prediction models. During an exhaled breath measurement, 64 × 36 data points are recorded for each sensor. In this way, each individual patient measurement comprises a data matrix with thousands of records'.[27]

A model calibrated with this technology could be applied to numerous other e-nose devices without requiring new calibration. This might lead to a practical implementation of the technique in clinical diagnosis of disease.

Head and Neck Cancer

Epidemiology

Head and neck cancer is the sixth most common form of cancer in the world.[28] In the United States alone there were 65,410 new cases in 2019.[29] Head and neck squamous cell carcinomas (HNSCCs) form 90% of head and neck cancers. Despite recent advances in diagnostic approaches and treatment modalities for HNSCCs, the 5-year survival rates have improved only marginally in the past decades.[30] In part, this can be attributed to the high rates of locoregional recurrences and second primary HNSCCs. Several authors report a 10% to 50% rate of recurrence, approximately 75% of which were found within 2 years after curative treatment.[31,32] The probability of developing a second primary HNSCC within 5 years after initial diagnosis is approximately 20%.[33-35]

Most second primary malignancies occur in the lung. These second primary lung cancers (SPLC) can occur synchronously with the HNSCC (0.8%) or can develop metachronously. The risk of a metachronous second primary lung cancer (SPLC) following HNSCC is reported to be 5.8%, 11.4%, and 16.4% at 5, 10, and 15 years, respectively.[36]

Another reason for the high mortality rates is the advanced tumor stage at first presentation. Around 66% of all patients present with a stage III or stage IV tumor.[37] Early detection is essential for successful treatment and to reduce cancer mortality and morbidity. Cancer screening in the asymptomatic but at-risk population might be a particularly promising way to reduce mortality rates. An essential condition for screening programs is a suitable, preferably non-invasive, test with a high level of accuracy.

Developing world

Cancer statistics for the developing world differ from those for the West. Cancer incidence and mortality rates are rising in developing countries, which already accounted for >50% of newly diagnosed cancers in 2010. This rate is estimated to increase to 70% by 2030.[38] In developing countries, the most common subsite for HNSCC is the oral cavity. According to the World Bank, lip and oral cavity cancer is the fourth most common cancer and the sixth most frequent cause of cancer deaths in low- and middle-income countries.[39] Risk factors for cancer of the oral cavity are predominantly associated with using tobacco, chewing betel quid, and drinking alcohol.[40] Besides the rising incidence of cancer there are other challenges in the developing world that hinder the proper diagnosis and treatment of patients with oral cancer. These are related to a lack of financial, infrastructural, and human resources.

The scarcity of resources is a good reason to try to involve the developing world in the early phase of e-health innovations. Using the Aeonose™ and interpreting its results require limited training and can be performed by non-medically trained personnel. The advantage of this low threshold is that highly skilled health workers thus remain available for the treatment of HNSCC. Cancer of the oral cavity seems to be a good starting point for the study and application of e-nose technology in the developing world because of its high incidence, the relatively easy access to histopathology confirmation, and the surgical treatment options that are available in these parts of the world.

Anatomy and tumor biology

Head and neck cancers are a heterogeneous group of malignancies of the upper aerodigestive tract, salivary glands, and thyroid. Ninety percent of them arise from the mucosal epithelium of the oral cavity (lips, buccal mucosa, hard palate, anterior tongue, floor of mouth, and retromolar trigone), nasopharynx, oropharynx (palatine tonsils, lingual tonsils, base of tongue, soft palate, uvula, and posterior pharyngeal wall), hypopharynx (the bottom part of the throat, extending from the hyoid bone to the cricoid cartilage), and larynx (supraglottis, glottis, and subglottis) (figure 6).

Tobacco-associated HNSCCs arise at all subsites, while human papilloma virus-associated HNSCCs arise primarily from the palatine and lingual tonsils of the oropharynx.

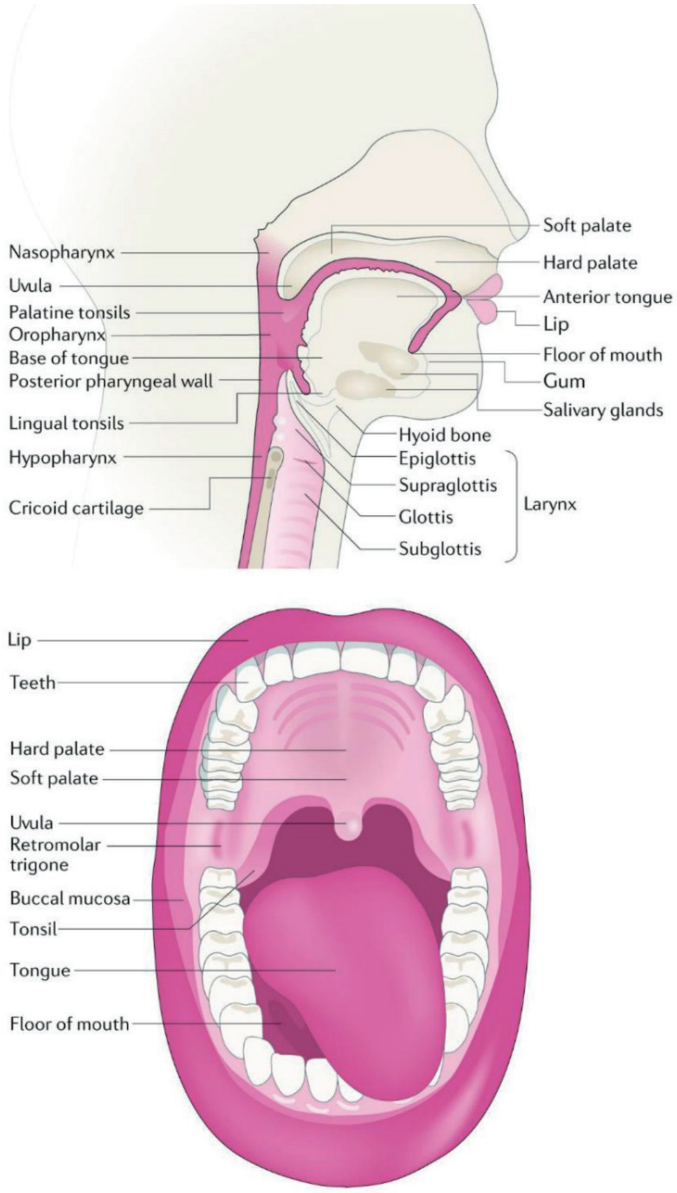

Figure 6. Anatomical sites of HNSCC development, Nature Reviews Disease Primers (Nat Rev Dis Primers) Reprinted by permission from Springer Nature: Springer Nature, ***Nature Reviews Disease Primers Head and neck squamous cell carcinoma****, Daniel E. Johnson et al), COPYRIGHT, 2020)*

Each subsite has unique features of anatomy, function, lymphatic drainage, etiology, and morphology. These factors determine the difficulty of diagnosing HNSCC. For example, malignant lesions of the oral cavity are usually detectable with a headlamp. Tumors of the vocal cords often present with complaints of hoarseness, unlike carcinomas of the oropharynx, sub- and supraglottic regions, and hypopharyngeal regions, which are often found in more advanced clinical stages. Therefore, the HNSCCs at different subsites have to be considered heterogenous groups of cancers.

This heterogeneity is confirmed by the observation that different HNSCC subsites produce different VOCs. A variety of VOCs such as ethanol, 2-propenenitrile and undecane dodecane, decanal, benzaldehyde, 3,7-dimethyl undecane, 4,5-dimethyl nonane, 1-octene, and hexadecane have been described as potential biomarkers for the diagnosis of HNSCC.[41,42] Bouza et al. described elevated concentrations of benzaldehyde, 3,7-dimethylundecane, and butyl acetate and proposed considering these VOCs as potential biomarkers for oral SCC. Garcia et al. reported 7 possible VOCs as biomarkers for laryngeal cancer: ethanol, 2-butanone, 2,3-butanediol, 9-tetradecen-1-ol, octane derivative compound, cycloheptane derivative compound, and cyclo-nonane derivative compound.[43]

Furthermore, recent clinical, genomic, and cellular studies demonstrate high levels of intra- and intertumoral heterogeneity of molecular biology and tumor immunology in HNSCCs.[44,45,46] Although the complex context of tumor heterogeneity lies beyond the scope of this thesis, it should be mentioned that different factors contribute to intra- and intertumoral differences, which could imply that tumors with the same origin and histologic classification would produce different VOCs.

The most important mechanisms contributing to the heterogeneity are tumor immunity, tumor hypoxia, metabolism of the tumor, and HPV status. Tumor immunity can differ strongly between different HNSCC subsites. Mainly, these differences are caused by infiltrating lymphocytes and the presence of cancer-associated fibroblasts (CAFs). These fibroblasts have a pro-tumorigenic effect on both the immune system and the tumor microenvironment.[44,47] Hypoxia is a common feature in head and neck tumors and is caused by a high demand and low supply of oxygen. These unfavorable hypoxic circumstances lead to neovascularization and angiogenesis, which in turn cause tumor progression, invasive growth, and spreading of cancer cells. Hypoxia has various biological, molecular, and genomic effects on the tumor and its micro-environment.[48] Malignant tumors require large quantities of energy and macromolecules, and that demand alters the metabolism of head and neck squamous cells. These changes include aerobic glycolysis, mitochondrial metabolism, and lipid and amino acid anabolism/catabolism.[49] Viruses are known to modulate cellular processes to facilitate infection.[50] Infection with HPV has been shown to phenocopy cancer-like metabolic changes that are maintained in HPV+ HNSCC.[51,52]

Although this overview is limited and surely incomplete, it gives an impression of the possible confounders of the use of VOCs in the diagnosis of HNSCCs.

There are differences in tumor microenvironment and biology between the subsites of HNSCC, but there are also similarities between these squamous cell carcinomas. They all arise from the squamous epithelia of the head and neck region and are in direct contact with the airways. One can find circulating tumor cells at distant places in the body.[53] In a previous study with Tedlar bags and a laboratory e-nose, we were able to discriminate 26 healthy controls from 36 patients diagnosed with HNSCC with a sensitivity of 90% and specificity of 80%.[18] As previously mentioned, we hypothesized that looking at a VOC pattern instead of the output of one specific VOC could lead to a more reliable method for detecting HNSCC.

Current detection methods of HNSCC

For a general practitioner, detection of a primary HNSCC is no easy task. What triggers most doctors to look for HNSCC are complaints of odynophagia, dysphonia, and dysphagia, especially when these are long-lasting (>6 weeks) and seen in patients using intoxicants such as alcohol and tobacco. This is different in HPV-positive tumors, where smoking and drinking do not seem to contribute much to the development of the tumor. Only the oral cavity can be properly examined by inspection with a head lamp and palpation. But if a tumor is suspected elsewhere in the pharynx or larynx, direct or indirect laryngo-pharyngoscopy (flexible or rigid) has to be performed, as well as a biopsy, if needed. Because these techniques require special equipment and training, they are not usually available in primary health care, and certainly not in developing countries.

Physical examination using flexible transnasal endoscopy has an accuracy of 92% in detecting primary laryngeal HNSCC. When using narrow-band imaging combined with transnasal endoscopy, the accuracy rises to 96%.[54] Table 1 provides an overview of methods of physical assessment of HNSCC.

Table 1. Methods of physical assessment of HNSCC

Method	Advantages	Disadvantages
Flexible transnasal endoscopy	Fast, cheap, limited resources needed.	No biopsy.
Panendoscopy under general anesthesia	No pain, good sight in hypopharynx. Biopsy possible. Better tactile assessment of tumor borders and need for resection.	Complications related to anesthesia or post-anesthesia, risk of bleeding, costs.
In-office panendoscopy	Fast. Direct biopsy. No general anesthesia. Cheap.	Risk of bleeding, required fasting state, discomfort, risk of non-representative biopsy.

The most commonly used imaging modalities for HNSCC diagnosis and staging are ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography/computed tomography (PET/CT). All have particular advantages and disadvantages (table 2).[55]

Table 2. Advantages and disadvatages of different imaging modalities

Imaging	Advantages	Disadvantages
Ultrasound	Easily available, no x-ray exposure, portable, cheap, direct cytology possible.	Operator dependency and experience, not transmitted through bone and air, large lesions are difficult to evaluate completely.
Computed tomography (CT)	Widely available, relatively cheap, quick, easy to perform, reproducible, fewer motion artefacts than an MRI scan, high resolution image acquisition allows high-quality multiplanar reconstructions with superior evaluation of bony structures and calcifications.	Radiation exposure, poorer soft tissue contrast compared to MRI, uses iodinated contrast medium to improve contrast with the risk of contrast-induced nephropathy, artefacts due to dental amalgam or orthopedic material, if present.
Magnetic resonance imaging (MRI)	Good soft tissue contrast, multiplanar scanning, evaluation of blood vessels without giving contrast media, no radiation exposure.	Higher costs, time-consuming, potential artefacts (motion artefacts, flow artefacts, field distortion artefacts due to metal or at air-bone interfaces or with blood products) which can mimic or obscure pathology. Gadolinium-containing contrast agents should be given to evaluate tumors and abscesses.
Positron emission tomography/ computed tomography with fluorine-18-Deoxy-D-Glucose (18FDG PET/CT)	Evaluation of the whole body, lymph node involvement, detection and exclusion of distant metastases.	Uptake is also increased in inflammation, expensive, time-consuming, requires patient to be fasting for 6 hours.

The detection of recurrent HNSCC is more challenging, largely because tumor recurrences and complications of radiotherapy (one of the major modalities used to treat primary HNSCC) can present with identical symptoms. In addition, the clinical picture, the radiological findings, and even histopathology cannot always differentiate between (radiation) necrosis and recurrent tumor. Furthermore, biopsies to detect recurrences are invasive and can cause serious complications like infection, chondritis, failure to heal, and further edema.[56,57] Ideally, diagnostic procedures for recurrent HNSCC would be non-invasive and accurate.

There is demand in both developing and developed countries for a non-invasive screening tool that could offer easy and reliable detection of HNSCC but also of other types of cancer. Both settings have their own challenges and specific needs.

Breath tests might be a particularly promising approach for non-invasive cancer diagnosis and/or screening. The analysis of VOCs in exhaled breath can provide information on pathologic metabolic processes in the body and therefore serve as an appropriate diagnostic tool.

Outline of this thesis

The general aim of this thesis was to study the diagnostic capabilities of e-nose technology in head and neck oncology.

Chapter 1: General introduction

Chapter 2: In this chapter we used e-nose technology in a clinical setting to detect primary HNSCC in general and in different subsites.

Chapter 3: While we were able to diagnose HNSCC with high sensitivity, we wanted to find out if e-nose technology differentiates between patients with HNSCC and bladder cancer, HNSCC and colon cancer, and colon cancer and bladder cancer.

Chapter 4: One of the major challenges in the follow-up of treated HNSCC patients is diagnosing recurrent HNSCC. In this chapter we used e-nose technology in detecting recurrent or second primary HNSCC.

Chapter 5: E-nose technology might be a valuable asset for countries in the developing world. We studied the use of the technique in Sudan, a low-income country in Africa.

Chapter 6: In this chapter we discussed the diagnostic properties of e-nose technology for the detection of lung cancer.

Chapter 7: General discussion

Summary

Impact

Chapter 8: Nederlandse samenvatting

Curriculum Vitae

List of publications

Dankwoord/acknowledgments

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CHAPTER 2

Detecting head and neck squamous carcinoma using a portable handheld electronic nose

van de Goor RMGE, van Hooren MRA, Henatsch D, Kremer B, Kross KW.
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Abstract

INTRODUCTION:
Detecting volatile organic compounds in exhaled breath enables the diagnosis of cancer. We investigated whether a handheld version of an electronic nose is able to discriminate between patients with head and neck squamous cell cancer (HNSCC) and healthy controls.

METHODS:
Ninety-one patients with HNSCC and 72 controls exhaled through an e-nose. An artificial neural network based model was built to separate between HNSCC patients and healthy controls. Additionally, three models were created for separating between the oral, oropharyngeal, and glottic subsites respectively, and healthy controls.

RESULTS:
The results showed a diagnostic accuracy of 72% at a sensitivity of 79%, specificity of 63% and area under the curve (AUC) of 0.75. Results for the subsites showed an AUC of 0.85, 0.82 and 0.83 respectively for oral, oropharyngeal, and glottic HNSCC.

CONCLUSION:
This feasibility study showed that this portable non-invasive diagnostic tool can differentiate between HNSCC patients and healthy controls.

Introduction

Each year, more than 600,000 individuals are diagnosed with head and neck squamous cell carcinoma (HNSCC).[1] These malignancies are associated with high morbidity and mortality rates.[2] Two-thirds of all HNSCC patients are diagnosed with advanced-stage disease at first presentation. Long-term survival rates for advanced HNSCCs are low and have not improved significantly over the last decades.[3] Early diagnosis of HNSCC increases the likelihood of treatment with a single modality, lowers the risk of mortality, decreases medical expenditure, and improves patients' quality of life.[4] For diagnosing HNSCC in an early stage, a reliable and cost-effective screening instrument is required. Furthermore, for implementation in first-line health care and rural areas, the instrument should be portable and easy-to-use. Currently, such a device is not available.

The electronic nose (e-nose) could meet these requirements and be implemented to diagnose HNSCC in an early stage. E-nose technology uses exhaled breath volatile organic compound (VOC) pattern analysis for classification. These VOCs are products of different metabolic processes, including cancer metabolism, that dissolve in the bloodstream and enter the respiratory tract through the alveoli.[5,6] Specific VOCs for HNSCC can be detected with e-nose technology using pattern recognition in which non-specific sensors are combined with machine learning techniques.[7-9] An artificial neural network (ANN) can be trained to classify individual breath patterns resulting into a model for diagnosing head and neck cancer.

Previous studies by our group demonstrated that a laboratory e-nose device operating with Tedlar bags is able to detect head and neck carcinomas, discriminating 36 smokers diagnosed with HNSCC from 26 healthy smokers, at a sensitivity of 90% and specificity of 80%.[8] Using the portable e-nose we found a diagnostic accuracy of 83% when differentiating between follow-up patients with locoregional recurrent or second (or third) primary HNSCC and controls without evidence of disease.[10] We also found that a portable handheld e-nose (without a Tedlar/mylar bag or container) can distinguish between lung cancer patients and a control group of healthy participants.[11] Finally, we showed that e-nose technology has the capability to discriminate between different types of cancers, including HNSCC, lung, bladder and colon cancer.[8,12]

In this follow-up study, the capability of a portable and point-of-care handheld e-nose was investigated to discriminate between HNSCC patients and a control group consisting of patients without a cancer history. If the e-nose is shown to function in a larger population, this study might pave the way for routine use of the device as a diagnostic tool in a regular outpatient setting.

Materials and Methods

Participants

This study was performed in a tertiary care referral hospital (Maastricht University Medical Center) from June 2013 to November 2017. Patients with pathohistological confirmed glottic, oropharyngeal or oral SCC were included as well as patients who visited the ear, nose and throat (ENT) department for benign conditions, here referred to as healthy controls.

Exclusion criteria were age under 18 years, current tracheostomy, having had any treatment for a current tumor, and a history of any other sort of cancer. Furthermore, patients were excluded if they could not complete the full 5 minutes of measurement or if they were unable to endure a nose clip during measurement, which was used to promote oral breathing through the e-nose. Their smoking habits and metabolic fasting state were documented. Non-smoking was defined as no smoking in the previous month. Tumor characteristics and medical history were collected from the clinical records during regular visits at our outpatient department. Side-effects or adverse events during or shortly after measurement were documented. The study protocol was approved by the medical ethics committee (PROTOCOL NUMBER 11407) and was designed in accordance with the declaration of Helsinki. Oral informed consent was obtained from all patients.

Materials

For this study we used four Aeonose™ devices (serial numbers 259, 309, 315, 362), using three micro hotplate metal-oxide sensors (AS-MLV sensors, Applied Sensors GmbH) and a Tenax tube. The combination of sensors and the Tenax tube ensures an optimal detection of the VOCs present, even at low concentration levels. The hotplates are periodically heated and cooled between 260 and 340 °C in 32 steps. During this process, exhaled air passes the sensors. The reduction and oxidation (redox) reactions of VOCs at the surface of the metal-oxide sensors cause changes in conductivity of the sensors. The conductivity values recorded represent a unique exhaled-breath pattern that can be analyzed.

Study design and participants

Both treatment and prognosis of HNSCC are dependent on the t-stage and subsite of the tumor. The most common subsites of head and neck carcinomas are the oral cavity, oropharynx and larynx. Based on these characteristics, four different models were created.[13] The first included healthy controls that were compared to patients with HNSCCs of all subsites. Models 2, 3 and 4 consisted of healthy controls and patients with HNSCCs of the oral cavity, oropharynx and glottis, respectively.

Before each measurement, patients were instructed to inhale and exhale into the e-nose for 5 minutes through a disposable mouthpiece. This mouthpiece contains a high-efficiency particulate arrestance (HEPA) filter, which protects the device to a large extent from contamination, e.g., from bacteria and viruses. Patients were instructed to close their lips over the mouthpiece at all times, and a nose clip was used to prevent nasal air passage. Test runs of in- and exhalations were performed so the patient could get acquainted with the device. Participants breathed through a carbon filter to limit the possibility that environmental VOCs would tamper with the measurement. For the first 2 minutes, the lungs were rinsed with clean filtered air that passed through the carbon filter without passing the sensors, and dead air space was removed. Afterwards, a valve was opened to ensure the passage of exhaled air over the sensors. The total measurement cycle lasted about 15 minutes, of which the patient in- and exhaled into the device for 5 minutes. The remaining time was used to measure any low-concentrated VOCs inside the Tenax tube and to regenerate the sensors with clean filtered air (for details see van Hooren et al. 2016).[12]

Patients did not receive individual diagnostic results from the e-nose analysis. The results from these measurements did not influence the regular diagnostic work-up or treatment of the participants.

Statistical analysis

Baseline group differences were determined using the independent sample *t* test, Fisher's exact test or Mann-Whitney *U* test. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.).

During one measurement, 64 times 36 data points were recorded for each sensor. To compress these data points of temperature, measurement cycle and sensors, a Tucker3-solution for tensor decomposition was used.[14] The resulting vectors combined with the classification (benign of malignant) were used to train an Artificial Neural Network (ANN). Data compression and ANN have been integrated in a proprietary software package (Aethena – the eNose Company, Zutphen, the Netherlands). ANN training was executed for a number of data scaling options, resulting in multiple ANN options for separating between benign and malignant conditions. Data was cross-validated using the Leave-10%-Out method. This method prevents to a large extent the fitting of data on artefacts instead of breath-profile classifiers.

We required at least 5 patients with HNSCC and 5 healthy controls to be measured per e-nose device to eliminate possible device dependencies.

The ANN model calculates a value between -1 and 1 for each breath pattern, related to the diagnosis of that patient. For each model a threshold (range -1 to 1) was determined by the ANN to obtain the best possible diagnostic accuracy. Individual predicted values above this threshold were classified as positive, and values below

this threshold were classified as negative. A Receiver-Operator-Curve is produced showing the performance of the model. By picking a position on this curve, a threshold is chosen for separating between positive and negative classified patients. In this way, data on sensitivity, specificity, area under the curve (AUC) and overall accuracy are obtained for each model.

Results

Baseline characteristics

Between May 2013 and October 2017, 72 healthy controls and 91 patients with primary HNSCC originating from the oral cavity (37), oropharynx (34) and glottis (20) were included. Baseline characteristics, shown in table 1, were comparable between both groups, except for 'currently smoking', which was significantly higher in patients with primary HNSCC. During this study, no adverse events were reported when using the Aeonose™.

Table 1. Baseline characteristics Model 1

	Healthy controls	All subsites
Number of patients	72	91
Age (mean years) *	63	64 (p= 0.401) *
Sex (male) †	57	68 (p=0.578) †
Currently smoking (yes) †	26	49 (p=0,027) †
Pack years (mean)#	32	29 (p= 0.915) *
Tumor stage (n)		
1	\	16
2	\	22
3	\	12
4	\	41

*Independent *t*-test
† Fisher's exact test
# Mann-Whitney *U* test

Data analysis

The ANN for model 1 calculated a value between -1 and 1 for each patient and the threshold value for the highest diagnostic accuracy was calculated and set at 0.07. This means that a patient with a value below 0.07 was marked as negative for HNSCC and with a value above 0.07 as positive for HNSCC. In this way, seventy-two out of 91 HNSCC patients were correctly diagnosed with HNSCC, irrespective of tumor origin. Out of the 72 healthy controls, 45 were classified as healthy (figure 1). This resulted in a diagnostic accuracy of 72%, sensitivity of 79% and specificity 63% for model 1.

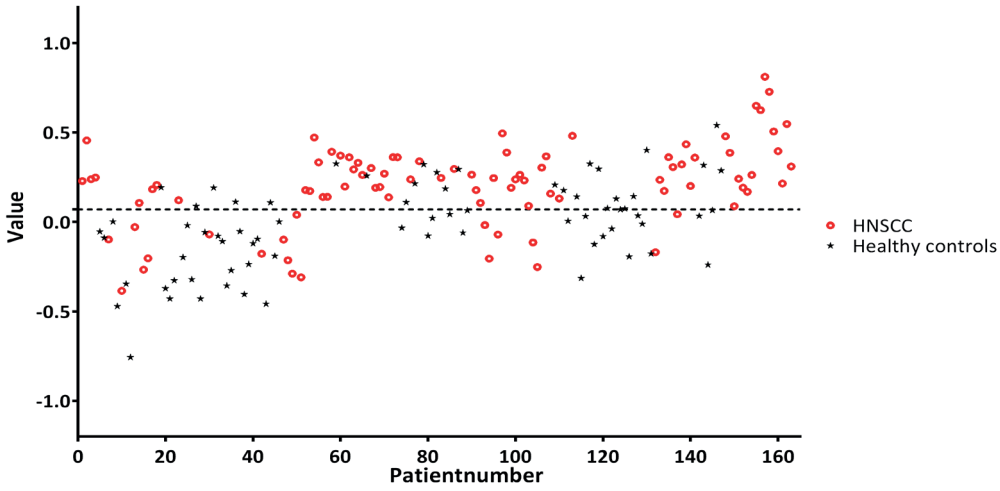

Figure 1. Healthy controls vs HNSCC of all subsites (model 1). The individual value of each patient and control calculated by the ANN is displayed. Values >0.07 are considered as positive for HNSCC. Red circles are patients with histopathologically confirmed HNSCC, and black asterisks represent healthy controls. ANN, artificial neural network; HNSCC, head and neck squamous cell cancer.

The thresholds for models 2, 3 and 4 were set at -0.07, -0.27 and -0.65 respectively. Analysis revealed that the sensitivity, specificity and AUC of the models 2 (oral cavity), 3 (oropharynx) and 4 (glottis) are all higher compared to model 1 (figure 2).

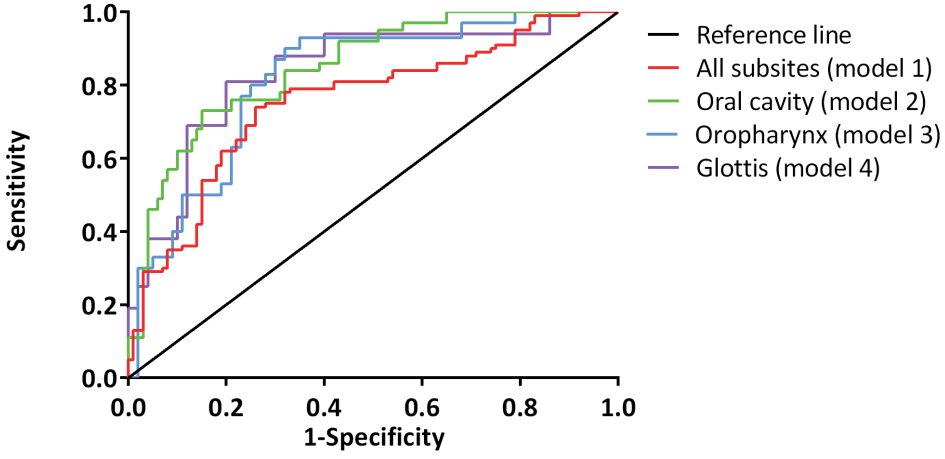

Figure 2. The ROC curve for each model. Black line represents the line of no-discrimination.

The sensitivity, specificity, overall accuracy and area under the curve of each model are shown in table 2.

Table 2. The sensitivity, specificity, overall accuracy and area under the curve of each model

	Model 1	Model 2	Model 3	Model 4
Sensitivity	79%	84%	87%	81%
Specificity	63%	67%	68%	76%
Accuracy	72%	72%	75%	77%
AUC	0.75	0.85	0.82	0.83

Model 1 (Healthy controls vs all HNSCC patients);
Model 2 (Healthy controls vs HNSCC subsite oral cavity);
Model 3 (Healthy controls vs HNSCC subsite oropharynx);
Model 4 (Healthy controls vs HNSCC subsite glottis).
AUC; area under the curve.

Discussion and conclusion

In this study, we investigated the ability of the portable e-nose to discriminate between patients diagnosed with HNSCC and healthy controls visiting our outpatient clinic for other benign diseases. We showed that the e-nose is capable to distinguish between HNSCC patients, including all subsites, and healthy controls with a diagnostic accuracy of 72%. When analyzing the different subsites of HNSCC, the sensitivity and specificity increases. This is probably due to the fact that the separate groups are more homogeneously than the combined one.

In recent years, investigations into the use of VOCs as potential biomarkers for head and neck cancer have drawn interest.[7] Most of these studies have used gas chromatography-mass spectrometry (GC-MS), a technique that detects individual VOCs based on their molecular weight. The disadvantages of GC-MS are the high costs, the need for specialized personnel to perform the analysis, and the need for an appropriate set of specific biomarkers for HNSCC. A variety of VOCs such as ethanol, 2-propenenitrile and undecane dodecane, decanal, benzaldehyde, 3,7-dimethyl undecane, 4,5-dimethyl nonane, 1-octene, and hexadecane have been described as potential biomarkers for the diagnosis of HNSCC.[7,15] Since GC-MS relies on the detection of one single biomarker, this is a major limitation for the use of GC-MS as a reliable screening instrument in the clinical setting. Bouza et al. described elevated concentrations of benzaldehyde, 3,7-dimethylundecane, and butyl acetate, measured by GC-MS with Tedlar bags, and proposed considering these VOCs as potential biomarkers for oral SCC. Interestingly, they found that a higher concentration of butyl acetate was significantly correlated with a higher histological degree of differentiation. A disadvantage of their study is that, before sample collection, subjects were asked to abstain from food and drink (except water) and asked not to smoke in the 6 hours before sample collection.[15] Garcia et al. reported 7 possible VOCs as biomarkers for laryngeal cancer: ethanol, 2-Butanone, 2,3-butanediol, 9-tetradecen-1-ol, octane derivative compound, cycloheptane derivative compound, and cyclononane derivative compound. In that study patients had to follow a strict protocol

and were not allowed to eat, drink or smoke 8 hours prior to testing. The technique they used was a combination of solid phase micro-extraction (SPME) with GC-MS.[16]

Our results show that the e-nose can discriminate between the different subsites of HNSCC, suggesting that each subsite of HNSCC has a different VOC profile. These differences between subsites have also been shown in a study by Gruber et al. Using an array of 6 nanomaterial-based sensors combined with discriminant factor analysis (DFA), they found an accuracy of 83%, sensitivity of 77% and specificity of 90% when comparing HNSCC patients with healthy controls. Even more interestingly, they found a sensitivity of 100% and specificity of 91% when comparing laryngeal squamous cell carcinoma with pharyngeal squamous cell carcinoma.[7] A major difference with our study is that they used Tedlar bags for breath sampling and they did not allow the patients to eat, drink alcohol or smoke in the 12 hours prior to the measurement. That protocol might not be suitable for every patient visiting the care facility.

The e-nose (Aeonose™) as applied in this study is a handheld, fast, easy-to-use and portable device. In the future, the e-nose might be incorporated in first-line healthcare or used as a screening instrument, for instance in developing countries. We did not perform a special hygienic protocol that interrupted the daily routine of the patient, which could improve compliance. The device is able to connect to the internet via WIFI and can run an unlimited number of validated models with only one measurement. Therefore, a patient at risk for HNSCC might be tested for glottic, oropharyngeal or oral cancer consecutively with models 2 to 4. Due to accurate temperature control of the sensors, these models can be easily transferred to an unlimited number of electronic noses. For the first time, mass application of electronic noses will be possible.[17]

Conclusion

We have shown that the e-nose could be a promising diagnostic tool for detecting HNSCC, particularly when specific models are used for different subsites. An interesting application area could be in first-line healthcare. Further investigation is warranted, notably of stage I and II tumors and larger groups of patients to allow modelling for each subsite.

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CHAPTER 3

Feasibility of electronic nose technology for discriminating between head and neck, bladder, and colon carcinomas

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Abstract

HYPOTHESIS and OBJECTIVE:
Electronic nose (e-nose) technology has the potential to detect cancer at an early stage and can differentiate between cancer origins. Our objective was to compare patients who had head and neck squamous cell carcinoma (HNSCC) with patients who had colon or bladder cancer in order to determine the distinctive diagnostic characteristics of the e-nose.

STUDY DESIGN:
Feasibility study. An e-nose device was used to collect samples of exhaled breath from patients who had HNSCC and patients who had bladder or colon cancer, after which the samples were analyzed and compared.

METHODS:
One-hundred patients with HNSCC, forty patients with bladder cancer, and twenty-eight patients with colon cancer exhaled through an e-nose for five minutes. An artificial neural network was used for the analysis, and double cross-validation was used to validate the model.

RESULTS:
In differentiating HNSCC from colon cancer, a diagnostic accuracy of 81% was found. When comparing HNSCC with bladder cancer, the diagnostic accuracy was 84%. A diagnostic accuracy of 84% was found between bladder cancer and colon cancer.

CONCLUSIONS:
The e-nose technique using double cross-validation is able to discriminate between HNSCC and colon cancer and between HNSCC and bladder cancer. Furthermore, the e-nose technique can distinguish colon cancer from bladder cancer.

Introduction

The leading cause of death worldwide is cancer, with about 14.1 million new cases and 8.2 million deaths in 2012.[1] The number of new cases is expected to rise to 22 million within the next two decades.[2] Only early detection and treatment can reduce the mortality rate. [3] That requires a quick, reliable, non-invasive, and inexpensive way to screen for cancer so that treatment might start at the earliest possible stage of the disease. Early diagnosis could lead to better radical treatment, less loss of function, and a higher survival rate.

Exhaled human breath contains hundreds of volatile organic compounds (VOCs) that can be detected by gas chromatography and mass spectrometry (GC-MS) on the compound level and by pattern recognition with Electronic Nose (e-nose) technology. There are three types of exhaled VOCs. Local VOCs arise directly in the alveoli or the airway lumen along the respiratory tract. Exogenous VOCs are 'inhaled' or absorbed through the skin. And some VOCs, originating from metabolic processes in the body, dissolve in the blood, subsequently exit the circulation, and enter the respiratory tract through the alveoli.[4]

Applications of e-nose technology are common in the food and beverage industry, in monitoring of air quality, and in the detection of explosive and chemical agents.[5] The interaction of VOCs with an array of partial selective chemical sensors (equivalent to the olfactory receptors in the human nose) results in a change in the resistance or conductance of the sensors. That change is transmitted to a processor. E-nose technology also has many healthcare applications; among others, it is used for diagnosing colon cancer, chronic obstructive pulmonary disease (COPD), asthma, lung cancer, and head and neck cancer.[6-10]

Recently, van Hooren et al. 2016 reported that the e-nose is able to discriminate between Head and Neck Squamous Cell Carcinoma (HNSCC) and lung cancer.[11] That study used a hand-held device with metal-oxide sensors that are periodically heated when processing the breath sample. Oxidation or reduction of the VOCs present in the breath sample is measured while the resistance changes as a function of temperature and time. Van Hooren et al. showed that e-nose technology, which uses VOC pattern recognition, was able to differentiate between HNSCC and lung cancer. Both types occur in the respiratory tract and share common risk factors such as smoking and male gender.[12]

To our knowledge, no studies have been published on the use of e-nose technology to differentiate between HNSCC and bladder or colon cancer by means of VOC pattern recognition. Crucially, if a tool is used to screen for primary malignancies, it should be able to differentiate between tumors of different types in different compartments of the human body. Moreover, no other studies on this topic have described the double cross-validation model. That is a strategy to optimize the complexity of regression models and make a realistic estimate of prediction errors when the model is applied to new cases.

Against that backdrop, the main objective of the present study is to determine whether the e-nose technique is able to discriminate between HNSCC and bladder or colon cancer using double cross-validation. The secondary objective is to investigate whether the E-nose is able to discriminate between colon and bladder cancer. As such, the e-nose has potential in healthcare as a screening tool for different origins of cancer.

Materials & Methods

Patients

This study was conducted in the Netherlands at a tertiary care referral hospital (Maastricht University Medical Center). It included patients with primary HNSCC originating from the oral cavity, pharynx, larynx, hypopharynx, nasopharynx, or nasal cavity. Also included were patients with primary cancer of the bladder or colon. The study protocol was approved by the medical ethics committee and was carried out in accordance with the declaration of Helsinki.

The exclusion criteria were age under 18 years, tracheostomy, any treatment for current tumor, and a history of cancer. Patients initially enrolled were subsequently excluded when they did not or could not complete the 5-minute measurement session or were unable to endure a nose clip. Malignancies of the salivary glands were also grounds for exclusion. The participants' smoking habits and metabolic fasting state were documented. The latter was defined as no food or drink four hours before the session, except for two units of non-caloric clear liquid two hours prior to measurement. Smoking was defined as smoking in the previous month. Tumor characteristics and medical history were collected from the clinical records. For tumor staging WHO classifications were used. Carcinomas in situ and non-invasive papillary bladder carcinomas were noted as stage 0 tumors. Any side or adverse effects during or shortly after measurement were documented. Informed consent was obtained from all patients.

Study design

To acquaint the patients with the device, they received instructions for a test run of in- and exhalations. After the instructions, all patients were asked to in- and exhale through the e-nose for five minutes. A clip was placed on the nose to prevent entry of non-filtered air. Patients were instructed to enclose the lips by the mouthpiece at all times.

E-nose readings were synchronous with the regular diagnostic work-up. Participants were not given any diagnostic information derived from their individual e-nose results. The routine diagnostic work-up was based on national cancer guidelines and was independent of e-nose measurements. The e-nose outcomes were compared with histopathology from biopsies.

Materials

The device used in this study (Aeonose; the eNose Company, Zutphen, the Netherlands) consists of three different micro-hotplate metal-oxide sensors (AS-MLV sensors; Applied Sensors GmbH.). During the measurement the hotplates are periodically heated and cooled between 260 and 340 °C in 32 steps during which the sensors are exposed to the exhaled breath. The reduction and oxidation (redox) reactions of the VOCs on the metal-oxide surface affect the conductivity of the sensors. Over time, these changes create a unique pattern of redox reactions. (See van Hooren et al. 2016 for more details on the method).[11] The measurements were performed with five Aeonoses (serial numbers 259, 309, 315, 362, and 379) in order to exclude possible machine-bound confounding factors.

Statistical analysis

Differences in baseline characteristics were determined with the independent sample *t* test, Fisher's exact test, or Pearson's chi-square test. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp.; Armonk, NY).

Each e-nose measurement generates 64 (temperature values) times 36 (measurement cycles) times 3 (sensor) data points, forming a multi-way dataset consisting of conductivity values. After preprocessing, the data are compressed using a TUCKER3 solution for tensor decomposition. The vectors representing the coded patient information are subsequently used to train an artificial neural network (ANN). This training is carried out for a number of data scaling options, yielding different models for separating 'HNSCC' from 'colon or bladder cancer' patients. Data compression and ANN have been integrated in a proprietary software package (Aethena) of the eNose company (Zutphen, the Netherlands). The binary results are presented in a scatter plot and a receiver operating characteristic curve (ROC curve). Matthews Correlation Coefficients (MCC) were calculated to determine the quality of the binary classifications, and 95% confidence intervals (CI) were given.

The data were labeled with the diagnosis of HNSCC, or colon cancer, or bladder cancer when processed in Aethena. Optimal results were obtained by combining multiple ANNs in the following sequence. First, one ANN separated all data into a positive and a negative group. Then each group was judged by three different ANNs, generating the average value of the ANN classifications (judge model). In order to calculate sensitivity, specificity, and overall accuracy for future, yet undefined breath samples, double cross-validation was performed. Using brute (computing) force, the optimal combination of available ANNs was determined. Double cross-validation ensures that comparable results can be expected when submitting blind data into the trained ANN.

Results

One hundred and sixty-eight patients were included in this study. They had histopathological proven HNSCC (N=100), bladder cancer (N=40), or colon cancer

(N=28). The tumor sites of the HNSCC patients were the oral cavity (N=28), oropharynx (N=23), nasopharynx/nasal cavity (N=4), hypopharynx (N=11), and larynx (N=34). All HNSCC patients were diagnosed with squamous cell carcinoma (including three patients with squamous cell carcinoma in situ). In table 1 the TNM stadiums of all HNSSC patients are shown.

Table 1. TNM staging of HNSCC patients

	0	CIS	1	2	3	4
T	/	3	28	30	19	20
N	60	/	13	25	2	/
M	95	/	5	/	/	/

HNSCC and colon cancer

The baseline characteristics of the HNSCC vs. colon cancer patients are listed in Table 2. There are several baseline differences between the two groups: age (p = 0.024), currently smoking (p = 0.000), packyears (p = 0.002), and tumor stage (p = 0.018). Of the 28 patients with colon cancer, 27 had an adenocarcinoma and one had a neuroendocrine carcinoma.

Table 2. Baseline characteristics HNSCC and colon cancer

	HNSCC	Colon	*p*-value	Test
Number of patients	100	28		
Age (years)	64	69	0.024´	†
Sex (male)	74	18	0.209	‡
Food intake <4 hours (“Yes”)	23	4	0.162	‡
Currently smoking	57	4	0.000´	‡
Packyears	34	17	0.002´	†
Aeonose serial number			0.276	*
259	27	6		
309	18	2		
315	18	10		
362	18	5		
379	19	5		
Tumor stage			0.018´	*
0	3	0		
1	26	6		
2	20	8		
3	16	11		
4	35	3		

* Pearson Chi-square
´ significant
‡ Fisher’s exact test
† Independent *t*-test

HNSCC and bladder cancer

The baseline characteristics of the head and neck vs. bladder cancer patients are listed in Table 3. Several baseline differences were found: age (p = 0.020), food intake (p = 0.038), smoking (p = 0.002) and tumor stage (p = 0.000). There were 24 patients with a stage 0 tumor. Four patients had a carcinoma in situ, respectively three with HNSCC and one bladder cancer patient. The remaining 20 patients had non-invasive papillary bladder carcinomas.

Table 3. Baseline characteristics HNSCC and bladder cancer

	HNSCC	Bladder	*p*-value	Test
Number of patients	100	40		
Age (years)	64	68	0.020 ´	†
Sex (male)	74	28	0.555	‡
Food intake <4 hours (“Yes”)	64	23	0.038 ´	‡
Currently smoking	57	10	0.002 ´	‡
Packyears	37	27	0.076	†
Aeonose serial number			0.223	*
259	27	5		
309	18	5		
315	18	8		
362	18	13		
379	19	9		
Tumor stage			0.000 ´	*
0	3	21		
1	26	6		
2	20	9		
3	16	2		
4	35	2		

* Pearson Chi-square
´ significant
‡ Fisher’s exact test
† Independent t-test

Bladder cancer and colon cancer

Only one significant difference in baseline characteristic was found: tumor stage (p = 0.000). The data are presented in Table 4.

Table 4. Baseline characteristics colon cancer and bladder cancer

	Colon	Bladder	*p*-value	Test
Number of patients	28	40		
Age (years)	68	69	0.535	†
Sex (male)	18	30	0.246	‡
Food intake <4 hours ("Yes")	23	24	0.065	‡
Currently smoking	4	11	0.160	‡
Packyears	40	28	0.106	†
Aeonose serial number			0.365	*
259	6	5		
309	2	5		
315	10	8		
362	5	13		
379	5	9		
Tumor stage			0.000´	*
0	0	21		
1	6	6		
2	8	9		
3	11	2		
4	3	2		

* Pearson Chi-square
´ significant
‡ Fisher's exact test
† Independent t-test

Data analysis

HNSCC and colon cancer

Figure 1 is a scatter plot of individual predictive values with a best fit of the data analyzed by the ANN. To obtain the best possible diagnostic accuracy of the data, the threshold was set to 0.00. This resulted in a sensitivity of 79% and specificity of 81%, with an overall accuracy of 81% (MCC: 0.56) in differentiating between colon cancer and HNSCC. Cross-validation data are shown in Figure 2.

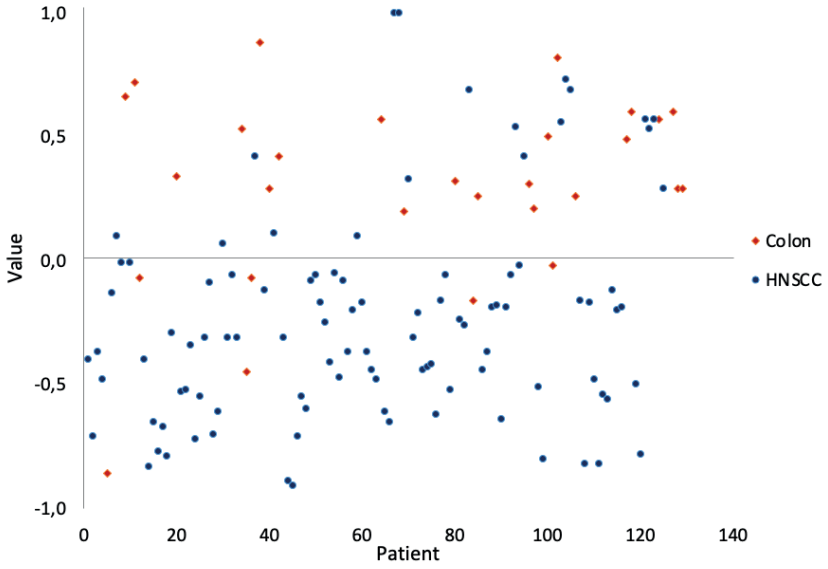

Figure 1. The individual E-nose value of each patient are plotted. Values > 0 are scored as being positive for Colon cancer. Values < 0 are scored as being positive for HNSCC. The Red dots are patients with histopathologic confirmed Colon cancer and the blue dots represent patients with histopathologic confirmed HNSCC cancer.

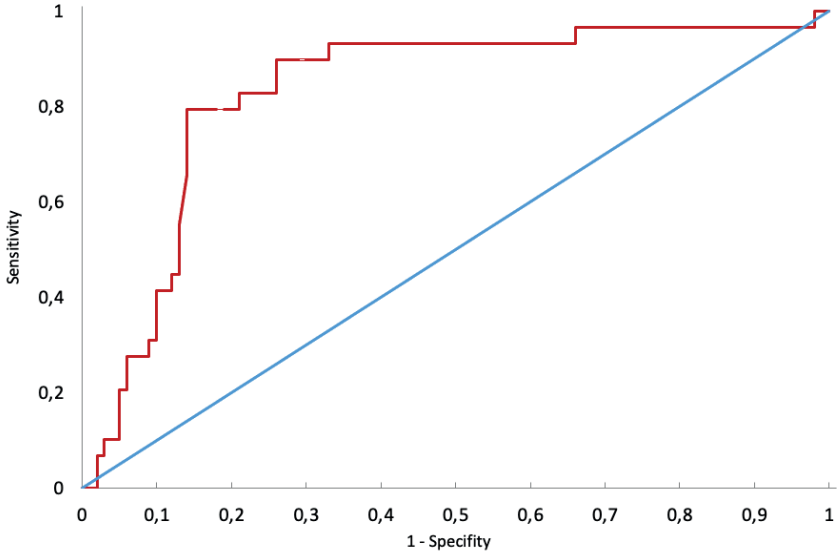

Figure 2. ROC curve of HNSCC and colon cancer. The ROC-curve illustrates the different sensitivities and specificities with altered thresholds of both the best fit of the data for double cross-validation (red line). The Blue line represents the line of no-discrimination. The area under the curve (AUC) is 0.83 (95% CI 0.74-0.92)

HNSCC and bladder cancer

Figure 3 is a scatter plot of individual predictive values with a best fit of the data analyzed by the ANN. To obtain the best possible diagnostic accuracy, the threshold was set to 0.00. The sensitivity was 80% and specificity was 86%, at an overall accuracy of 84% (MCC: 0.66) in differentiating between colon carcinoma and HNSCC. Cross-validation data are given in Figure 4.

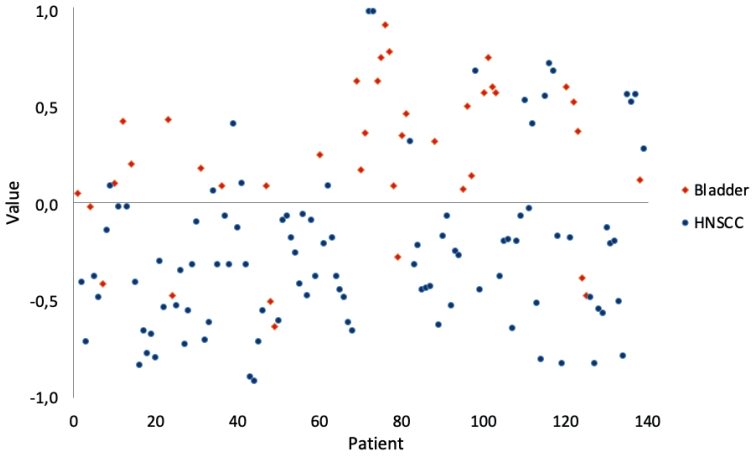

Figure 3. The individual E-nose value of each patient. Values > 0 are scored as being positive for Bladder cancer. Values < 0 are scored as being positive for HNSCC. The Red dots are patients with histopathologic confirmed bladder cancer and the blue dots represent patients with histopathologic confirmed HNSCC.

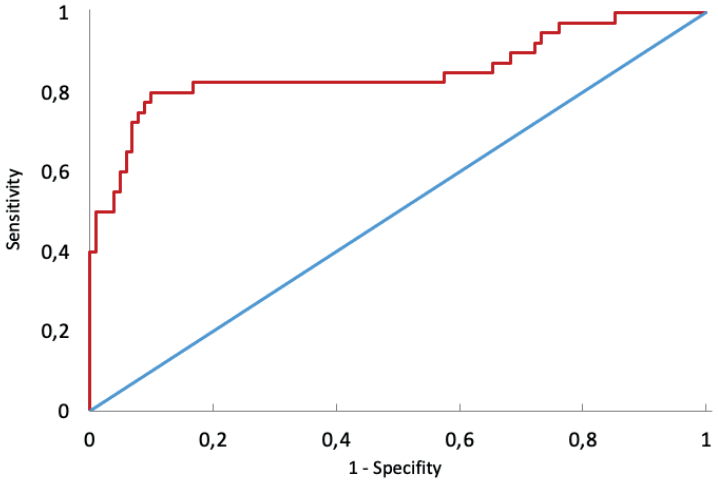

Figure 4. ROC curve of HNSCC and bladder cancer. The ROC-curve illustrates the different sensitivities and specificities with altered thresholds of both the best fit of the data for double cross-validation (red line). Blue line represents the line of no-discrimination. The area under the curve (AUC) is 0.85 (95% CI 0,76-0,94)

Bladder cancer and colon cancer

The scatter plot in Figure 5 displays the individual predictive values with a best fit of the data analyzed by the ANN. For the best possible diagnostic accuracy, the threshold was set to 0.00. This resulted in a sensitivity of 88% and specificity of 79%, and an overall accuracy of 84% (MCC: 0.69) in differentiating between colon carcinoma and bladder carcinoma. Cross-validation data are shown in Figure 6.

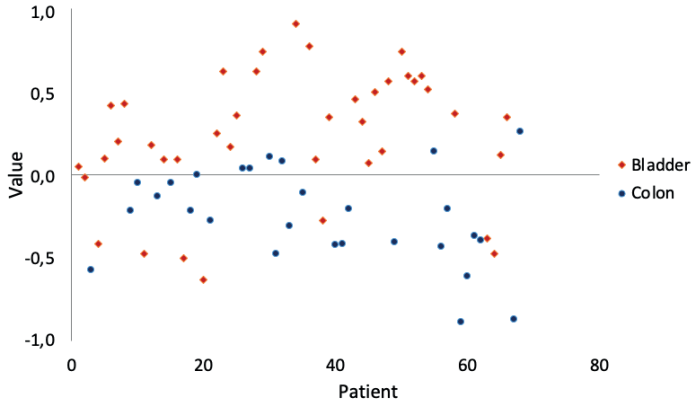

Figure 5. The individual E-nose value of each patient. Values > 0 are scored as being positive for Bladder cancer. Values <0 are scored as being positive for Colon cancer. The Red dots are patients with histopathologic confirmed bladder cancer and the blue dots represent patients with histopathologic confirmed Colon cancer.

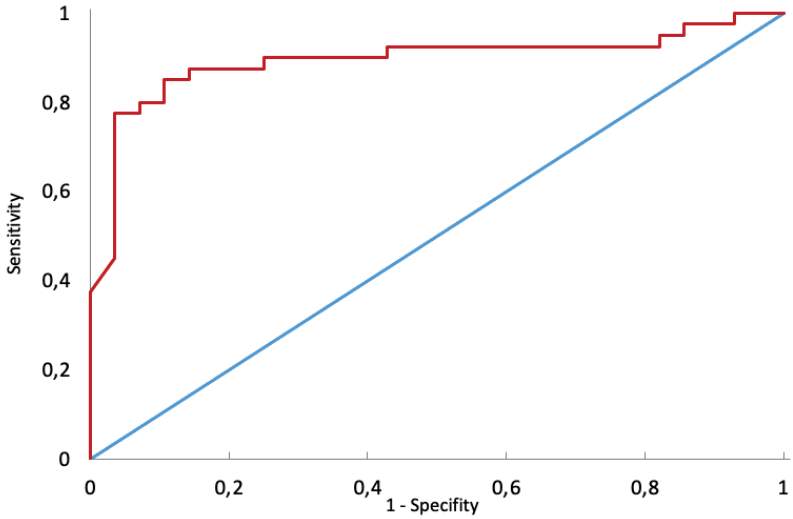

Figure 6. ROC curve of bladder and colon cancer. The ROC-curve illustrates the different sensitivities and specificities with altered thresholds of both the best fit of the data (blue line) as the double cross-validation (red line). The area under the curve (AUC) is 0.90(95% CI 0,81-0,98)

Discussion

In this feasibility study, the breath VOC patterns of patients with HNSCC were compared to the patterns of patients with colon or bladder cancer. Our results show that breath VOC pattern analysis with the e-nose is feasible. The technique exhibits a reasonable degree of sensitivity and specificity for double cross-validation when comparing HNSCC with colon cancer or bladder cancer.

Interest in the use of VOCs in diagnosing primary carcinomas has been growing. Meij et al.[6] tested 157 stool samples (40 patients with colon cancer, 60 patients with advanced adenomas, and 57 healthy controls). They found that the VOC profiles of patients with colon cancer differed significantly from those of controls without cancer (AUC 0.92, sensitivity 85% and specificity 87%). Amel et al.[13] evaluated breath VOC pattern analysis by testing 65 patients with colon cancer and 122 healthy controls. Their sensor analysis distinguished colon cancer from the healthy control group with 85% sensitivity, 94% specificity, and 91% accuracy. Comparing HNSCC patients with healthy subjects, Gruber et al.[14] used an e-nose to analyze breath samples of 22 patients with malignant larynx or pharynx tumors, twenty-one healthy controls. They were able to distinguish HNSCC patients from healthy controls as well as from individuals with benign tumors at a sensitivity of 77%, specificity of 90%, and overall accuracy of 83%. Our group used an e-nose to evaluate VOC patterns in exhaled breath of 36 HNSCC patients and 23 patients without malignant disease and found 90% sensitivity and 80% specificity in diagnosing HNSCC.[10] Using pattern recognition and principal component analysis (PCA), Peng et al.[15] showed that an e-nose can distinguish different tumors in different tracts (lung, colon, breast, prostate). Against that background, the innovative aspect of the present study is that double cross-validation is shown to improve the diagnostic accuracy when the generated Judge model is applied to new cases. Furthermore, this generated model can be translated to different Aeonose device.

Double cross-validation showed a sensitivity of 79% and specificity of 81% when HNSCC was compared with colon cancer using breath samples and the e-nose. When comparing HSCNN with bladder cancer, this study found a sensitivity of 80% and specificity of 86%. And it showed a sensitivity of 88% and specificity of 79% when comparing bladder cancer with colon cancer.

In the past decade, diagnosis of primary cancers with VOCs has shown promising results. Among the various methods to analyze VOCs, one uses GC-MS and is able to identify specific volatile organic compounds for diseases of interest. However, this method has some disadvantages: cost, its time-consuming procedure; and the need for well trained personnel to collect and analyze the samples. Furthermore, the identification of detected compounds is not straightforward; reference libraries have to be checked and validated using the mass and retention time of known standards.

Another method, the one used in this study, is e-nose technology, which is based on pattern recognition. The e-nose needs to be 'trained' to build a database for recognition, after which it can be used to classify blind samples. The crucial factors of meaningful pattern recognition are the size of the training set and representativeness of the sample for the populations to be tested. The advantage of the e-nose used in this study (Aeonose) is that it is a portable hand-held device, making it easily applicable in an outpatient setting. Furthermore, the method is quick and fairly cheap.

Limitations

The design of this feasibility study entailed some limitations; therefore, some caution should be taken when interpreting our results. First, there were significant baseline differences in both the HNSCC vs. colon cancer analysis and the HNSCC vs. bladder cancer analysis. These differences reflect the clinical setting: the majority of patients with HNSCC are smokers with an advanced tumor stage at first presentation.[16] Second, none of the patients with bladder or colon carcinoma had received a panendoscopy or any other diagnostic procedure to exclude HNSCC, as no clinical symptoms were present at the time of sample collection.

Conclusion

The e-nose technique, using double cross-validation, is able to discriminate between HNSCC and colon cancer (sensitivity 79%, specificity 81%) and between HNSCC and bladder cancer (sensitivity 80%, specificity 86%). Furthermore, the e-nose can distinguish colon cancer from bladder cancer (sensitivity 88%, specificity 79%). Large, preferably blinded studies should be conducted to determine the role that the e-nose could play as a diagnostic tool in primary cancer diagnostics and management.

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CHAPTER 4

Detecting recurrent head and neck cancer using electronic nose technology: A feasibility study

van de Goor RMGE, Hardy JCA, van Hooren MRA, Kremer B, Kross KW.
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Abstract

BACKGROUND:
The aim of this feasibility study was to assess the diagnostic performance of an electronic nose (e-nose) as a non-invasive diagnostic tool in detecting locoregional recurrent and/or second (or third) primary head and neck squamous cell carcinoma (HNSCC) after curative treatment.

METHODS:
Using an e-nose (Aeonose™, The eNose Company, Zutphen, The Netherlands), breath samples were collected from patients after curative treatment of an HNSCC with a locoregional recurrence or second (or third) primary tumor (N= 20), and from patients without evidence of recurrent disease (N= 20). Analyses were performed utilizing artificial neural networking based on patterns of volatile organic compounds.

RESULTS:
A diagnostic accuracy of 83% was observed in differentiating follow-up patients with locoregional recurrent or second (or third) primary HNSCC from those without evidence of disease.

CONCLUSION:
This study has demonstrated the feasibility of using an e-nose to detect locoregional recurrent and/or second (or third) primary HNSCC.

Introduction

Head and neck cancer (HNC) has a major impact on global health, being the seventh most common cancer worldwide.[1] The vast majority of head and neck cancers are squamous cell carcinomas (HNSCC). Despite recent advances in diagnostic approaches and treatment modalities for HNC, the 5-year survival rates have improved only marginally in the past decades.[2] In part, this can be attributed to the high rates of locoregional recurrence and second primary HNSCC. Several authors report a 10% to 50% rate of recurrence,[3,4] approximately 75% of which found within 2 years after curative treatment. The probability of developing a second primary HNSCC within 5 years after initial diagnosis is approximately 20%.[5,6]

Diagnosing locoregional recurrence and/or second primary HNSCC has proven to be challenging. Tumor recurrence and complications following radiotherapy, one of the main (adjuvant) treatment modalities, often present with identical clinical symptoms. Furthermore, oncological treatment often severely alters both anatomy and physiology. Consequently, it is difficult to assess the indication for an endoscopic procedure solely by clinical examination and diagnostic imaging. In addition, pathologic differentiation between (radio) necrosis and tumor can be difficult. As a result, less than half of endoscopic procedures correctly differentiate a recurrent HNSCC from post-irradiation complications at first attempt, with relatively high rates of false-negative biopsies.[7,8] It is therefore necessary to improve the diagnostic approach for previously treated HNSCC patients with suspected locoregional recurrence and/or second primary HNSCC. The need to develop new strategies is urgent, in that early diagnosis could lead to higher survival rates and fewer futile endoscopic procedures under general anesthesia.

A promising diagnostic and screening tool for this purpose is volatile organic compounds (VOCs) analysis. VOCs are gaseous products of both physiological and pathological processes in the human body. Disease is often associated with altered metabolism, resulting in a modified VOC output with a distinctive fingerprint.[9] Several techniques have been used to assess VOCs. One combines gas chromatography with mass spectrometry (GC-MS); another, called electronic nose technology (e-nose), is based on pattern recognition rather than component identification.[10] VOCs have been found in faeces, urine, headspace of micro-organism cultures, and exhaled breath. The compounds have been associated with respiratory,[11] urogenital,[12] and neurological disease,[13] as well as with malignancies of lung,[14,15] colorectal,[14,16] and head and neck origin.[14,17-20]

The present study used an Aeonose™ (The eNose Company, Zutphen, the Netherlands), a low-cost, rapid, portable, handheld and non-invasive diagnostic tool for VOC pattern recognition in breath samples. Using this device, our group illustrated the ability of an e-nose to differentiate healthy patients from patients with primary HNSCC[18] and lung carcinoma,[21] and to discriminate patients with primary

HNSCC from those with bladder cancer, colon carcinoma, and lung carcinoma.[14,19] To our knowledge, no other studies have been performed to investigate the use of e-nose technology in diagnosing recurrent and/or second primary HNSCC.

The aim of this feasibility study was to determine the diagnostic performance of an e-nose as a non-invasive diagnostic tool in detecting locoregional recurrent and/or second (or third) primary HNSCC after prior curative treatment.

Materials and methods

Participants

This study was conducted at a tertiary care referral hospital: the Maastricht University Medical Center (MUMC). Patients with histopathologically or cytologically confirmed locoregional recurrent and/or second (or third) primary HNSCC were included along with a follow-up group (i.e., patients who had previously been curatively treated for HNSCC and showed no evidence of recurrent disease). All enrolled patients without evidence of recurrent HNSCC at the time of e-nose measurement remained tumor-free at least up till the end of the study, with a minimal follow-up duration of 6 months. Only patients with HNSCC originating from the oral cavity, oropharynx, hypopharynx, or larynx were included. Locoregional recurrence was defined as a newly diagnosed HNSCC at a distance of less than 2 centimeters from the primary tumor site or in an adjacent regional lymph node after a disease-free period of 6 months or more but less than 2 years. A tumor arising farther than 2 centimeters from a primary tumor site or after a disease-free period of more than 2 years was considered as a second primary HNSCC. A tumor arising less than 6 months after curatively intended treatment was considered as residual disease and therefore such patients were not included in the present study.[22] This study protocol is approved by the METC. Project registration number 11407. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Exclusion criteria were: age under 18, current tracheostomy, supplemental oxygen, current carcinoma in situ (CIS), having had any treatment for current tumor or a history of any other form of cancer. Patients unable to complete an e-nose measurement or to endure a nose clip were excluded as well. Follow-up patients having had any oncological treatment within 6 months prior to measurement were excluded to rule out any adverse short-term effects of treatment on VOC output. A past history of cutaneous squamous-cell or basal-cell carcinoma was permitted due to the fact that the vast majority of such cases represent localized disease with an extremely low incidence of metastasis.[23]

Tumor characteristics and medical history were gathered from the clinical records. TNM stages were established by an experienced head and neck tumor board using the American Joint Committee on Cancer Staging Manual (7th edition). Patients' smoking habits were documented. Their smoking history was reported in pack years, which were calculated using an online pack year calculator.[24] Smoking cessation was defined as no smoking for at least 1 month. Side effects or adverse effects during or shortly after measurement were documented. Oral informed consent was obtained from all patients. The study protocol was approved by the local medical ethics committee in accordance with the Declaration of Helsinki.

Study design

All patients were asked to inhale and exhale through the e-nose for 5 min. Before starting the measurement, they were instructed to breathe through the device to familiarize themselves with the procedure. To prevent entry of non-filtered air, a nose clip was placed on the nose. Participants were instructed to close their lips over the mouthpiece at all times, not to talk, and, if possible, not to sneeze or cough during the procedure. Measurements of patients not meeting these standards were not used for analysis.

E-nose measurements took place in accordance with "standard cancer care" based on national guidelines for diagnostic routines in cancer patients. To eliminate potential interference in the diagnostic process, no individual e-nose outcomes were given to the participants or the medical practitioners. The results were labeled 'sick' or 'healthy'.

We then determined the diagnostic performance of an e-nose in detecting locoregional recurrent and/or second (or third) primary HNSCC after prior curative treatment. This was achieved by analysis of the e-nose measurements, whereby breath patterns of follow-up patients without evidence of disease were compared to follow-up patients with histopathologically or cytologically confirmed locoregional recurrent or second (or third) primary HNSCC. To reduce the long-term effects of oncological treatment on the analysis of e-nose measurements, participants with recurrent or second (or third) primary HNSCC were randomly matched to patients (without evidence of disease during measurement) who had received similar oncologic treatments for prior HNSCC. This was accomplished by means of 'Case-control Matching' analysis performed with a statistical software package of IBM SPSS Statistics for Windows, Version 24.0 (Armonk, NY: IBM Corp.). The matching resulted in a cohort of follow-up patients with locoregional recurrent or second (or third) primary HNSCC (N=20) and follow-up patients without evidence of disease (N=20), whereby the variances in received oncological treatment modalities were equal for the two groups.

Materials

The e-nose used in this study (Aeonose™; The eNose Company, Zutphen, the Netherlands), harbors three micro hotplate metal-oxide sensors (AS-MLV sensors,

Applied Sensors GmbH). During measurement, the hotplates are heated and cooled in 32 steps, accurately regulating temperature between 260 and 340 °C. While the sensors are exposed to exhaled air, temperature-dependent reduction and oxidation (redox) reactions of VOCs on the metal-oxide surface affect the conductivity of the sensors. The registered changes in conductivity represent a unique VOC pattern. A full measurement procedure lasts about 15 min, of which 5 min are spent on inhalation and exhalation. The remaining time is used for sensor regeneration and detecting possible low-concentrated VOCs. (For a more detailed discussion on this point-of-care device, see van Hooren et al., 2016).[19] To eliminate possible device-related confounding factors, two Aeonoses™ (serial numbers 309 and 362) were used in this study.

Statistical analysis

Differences in baseline characteristics were determined using independent sample *t*- test, Fisher's exact Test, and Mann- Whitney U test. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0 (Armonk, NY: IBM Corp.).

Each e-nose measurement yields 64 (temperature values) times 36 (measurement cycles) times 3 (sensors) data points, generating a multi-way data set consisting of conductivity values. After pre-processing, the data is compressed using a TUCKER3-like solution generating low-dimensional vectors for each measurement. Subsequently, these vectors, representing unique VOC patterns, are used to train an artificial neural network (ANN). The combination of several pre-processing techniques, vector lengths, and neural network topologies results into several models for separating 'sick' from 'healthy' patients. A model showing proper diagnostic accuracy was selected. Pre-processing, data compression, and ANN analysis have been integrated in a proprietary software package (Aethena; The eNose Company, Zutphen, the Netherlands). 'Leave-10%-out' cross-validation was applied to prevent fitting of the data on artefacts instead of breath profile classifiers. All data were labeled either 'sick' (i.e., follow-up patient with recurrent, second, or third HNSCC) or 'healthy' (i.e., follow-up patient without evidence of disease) when processed by Aethena.

The individual predictive values (ranging from -1 to 1) were presented in a scatterplot and subsequently used to assemble a receiver operating characteristics curve (ROC curve).

Results

A total of 40 patients were enrolled in this study. The collection of breath samples did not result in any adverse effects requiring medical attention. The study included follow-up HNSCC patients without evidence of disease (N=20), and follow-up patients with histopathologically or cytologically confirmed locoregional recurrent or second (or third) primary HNSCC (N=20). Baseline characteristics are shown in Table 1 and 2. A significant difference in mean age was observed between the two groups.

Table 1. Baseline characteristics (demographics, and features of first tumor)

Parameter	Follow-up without HNSCC	Follow-up with HNSCC	*p*-values
No. of patients	20	20	
Age (mean years ± SD)	62 ± 9	69 ± 10	0.015†
Male sex (%)	14 (70)	17 (85)	0.451‡
Currently smoking* (%)	8 (40)	6 (30)	0.741‡
Pack years (median)	31	45	0.714§
Missing	0	2	
Location of first tumor			0.503‡
Oral cavity (%)	7 (35)	4 (20)	
Oropharynx (%)	6 (30)	7 (35)	
Hypopharynx (%)	0	2 (10)	
Larynx (%)	7 (35)	7 (35)	
Stage of first tumor			0.966‡
I (%)	6 (30)	4 (22)	
II (%)	3 (15)	2 (11)	
III (%)	4 (20)	4 (22)	
IV (%)	7 (35)	8 (44)	
Missing	0	2	
History of oncologic treatment			1.00‡
Radiotherapy (%)	5 (25)	5 (25)	
Surgery (%)	5 (25)	5 (25)	
(Chemo)radiotherapy (%)	4 (20)	4 (20)	
Surgery and (chemo) radiotherapy (%)	3 (15)	3 (15)	
Combination of above (%)	3 (15)	3 (15)	
Days after treatment (median ± range)	663 ± 2508	964 ± 7569	0.274§
Missing	1	1	

*Defined as no smoking for at least one month.
† Independ t-test
‡ Fishers exact test
§ Mann-Whitney U test

Table 2. Baseline characteristics (features of current tumor)

Location of current tumor	**N (%)**
Oral cavity	7 (35)
Oropharynx	4 (20)
Hypopharynx	1 (5)
Larynx	7 (35)
Regional lymph node	1 (5)
Stage of current tumor	
I	3 (17)
II	2 (11)
III	4 (22)
IV	9 (50)
Unknown*	2
Type of follow-up tumor	
Local recurrence	5 (25)
Regional recurrence	1 (5)
Second primary	12 (60)
Third primary	2 (10)

* In two patients the existence of metastatic disease was unknown.

Follow-up patients with locoregional recurrent or second (or third) primary HNSCC were compared to follow-up patients without evidence of disease. Figure 1 displays a scatterplot of individual predicted values as calculated by ANN on the basis of e-nose measurements. To obtain the best possible diagnostic values, the threshold was set to -0.06. Individual predicted values above this threshold were classified as positive, and values below this threshold were classified as negative for recurrent or second (or third) primary HNSCC. Substantial variances in individual predicted values were observed; approximately 80% of the predictive values were located between -0.5 and 0.5.

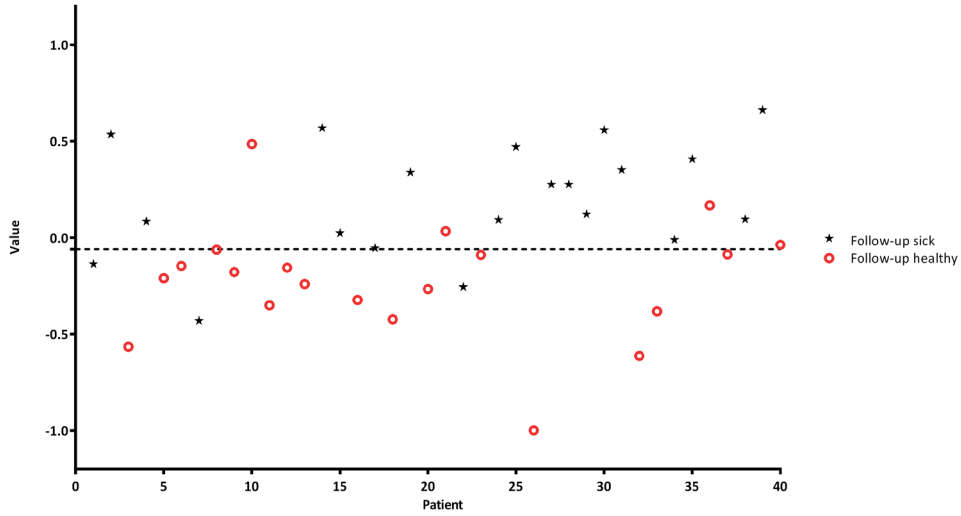

Figure 1. Scatterplot of individual predictive values of each patient (threshold -0.06). Values > -0.06 are scored as being positive for cancer. The black asterisks are follow-up patients with histopathologically confirmed head and neck cancer, the red circles represent healthy tumor-free follow-up patients.

A sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPV), and overall diagnostic accuracy (ACC) of respectively 85%, 80%, 81%, 84%, and 83% were achieved. The corresponding ROC curve, with an area under the curve (AUC) of 0.85, is presented in Figure 2.

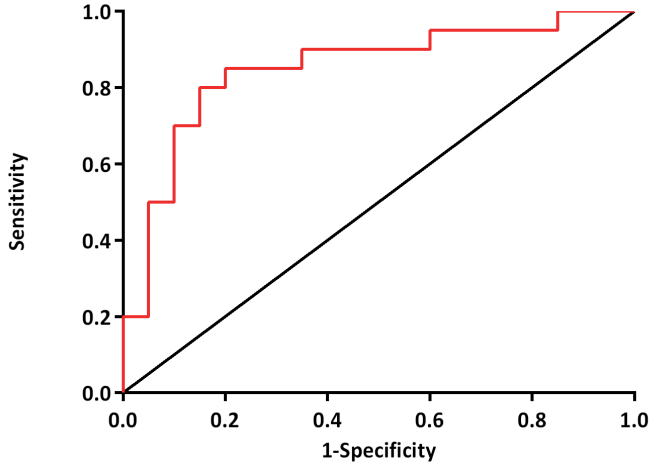

Figure 2. ROC curve, diagnostic performance of an e-nose to discriminate between follow-up patients with and without HNSCC; AUC = 0.85.

Discussion

In this feasibility study, we examined the ability of an e-nose to discriminate between follow-up patients with locoregional recurrent or second (or third) primary HNSCC, on the one hand, and follow-up patients without evidence of disease, after prior curative treatment, on the other. To attenuate the effects of oncological treatment on e-nose VOC output, participants were randomly matched based on oncological treatment undergone previously. The substantial variances in individual predicted values indicate that, besides having (had) malignant disease and oncologic treatment, a complex interplay of multiple factors contributes to VOC output, as visualized in Figure 1. Nonetheless, our results show high diagnostic accuracies in differentiating follow-up patients *with* from those *without* recurrent or second (or third) primary HNSCC. Our findings illustrate the feasibility of using an e-nose for diagnosing recurrent and second (or third) primary HNSCC after prior oncological treatment.

In the present study, participants with locoregional recurrent HNSCC as well as participants with second (or third) primary HNSCC were analyzed together. Combining these groups could possibly mispresent the true diagnostic performance of an e-nose in detecting either local or regional recurrence, or second (or third) primary HNSCC. However, during the diagnostic work-up of follow-up patients suspected of HNSCC, the first concern of the medical practitioners is to detect (any) HNC rather than to discriminate between local or regional recurrence and second primary tumors. General practice would benefit most from an e-nose that could detect follow-up patients with HNSCC and discern those without malignant disease, a performance outcome that is in line with the present study design.

The ability of an e-nose to discriminate follow-up patients with locoregional recurrent or second primary HNSCC from those without disease might depend on previous oncologic treatment. There is evidence suggesting that irradiated normal tissue is subjected to persistent injury at molecular and cellular level (e.g., oxidative stress, hypoxia, inflammation), resulting in metabolic derangements[25] and complications of radiotherapy (e.g., mandibular osteoradionecrosis).[26] Furthermore, recent studies propose the possibility of a self-sustaining immunologic response to irradiated normal tissue, similar to an autoimmune reaction, following radiotherapy.[25,27,28] Such a response has the potential to modify VOC output in irradiated patients. This may imply that separate predictive models based on prior treatment modalities need to be constructed in order to achieve the best possible diagnostic accuracies by means of an e-nose. No studies have been conducted yet to evaluate the association between long-term metabolic and immunogenic alterations following radiotherapy in relation to VOC output. Further research is needed in order to gain more insight in their potential role in VOC metabolism.

This is the first study to describe the potential role of an e-nose in diagnosing locoregional recurrent or second (or third) primary HNSCC. A recent systematic

review evaluated the diagnostic accuracy of ^{18}FDG-PET and ^{18}FDG-PET/CT in detecting locoregional recurrent HNSCC at least 12 months following curative treatment. In their paper, the authors found a pooled sensitivity and specificity of 92% and 91%, respectively, of which the latter significantly increased with time after primary treatment.[29] Similar diagnostic accuracies were found for ^{18}FDG-PET/MRI.[30] A recent review described the potential role of apparent diffusion coefficients (ADC) using MRI with diffusion-weighted imaging (DWI). That review reported sensitivities and specificities ranging from 85% to 95%, and 69% to 100%, respectively, for the detection of locoregional HNSCC at least 3 months after initial treatment.[31] Nonetheless, these imaging techniques have disadvantages that should not be ignored: the use of ionizing radiation and/or contrast medium, limited use within the first weeks after radiotherapy, and high costs. Narrow-band imaging (NBI) during flexible transnasal endoscopy is a relatively new optical method that is potentially suitable for detecting recurrent HNSCC. Different studies report promising diagnostic accuracies, with sensitivities and specificities ranging from 88% to 100%, and 92% to 98%, respectively.[32-34] However, it should be kept in mind that diagnostic performance is dependent on the clinicians' experience. In addition, for reliable examination of the laryngeal mucosa, direct laryngoscopy under general anesthesia is required. Furthermore, a regional lymph node recurrence and distant metastasis cannot be detected by NBI. An e-nose might be useful in overcoming the common disadvantages of modern imaging techniques and NBI. Potentially, the device could be used to identify patients suspected of recurrent or second primary HNSCC who could benefit from examination under general anesthesia (with biopsies taken). Moreover, the e-nose as a rapid, real-time, and low-cost diagnostic procedure might be particularly useful as a screening tool in primary healthcare and/or in less developed countries.

This is the first study to illustrate the diagnostic performance of an e-nose in diagnosing locoregional recurrent or second (or third) primary HNSCC. An e-nose seems to have potential as a rapid, real-time, and non-invasive tool for diagnosing recurrent or second (or third) primary HNSCC. A larger study, including a blinded group for validation, would be needed to determine whether an e-nose can be incorporated in the follow-up of HNSCC patients.

Limitations

This feasibility study has some limitations due to its design, and the results have to be interpreted accordingly. Due to matching, half of the participants were follow-up patients without evidence of disease whose history of oncological treatment was similar to that of follow-up patients with malignant disease. As a result, the group of follow-up patients without evidence of disease may not be an authentic representation of this population in a tertiary care hospital.

A possible limitation of this study is related to the use of ANN to determine the diagnostic performance of an e-nose. The models created by this technique could have been based partially on artefacts that are not directly related to malignant disease. The level of alcohol consumption and/or (history of) alcohol abuse was not documented, and mean age differed significantly between the two groups, possibly contributing to artefacts. Cross-validations were done to reduce the influence of these issues but cannot exclude it entirely.

The group of patients with recurrent or second (or third) primary HNSCC was relatively small, possibly restricting the potential of ANN to calculate the predictive values of both groups. The vast majority of follow-up patients with malignant disease had second (or third) primary HNSCC, making the results less applicable for detection of locoregional recurrence of HNSCC. Also, due to the small number of participants in the current study, local and regional recurrences of HNSCC were not analyzed separately, thereby possibly limiting the diagnostic potential of an e-nose. Furthermore, a subgroup analysis for patients having stage I/II tumours might be relevant for the clinical use of the e-nose. However, the number of patients diagnosed with stage I/II tumours in our study population was not sufficient to create a reliable model with the ANN.

None of the patients without malignant disease received a diagnostic work-up to exclude cancerous disease, as no clinical symptoms were present at the time of sample collection.

Conclusion

This is the first study to illustrate the potential of an e-nose as a non-invasive diagnostic tool in the follow-up of HNSCC patients. With a diagnostic accuracy of 83%, an e-nose is regarded as playing a feasible role in detecting locoregional recurrent or second (or third) primary HNSCC, after prior curative treatment. A larger study, including a blinded group for validation, is needed to determine whether an e-nose could be incorporated in the follow-up of HNSCC patients.

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CHAPTER 5

Feasibility of a portable electronic nose for detection of oral squamous cell carcinoma in Sudan

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Abstract

BACKGROUND:
Oral squamous cell carcinoma (OSCC) is increasing at an alarming rate particularly in low-income countries. This urges for research into noninvasive, user-friendly diagnostic tools that can be used in limited-resource settings. This study aims to test and validate the feasibility of e-nose technology for detecting OSCC in the limited-resource settings of the Sudanese population.

METHODS:
Two e-nose devices (Aeonose™, eNose Company, Zutphen, Netherlands) were used to collect breath samples from OSCC (n=49) and control (n=35) patients. Patients were divided into a training group for building an artificial neural network (ANN) model, and a blinded control group for model validation. The statistical software package SPSS was used for the analysis of baseline characteristics and regression. Aethena proprietary software was used for data analysis using artificial neural networks based on patterns of volatile organic compounds.

RESULTS:
A diagnostic accuracy of 81% was observed, with 88% sensitivity and 71% specificity.

CONCLUSION:
This study demonstrates that e-nose is an efficient tool for OSCC detection in limited resource settings, where it offers a valuable cost-effective strategy to tackle the burden posed by OSCC.

Introduction

Disease-associated odor is an old phenomenon, which was first mentioned by Hippocrates of Kos (460–370 BC), who described 'fetor oris' and 'fetor hepaticus'. The interest for this phenomenon developed over time throughout Antoine Lavoisier studies in the 18th century.[1,2] Modern era of breathomics started in 1970s with the pioneering research of Linus Pauling on analyzing urine vapor utilizing gas chromatography.[3]

Gas chromatography-mass spectrometry (GC-MS) is the gold-standard platform that identifies individual volatile organic compounds (VOCs) according to their physical features when compared to a reference library. Unfortunately, its clinical use is not feasible since it is not real-time, needs a long time for sample processing, relies on non-portable devices and needs constant calibration for specific analytes. Other MS-hybrid methodologies were also proved to be useful.[4,5]

Electronic noses are devices that also allow detection and identification of various volatile compounds or odors based on gas sensor arrays to simulate the function of the human olfactory system.[6] Concomitant with rapid developments in sensor technology, there is a plethora of electronic noses (e-noses) technologies that appeared lately[7], displaying different sensor chemistries.[8,9,10-13] As such, sensor arrays used in e-noses include many types of electrochemical sensors and different types of sensor-coating materials which are classified according to additive doping materials, the type and nature of the chemical interactions, the reversibility of the chemical reactions and running temperature. Those includes electrochemical (EC), metal oxide semiconductor (MOS), nondispersive infrared sensors (NDIR), thermal sensor, and photoionization sensor (PID).[14] The most widely used class of gas sensors are MOSs. The selectivity of these sensors can be changed by using different noble metals or by changing the operating temperature. They are very sensitive, robust, humidity resistant and durable, although they can suffer from drift over time.[7,15]

In addition to sensors, there are different transducers recording devices according to what they measure as output from the sensor, *e.g.* electrical measurement or optical measurement.[10] The output signal of a sensor in a gas sensor arrays, represents components of a vector, which is normally processed by feature extraction first, followed by preprocessing and normalization; then explanatory techniques are applied.[8,16,17] The measurement data from sensor arrays are multidimensional, therefore dimensionality reduction and classification methods are used to furthermore process the data.[8] The cross-reactive sensor arrays are usually coupled with a pattern recognition algorithm to detect VOC fingerprint patterns resulted from the combination of signals produced by different sensors.[16]

E-nose technology can use exhaled breath VOC pattern analysis in order to detect specific pathological conditions in different hosts and to create a prediction model.[6,17,18] These VOCs are products of different metabolic processes, including cancer

metabolism, that dissolve in the bloodstream and enter the respiratory tract through alveoli.[19] It has been demonstrated that specific VOCs for head and neck squamous cell carcinoma (HNSCC), including a subset of oral squamous cell carcinoma (OSCC), can be detected with e-nose technology by using pattern recognition in which a-specific sensors are combined with machine learning.[20] Using an artificial neural network (ANN) technique, these individual patterns can be transferred to a model for diagnosing HNSCC, including OSCC.

While significant improvement has been made in the detection and treatment of most malignancies, the prognosis of OSCC has remained relatively unchanged.[21,22] In Sudan, OC is the sixteenth most common cancer[21], with an incidence of 775 cases per year[22], most cases presenting at stages III or IV.[23]

Full diagnosis of OSCC requires a specialized setting where clinical examination is followed by contrast-enhanced computerized tomography (CT) and/or magnetic resonance imaging (MRI) in addition to the histopathological examination of biopsies, which is essential for proper diagnosis[24]. Current diagnostic tools for OSCC are invasive and expensive, posing difficulties in Sudan, where the specialized diagnostic centers are sparse and located in the main cities only.

There, devices based on e-nose technology could meet the need for low-cost, easy-to-use tools for diagnosis of OSCC at early stages, which could substantially prolong life expectancy and reduce the costs of treatment. The main advantage of the e-nose is its user-friendly design. This portable handheld device does not require specialized facilities or personnel. The e-nose could provide results in just 15 minutes after the patient starts exhaling through the device and if the device is connected to an internet network. However, the e-nose is not intended to replace the histopathological examination of oral biopsies, which should still be the gold standard for the final diagnosis and staging of OSCC.[25] It is conceived that it will help to reduce the workload demanded by the still invasive and time-consuming conventional biopsy procedure by better selection of malignant suspicious cases since it is well recognized that conventional oral examination (COE) is not predictive of histological diagnosis of oral lesions OSCC.[26] Add to that the inter- and intra- observer reliability in reporting[27], utilization of e-nose methodology is meant to be used in addition to COE for better selection of cases for biopsies, which will be of value particularly in the limited-resource settings of low-income countries such as Sudan.

The present study aimed to test the feasibility of e-nose technology for detecting OSCC in the limited-resource settings of the Sudanese population. Similar studies testing the feasibility of e-nose have been carried out on Western populations.[28] However, there are significant differences between the Sudanese and a Western population, not only in terms of the type of the tobacco consumed[29,30], but also in the availability of resources and health-care networks, differences justifying the present study.

Materials and Methods

Study cohort

This study was conducted at Khartoum Teaching Dental Hospital in Khartoum, Sudan (tertiary care referral hospital) from July 2016 through October 2018. The study protocol (no. 2016/4) was approved by the Medical Ethics Committee at the Ministry of Health, Sudan and all methods were performed in accordance with the relevant guidelines and regulations. Inclusion criteria were consecutive patients who had histologically confirmed primary OSCC (C00 – C06) during that period and healthy non-cancer controls, who consented to participate in the study. As far as possible, the healthy controls were selected among age- and sex-matched individuals who visited the out-patient clinic of the same hospital for routine dental treatments. Exclusion criteria were being younger than 18 years of age, having any previous or current cancer diagnosis, any treatment for the current tumor or a history of cancer, and other histological types of lesions/tumors than OSCC. Six cases later confirmed histologically as adenocarcinoma (two), oral cavity aspergillosis (two), and verrucous hyperplasia (two) were excluded from further analysis. Tumor characteristics and medical history were collected from the clinical records. TNM stage was registered according to version 7.0 of the American Joint Committee on Cancer Guidelines. Information on current smokeless tobacco (*toombak)* use, smoking habits, and history of smoking was collected and reported in pack-years, with calculations for *toombak* consumption adjusted according to the average of manually prepared portions in Sudan. [31] Nonsmoking was defined as no smoking during the previous month. Fasting was defined as ingesting no food for the last 3 hours and just water or clear tea without additives for the last hour.

To obtain a reliable model valid for ANN analysis using Aethena software, the sample size was calculated using the software PASS 2020, v20.0.3. The minimum number of n=62 would give a power of 0.9 (alpha 0.15 and beta 0.099).

Study design

Before each measurement, patients were instructed to inhale and exhale through a disposable mouthpiece in the e-nose for five minutes. This mouthpiece contains a high-efficiency particulate arrestance (HEPA) filter which protects the device from contamination, e.g., by bacteria and viruses. Patients were instructed to close their lips over the mouthpiece, and a nose clip was used to prevent nasal air passage (Figure 1).

Figure 1. Taking a measurement from a patient using Aeonose device.

Test runs of in- and exhalations were performed so the patient could get acquainted with the device. Participants were breathing through a carbon filter to limit the possibility that environmental VOCs would tamper with the measurement. For the first 2 minutes, their lungs were rinsed with clean filtered air that passed through the carbon filter without passing the sensors and dead air space was removed. Afterwards, a valve was opened to allow exhaled air to interact with the sensors. The total measurement cycle lasted about 15 minutes, during which time the patient in- and exhaled through the device for 5 minutes. The remaining time was used to measure any low-concentrated VOCs inside the Tenax tube and to regenerate the sensors with clean filtered air (for details see van Hooren).[32]

Patients did not receive individual diagnostic results from the e-nose analysis. The results of these measurements did not influence the regular diagnostic work-up or treatment of the participants. All measurements were performed in the same room by the same operators.

Materials

The e-nose device (Aeonose™; The eNose Company, Zutphen, the Netherlands) contains micro hotplate metal-oxide based sensors (AS-MLV sensors, Applied Sensors GmbH), which are heated and cooled in 32 steps with accurate regulation of temperature between 260 and 340 °C during the measurements. The change in the sensors' conductivity follows the temperature-dependent reaction of VOCs from breathing air (redox reactions) and produces a unique VOC pattern, as previously described.[32,33] The measurement takes, in total, about 15 minutes, including 5 minutes spent on respiration with the patient holding the device (Figure 1). The next 10 minutes are used for sensor regeneration and detecting possible low-concentrated

VOCs. For a more detailed discussion on this point-of-care device, see van Hooren et al, 2016.[32] Two Aeonose™ devices (serial numbers 257 and 372) were used in this study to reduce any possible device-related confounding factors.

Statistical analysis

Baseline group differences were determined using independent sample t-test, Fisher's exact test, or Mann-Whitney U test according to data characteristics. Logistic regression has been also performed on the data including other clinical parameters such as gender, age, smoking (total pack-year) and toombak. All statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) for Windows, Version 25.0 (Armonk, NY: IBM Corp.). Logistic regression was done using forward-stepwise (conditional) method. The subject group (patient or control) was assigned as the dependent variable, while age, gender, toombak use and smoking were assigned as independent variables. All categorical data was coded as 0 and 1. The predicted probability produced from each step in the model used to generate the ROC curves.

During one measurement, 64 times 36 data points were recorded for each of the three sensors. A Tucker3-like solution for tensor decomposition was used to compress these data points of temperature, measurement cycle, and sensors [32]. In brief, the raw data points are normalized, per participant, between 0 and 1. Then spikes were removed by peak shaving. Fourier transformation was applied to compensate for the clean air signal, and then Fourier back transform was applied. Following that the e-power was applied to all data points. NOx-sensor was only selected. Feature extraction was done to end up with only 19 element vector per participant. The resulted vectors were normalized between -1 and +1 (Supplementary data file available online at https://www.mdpi.com/article/10.3390/healthcare9050534/s1.).

The compressed data were pre-marked as either benign or malign and used to train the Artificial Neural Network (ANN). Data compression and ANN have been integrated into a proprietary software package (Aethena, The eNose Company, Zutphen, the Netherlands). A resilient backpropagating ANN training was executed for a number of data scaling options, resulting in multiple ANN options for separating benign from malignant conditions. The following parameters were used to train the ANN: Max Epoch : 5000, Max Retries : 25, Max Same Error : 30, Max Error Inc : 15, Minimal Error : 0.0005, Learn Rate : 0.0010, Alpha : 0.0500, Topology : 17x7. Data were cross-validated by the Leave-10%-Out method. This method prevents to a large extent the fitting of data on artefacts instead of on breath profile classifiers.

To exclude possible block size device dependencies, no more than 5 consecutive measurements of healthy controls or patients with OSCC were allowed. Meaning that, for example, a sixth consecutive OSCC patient measured is excluded when building the ANN for that specific model. This continues till a healthy control sample is measured. All patients excluded due to block size dependency were used to create the blinded group.

The ANN model calculates a value between -1 and 1 for each patient, corresponding with the diagnosis for that patient. These data result in a ROC-curve for each ANN showing accuracy values that can be obtained by that specific model. These calculations were performed for each model separately and resulted in data on sensitivity, specificity, the area under the curve (AUC), and overall accuracy. The flow of data processing is described step by step in the Supplementary Materials and Methods (available online at https://www.mdpi.com/article/10.3390/healthcare9050534/s1.).

Results

Cohort characteristics

A total of 84 patients with histologically confirmed OSCC and healthy controls (age range: 21-82; mean=50.6 years; median=50.5 years) were included in the study. The collection of breath samples did not result in any adverse effects. Healthy controls were younger and reported more *toombak* consumption and/or smoking behaviour than OSCC patients (Table 1). The localization of OSCC lesions was predominantly lower buccal or labial (51%); only 14.3% were localized on the tongue. Of all OSCC patients, 69.3% presented with locoregional lymph node metastases at the time of diagnosis. Only 4% of the OSCC cases presented at early stages; nearly all OSCC patients (85.8%) presented at a late stage.

Table 1. Cohort demographics and clinical characteristics of the cohort

		Non-OSCC patients	**OSCC patients**
Number of individuals		35 82.9 % males (29) 17.1 % females (6)	49 49 % males (24) 51 % females (25)
Age*	Males	48.4 y (24-68)	55.6 y (21-82)
	Females	33.5 y (27-64)	52.2 y (27-80)
Tobacco history and mean pack-years (PY) *		65	35
Clinical findings for OSCC patients			
Tumour location	Number of cases	Tumour stage	
		Stage	Number (%)
Buccal lower	26.5% (13)	I	2% (1)
Labial lower	24.5% (12)	II	2% (1)
Tongue	14.3% (7)	III	22.5% (11)
Palate	8.2% (4)	IV	63.3% (31)
Other sites	12.2% (6)	Missing staging: 10.2% (5)	
Missing sites data	14.3% (7)		

*Statistically significant differences; $p < 0.05$, Mann-Whitney U test

Feasibility analysis of e-nose measurements

A scatterplot of individual predicted values as calculated by ANN on the basis of e-nose measurements is presented in Figure 2. In order to obtain a high sensitivity combined with an acceptable specificity, the threshold was set to -0.21. Individual predicted values above this threshold were classified as positive, and values below this threshold were classified as negative primary OSCC. Substantial variances in individual predicted values were observed; approximately 80% of the predictive values were located between -0.5 and 0.5.

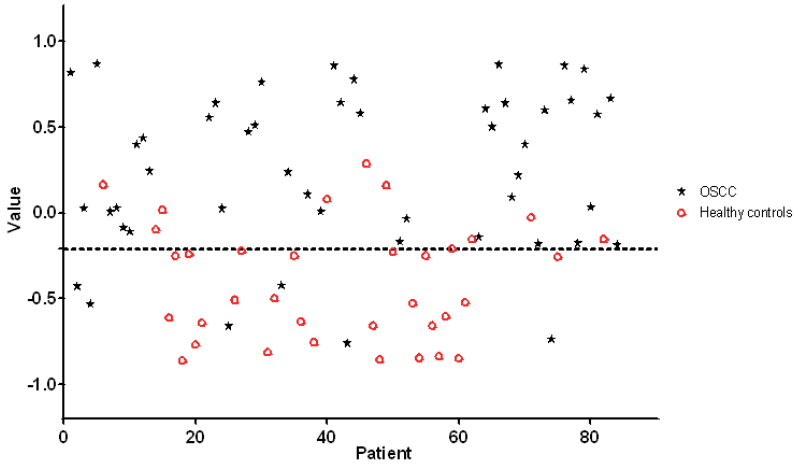

Figure 2. Individual e-nose values for each patient and control of the training set are plotted. Values > -0.21 are scored as being positive for OSCC. The black asterisks represent patients with histopathologically confirmed OSCC. The red circles represent healthy controls.

We obtained a sensitivity (SE) of 88% with a corresponding specificity (SP) of 71%. Furthermore, a positive predictive value (PPV) of 81% was calculated with a corresponding negative predictive value of 81%. The overall diagnostic accuracy was calculated to be 81%. The corresponding ROC curve with an area under the curve (AUC) of 0.86 is presented in Figure 3. Logistic regression performed on the data revealed that including gender, age, smoking (total pack-year) and toombak use increased the predictive probability of the e-nose test measurements to 92.9% (Figure 4).

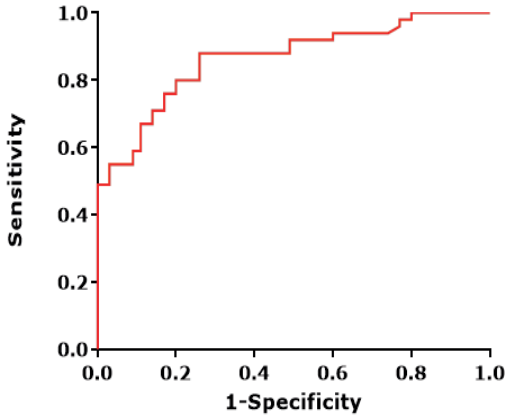

Figure 3. The ROC curve depicting the different sensitivities and specificities with altered thresholds of both the best fit of data for cross validation (red line). The area under the curve is 0.86.

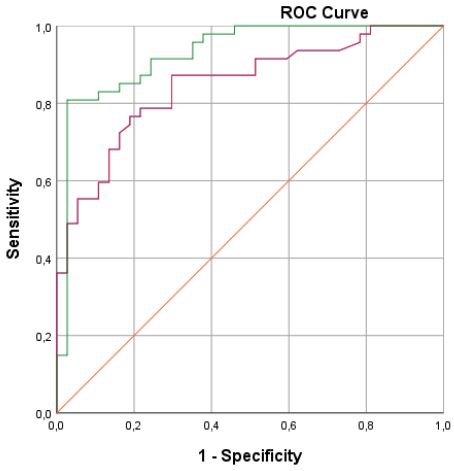

Figure 4. ROC curves showing the best fit of data for cross-validation when including in the statistical analysis the clinical parameters of gender, age, smoking (total pack-year) and toombak use (green line; the area under the green curve is 0.92) compared to the best fit for cross-validation when only the e-nose test values are analyzed (red line).

Blinded control group

Some cases were automatically (following the mentioned block size device dependencies rules) assigned to a blinded group by the Aeonose software for the validation of the model. Ten patients with OSCC and 17 healthy controls were assigned by the software to this group. The results are as follows: True positive n = 8; True negative n = 13; False positive n = 4; and False-negative n = 2, leading to a PPV of 67% and an NPV of 87%. This shows a sensitivity of 80%, a specificity of 77%, and an accuracy of 79%, which aligns with the values obtained on the validation set. Logistic regression

performed on the data for the blind set showed that after the inclusion of the same covariates, *i.e.,* gender, age, smoking (total pack-year) and toombak use, the predictive probability of the e-nose test measurements to 86.9% (Figure 5).

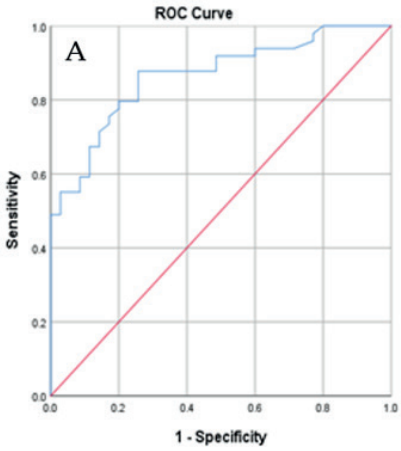

Figure 5 A

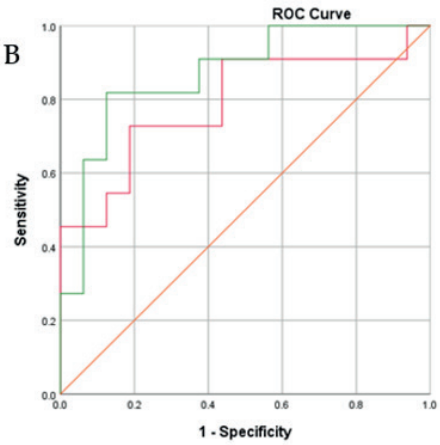

Figure 5 B

Figure 5. A) The ROC curve for the blind set. The diagonal red line represents the line of no-discrimination, while the blue curve represents different sensitivities and specificities with altered thresholds. The area under the curve is 0.882. B) ROC curves showing the best fit of data for cross-validation when including in the statistical analysis the clinical parameters of gender, age, smoking (total pack-year) and toombak use (green line; the area under the green curve is 0.92) compared to the best fit for cross-validation when only the e-nose test values are analyzed (red line).

Discussion

In this study, we reviewed the ability of a portable e-nose to discriminate with an accuracy of 81% between patients diagnosed with OSCC and patients visiting the outpatient clinic for other benign diseases in Sudan, a low-income country in Africa. Of note, the predictability of the e-nose measurements was increased by including gender, age, smoking (total pack-year) and toombak in the statistical model for data analysis, indicating that the ability of e-nose to detect OSCC can be improved even more. Its potential as a diagnostic tool should be further explored on bigger cohorts since more parameters included in the analysis require a higher number of cases available for analysis.

In recent years, the use of VOCs as potential biomarkers for cancer in general, and HNSCC in particular, has drawn increasing interest. Gas chromatography-mass spectrometry (GS-MS) has been used in most of the recent research. This technique allows detection of individual VOCs based on their molecular weight. The disadvantages of GS-MS are its high cost, the need for specialized personnel to perform the analysis, and the lack of one specific biomarker for OSCC. Bouza et al[33] utilized GC-MS

methodology and identified several VOCs such as ethanol, 2-propene-nitrile, and undecane dodecane, decanal, benzaldehyde, 3,7-dimethyl undecane, 4,5-dimethyl nonane, 1-octene, and hexadecane as potential biomarkers for the diagnosis of OSCC. Interestingly, they found that butyl acetate was significantly correlated with the histological degree of differentiation. The fact that GS-MS relies on the detection of one single biomarker limits its use as a reliable screening instrument in a clinical setting, particularly in the resource-limited setting of a low-income country such as Sudan. Furthermore, Hakim et al found an electronic nose containing nanoparticle-based sensors to be superior to GC-MS in distinguishing between HNSCC, lung cancer patients, and healthy controls.[34] Leunis et al utilized an e-nose with metal-oxide based sensors and confirmed that the resistance patterns of VOCs differed between patients diagnosed with HNSCC and a control group, with a sensitivity of 90% and a corresponding specificity of 80%.[28] Shigeyama et al. identified a signature of 12 VOCs extracted from saliva of OSCC patients as potential OSCC biomarkers.[35] Hartwig et al confirmed the absence of cancer-associated VOCs in the breath after therapy for HNSCC.[36] However, all these studies on HNSCC including OSCC subsets have been performed on Western populations, in which smoking and alcohol are the major etiological factors and the tongue is the most common site.[37]

The present study was performed on a cohort containing a small subset of *toombak*-related OSCC lesions, as indirectly also demonstrated by the preferential buccal and labial localization. Here we present results indicating that the e-nose might be feasible as a diagnostic tool for populations that have different demographic characteristics and use other types of tobacco than Western populations.

The e-nose (Aeonose™) used in this study is a handheld and easy-to-use detector. It can be used in areas where high-tech machinery and/or specialized health care workers might not be available, e.g., in rural parts of low-income countries. Therefore, the results of this study provide data to substantiate proposing this device as a feasible solution for OSCC detection in resource-poor areas, such as the rural areas of Sudan, where cancer diagnostic services are unavailable. In principle, to use this device, one would need only a (portable) computer to download the data for calculation, which could be accomplished anywhere in the world where these facilities are available. We believe therefore that in the future the e-nose might be used as a screening instrument in resource-limited areas where OSCC poses a major burden of disease. We did not perform a special hygienic protocol that would have interrupted the daily routine of the patient. The newer devices can connect to the internet via Wi-Fi and run an unlimited number of validated models with a single measurement. Once further developed and tested, these calibrated models can be easily transferred to an unlimited number of electronic noses.

Conclusions

This study shows that e-nose is a feasible technology for detection of OSCC in the Sudanese population, in a cohort with different demographic features than the Western populations previously investigated. It provides further evidence for considering e-nose as a potential tool for early detection of OSCC in resource-limited areas that lack health care infrastructure.

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CHAPTER 6

Training and validating a portable electronic nose for lung cancer screening

van de Goor RMGE, van Hooren M, Dingemans AM, Kremer B, Kross K.
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Abstract

INTRODUCTION:
Profiling volatile organic compounds in exhaled breath enables the diagnosis of several types of cancer. In this study we investigated if a portable point-of-care version of an electronic nose (Aeonose™) is able to discriminate between lung-cancer patients and healthy controls, based on their volatile organic compound pattern.

METHODS:
In this study, we used five e-nose devices to collect breath samples from lung-cancer patients and healthy controls. Sixty lung-cancer patients and 107 controls exhaled through an e-nose for five minutes. Patients were assigned either to a training group for building an artificial neural network model, or to a blinded control group for validating this model.

RESULTS:
For differentiating lung-cancer patients from healthy controls, the results showed a diagnostic accuracy of 83% with a sensitivity of 83%, specificity of 84% and area under the curve of 0.84. Results for the blinded group showed comparable results with sensitivity of 88%, specificity of 86% and diagnostic accuracy of 86%.

CONCLUSION:
This feasibility study showed that this portable e-nose can properly differentiate between lung-cancer patients and healthy controls. This result could have important implications for future lung cancer screening. Further studies with larger cohorts, including also more early-stage tumor participants, should be performed to increase the robustness of this non-invasive diagnostic tool, and to determine its added value in the diagnostic chain for lung cancer.

Introduction

Lung cancer has a high mortality rate and is one of the most common cancers worldwide, causing approximal 9% of all cancer-related deaths.[1] However, in only 15% of newly diagnosed cases, it is being detected at an early stage.[1,2]

Early diagnosis could substantially prolong life expectancy.[1,3] Since current diagnostic tools for lung carcinoma are either expensive or invasive, and therefore not ideal for screening purposes, a low-cost, non-invasive handheld portable tool such as the electronic nose (e-nose) could be useful in clinical practice.[2]

Screening and early detection of cancer are prominent in the WHO cancer diagnosis and treatment program. For lung cancer, the National Lung Screening Trial (NLST) demonstrated a 6.7% improvement of overall survival and a 20% reduction of mortality for patients screened with computed tomography (CT).[4] Known disadvantages of screening programs are false positive results which lead to high cost, concerns for the patient, and possible adverse events in follow-up investigations.[4]

Electronic nose (e-nose) technology, using the Aeonose™, has proven its ability to detect cancer.[5,6] Lung cancer could also be detected from exhaled-breath analysis, e.g., using an e-nose, a new diagnostic tool for Volatile Organic Compound (VOC) pattern analysis. VOCs are present in feces, urine, and exhaled breath and are a product of metabolic processes, including cancer metabolism.[7] These compounds interact with a-specific sensors in the Aeonose™, and influence the conductance, thereby producing a specific profile. With an artificial neural network (ANN), the data can be analyzed and prepared for pattern recognition. The VOC patterns detected by e-nose technology could then be used in diagnosing a variety of diseases such as lung cancer.[8-12]

Various studies report the use of this tool for VOC pattern analysis to detect lung malignancies, demonstrating fairly high diagnostic accuracies.[13-19] Yet, no large-scale implementation studies using an e-nose have been reported. One reason for the absence of blinded studies in literature could be the reproducibility of apparently similar sensors used in different e-nose devices.[20] There appears to be an inter-sensor difference, mainly due to small differences in the sensing layer and the absence of accurate temperature control.[20] Reproducibility would be improved if VOC patterns could be compared without building a new model for each device, enabling large-scale application. Another impediment to reproducibility could lie in the statistic or mathematical methods used to detect VOC patterns in exhaled breath.[2] These methods could compensate for the inter-device differences.[21,22]

This feasibility study investigated the added value of implementing an e-nose in diagnosing lung carcinoma. We used ANN for VOC pattern recognition in differentiating patients with benign conditions from patients with lung malignancies. In

contrast to other studies, we used an Aeonose™ (the eNose Company, Zutphen, the Netherlands) with thermo-cycled metal-oxide sensors.

Five similar e-noses were used interchangeably. The aim was to create a model for detecting lung cancer and validate it with a blinded set. The model calibrated with these devices could then be applied to numerous other e-nose devices without requiring new calibration.

Materials & Methods

Participants

Patients with histologically proven primary lung cancer and patients who visited the ear, nose and throat (ENT) department for benign conditions, further referred as healthy controls, were recruited in a tertiary care referral hospital, Maastricht University Medical Centre (MUMC). Exclusion criteria were age < 18, any treatment for current tumor or past history of cancer. Tumor characteristics and medical history were collected from the patients' clinical records. TNM stage was noted according to version 7.0 of the American Joint Committee on Cancer guidelines. Both patients with small cell lung carcinomas and non-small cell lung carcinomas were included. Information on current smoking habits and history of smoking was collected and reported in pack years. Non-smoking was defined as no smoking in the previous month. Any side or adverse effects during or shortly after measurement were documented. Measurements were performed at the outpatient clinic of the ENT and lung department. Oral informed consent was obtained from all patients. The study protocol was approved by the medical ethics committee.

Materials

For this study, we used five Aeonoses (serial numbers 259, 309, 315, 362, 379). The Aeonose™ consists of three micro hotplate metal-oxide sensors with different surface properties (AS-MLV sensors, Applied Sensors GmbH), and a Tenax tube. The combination of sensors and the Tenax tube enables breath profiling. The hotplates are periodically heated and cooled between 260 and 340 °C in 64 steps during an interval of approx. 20 seconds. A measurement consists of 36 of these intervals. During this process, exhaled air passes the sensors. Redox reactions of the VOCs at the surfaces of the metal-oxide sensors cause conductivity changes. In this way, a VOC profile is being recorded for each patient.

Study design

Before measurement, patients were instructed to gently inhale and exhale for five minutes through a disposable mouthpiece in the Aeonose™. This mouthpiece contains a high-efficiency particulate arrestance filter which protects the device from contamination by bacteria and viruses. The patient's lips were to be closed over

the mouthpiece at all times and a nose clip was used to prevent nasal air passage. A short test run of in- and exhalations was performed so the patient could get acquainted with the device. Carbon filters were used to diminish the possibility that environmental VOCs would tamper with the measurement. For the first two minutes, the lungs were rinsed with clean filtered air that passed through the carbon filters without passing the sensors, and dead air space was removed. Afterwards, a valve was opened to ensure the passage of exhaled air over the sensors. The total measurement cycle lasted about 15 minutes, of which the patient in- and exhaled into the device for five minutes. The remaining time was used to measure any low-concentrated VOCs inside the Tenax tube, and to regenerate the sensors with clean filtered air (for details see van Hooren et al. 2016).[23]

Performing these measurements did not influence the regular diagnostic work-up. Patients did not receive individual diagnostic results from the e-nose analysis.

Both patients and healthy controls were divided into a training set and a blinded set for validation.

Statistical analysis

Baseline group differences were determined using independent sample *t* test or Fisher's exact test. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.).

During one measurement, 64 times 36 data points were recorded for each sensor. To compress these data points of temperature, measurement cycle, and sensors, a Tucker3-like solution for tensor decomposition was used.[24] The resulting vectors of the compressed information were used as input for an ANN. Data compression and ANN have been integrated in a proprietary software package (Aethena – the eNose Company, Zutphen, the Netherlands). ANN training was executed for a number of data scaling options and network topologies, resulting in multiple ANN options for separating benign from malignant conditions. Part of the data was pre-marked as either benign or malignant to train the ANN. Data were cross-validated using the Leave-10%-Out method. This method prevents to a large extent the fitting of data on artefacts instead of VOC breath profile classifiers. After the training, the model created by the ANN was used to score the blinded set of patients with benign conditions or lung cancer. For more details of the statistical analysis see Kort et al. 2017.[25]

As a rule of thumb, at least 20 patients and 20 controls are needed to create an ANN model. Since this model is based on pattern recognition, more measurements lead to improved robustness of the model.

The individual e-nose classification values are presented in a scatterplot and a receiver operating characteristic curve (ROC curve) enabling to choose a sensitivity / specificity

combination. For the training group a threshold (range -1 to 1) was determined to obtain the best possible diagnostic accuracy. We report the sensitivity (SE), specificity (SP), area under the curve (AUC), negative predictive value (NPV), positive predictive value (PPV), and overall accuracy of the training set and the blinded set.

Results

Baseline characteristics

Between May 2013 and January 2016, we included 107 patients with benign disease and 60 patients with histologically proven lung carcinoma. No adverse effects were observed when using the Aeonose™. The ANN was trained with measurements from 52 patients with lung cancer and 93 healthy controls. The remaining measurements (from 14 healthy controls and 8 lung-cancer patients) were used for the blinded validation set. A cohort flowchart is presented in figure 1.1 and baseline characteristics are given in Table 1. No significant base line differences were found for age, pack years and currently smoking.

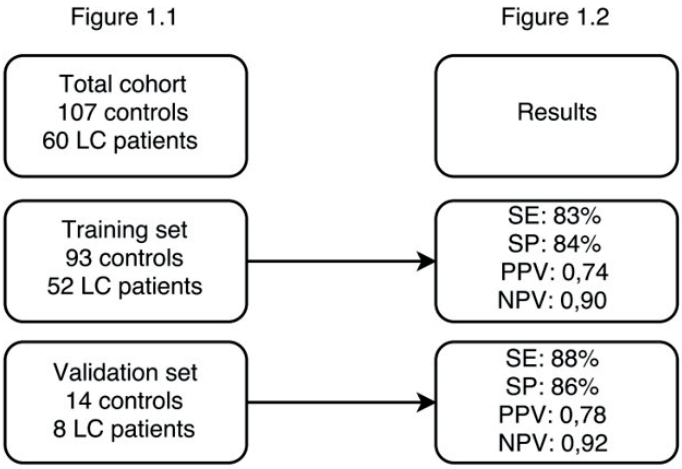

Figure 1. Cohort flowchart. Figure 1.1 shows the different groups and figure 1.2 their outcomes. Sensitivity, specificity, positive predictive value, and negative predictive value of each set are presented. LC, lung cancer; SP, specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 1. Baseline characteristics

	Healthy controls	Lung cancer	P value
Number of patients	93	52	
Age (mean years) *	63	65	0,355
Sex (male) †	61 (65,6%)	29 (55,8%)	0,286
Currently smoking (yes) †	30 (32.3%)	25 (42.4%)	0,075
Pack years (mean)*	33	45	0,468
Tumor stage (n)			
1		4 (7,7%)	
2		2 (3,8%)	
3		18 (34,6%)	
4		28 (53,9%)	
Histology (n)			
Small cell lung carcinoma		7 (13.9%)	
Non-small cell lung carcinoma		44 (84,6%)	
Mixed		1 (1,9%)	

*Independent t-test
† Fisher's exact test

Data analysis

For each patient the model calculated an individual value between -1 and 1, -1 meaning 'benign', and +1 meaning 'malignant'. In order to obtain the best possible diagnostic accuracy of this set a threshold of -0,70 was imposed. Patients with values above that level were scored as positive for lung cancer and those with values below -0,70 as negative.

The individual classification of all lung-cancer patients and healthy controls in the training set are presented in a scatter plot (Figure 2). Analysis of this data revealed a sensitivity of 83% and a specificity of 84%, with an overall accuracy of 83% in differentiating lung-cancer patients from healthy controls. The ROC curve of the cross-validation data is plotted in Figure 3. The model of the training set has an AUC of 0.84.

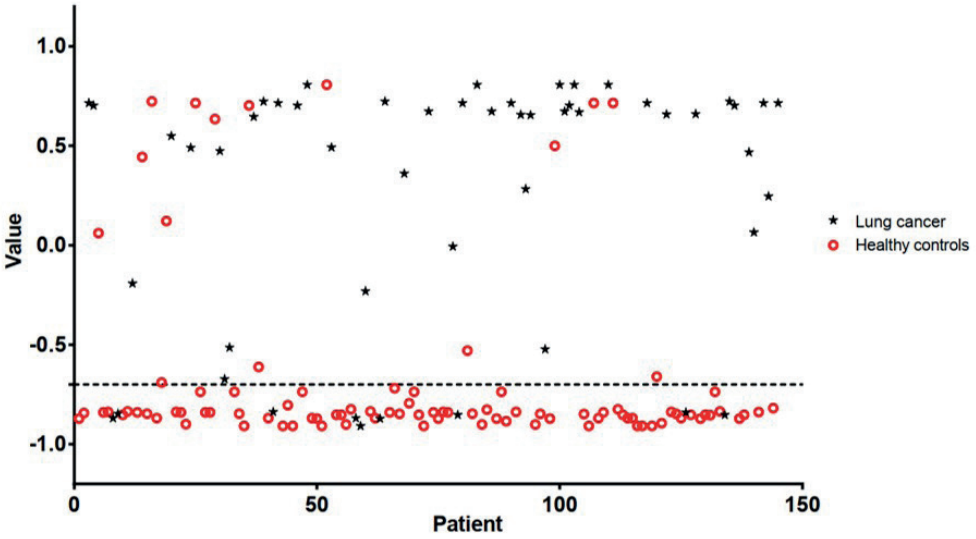

Figure 2. The individual e-nose value of each patient and control is plotted. Values > -0,70 are scored as being positive for lung cancer. The black asterixis are patients with histopathological confirmed lung cancer, the red circles represent healthy controls

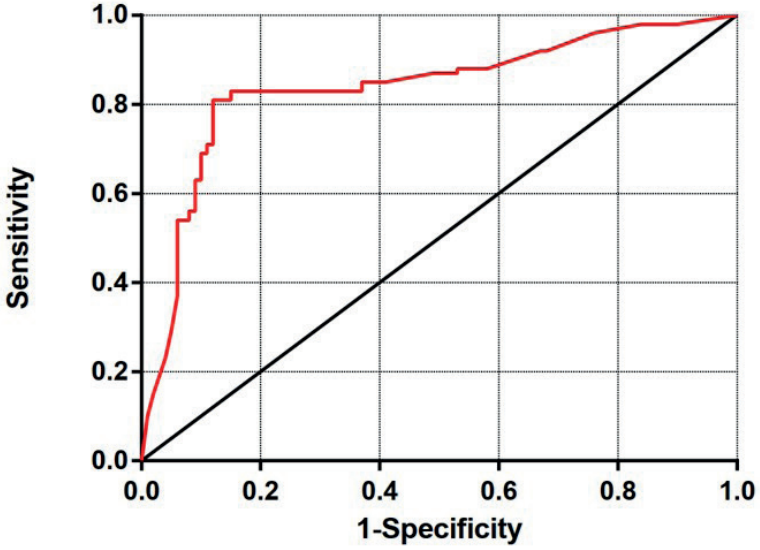

Figure 3. The ROC curve illustrates the different sensitivities and specificities with altered thresholds of both the best fit of data for cross validation (red line). Black line represents the line of no-discrimination. The area under the curve is 0,84

Figure 4 shows the individual classification values of the blinded validation set. Two false positive and one false negative result were identified. This reveals a sensitivity of 88%, a specificity of 86%, a positive predictive value of 0,78, a negative predictive value of 0,92 and an overall accuracy of 86%. The results for both the training set and the blinded set are shown in Figure 1.2.

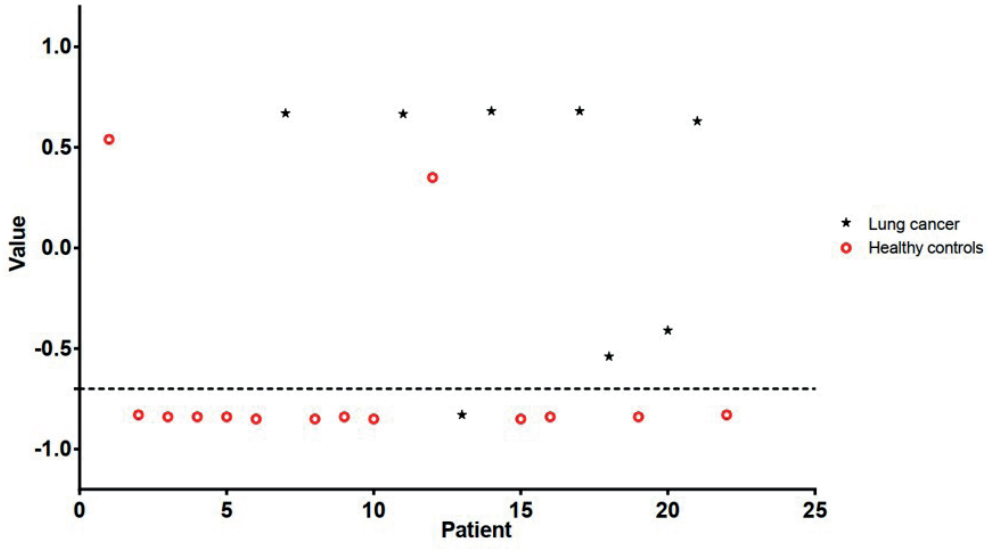

Figure 4. The individual e-nose value of each patient and control is plotted. Values > -0,70 are scored as being positive for lung cancer. The black asterixis are patients with histopathological confirmed lung cancer, the red circles represent healthy controls.

Discussion

Over the last few years numerous studies have examined the use of VOCs for diagnosing lung cancer. The main detection technique is gas chromatography–mass spectrometry (GS-MS). GS-MS is able to detect VOCs at the level of their chemical structure but it requires skilled personnel and is expensive and time consuming. Furthermore, more than one VOC has been found for lung cancer. Saalberg et al. provided a good summary of the various VOC biomarkers for lung cancer, noting that 33 different VOCs were reported in more than one study.[26] This limits the diagnostic value of the VOC biomarkers found by GS-MS.

The e-nose technology as used in this study is based on pattern recognition, and therefore, the device needs to be trained. Accordingly, the accuracy of this method depends on the size of the training set, representativeness of the sample population and reliability of the golden standard (e.g., biopsy). After external validation, the model created by the ANN can be used by other e-nose devices without the need

for a new training set. An advantage for the practitioner is that the handheld e-nose used in this study (Aeonose™) is quick to administer, easy-to-use in the outpatient clinic, and low-cost. An advantage for the patients is that it's noninvasive, they do not have to interrupt their smoking behavior, and are allowed to eat and drink before the measurement. As a result, patients can be checked without interruption of their daily routine and without any specific preparations. In the near future, this device will also be able to provide a classification within seconds after completing the measurement. This is a unique feature of the e-noses used in this study. In fact, the newest version of the Aeonose™ has the technology on board for online classification.

Gasparri et al., using e-nose technology, found a sensitivity of 81% and a specificity of 91% when comparing 70 lung-cancer patients with 76 healthy controls. All patients followed a strict hygienic protocol; this meant no smoking the night before the measurement, no food or drink (except water) in the previous eight hours, and no perfume or scented soap in the preceding 24 hours.[27] A study by D'Amico et al. used gas chromatography–mass spectrometry (GS-MS) and found a sensitivity of 85% and a specificity of 100%.[14] Rocco et al. found a sensitivity and specificity of respectively 86% and 95% using e-nose technology. Patients were asked to breathe into a special cartridge, which was then sent to the lab for gas chromatography.[28] Phillips et al. investigated VOC profiles using GS-MS with 95 histologically confirmed lung-cancer patients and 82 controls; they added a blinded control set and obtained similar results.[12] Shlomi et al found a sensitivity and specificity of respectively 75% and 93.3% using nanoarray sensors with cartridges. VOC analysis took place in a lab. All patients and controls followed a hygiene protocol and results were not compared with a blinded validation set. This study could also distinguish patients with EGFR mutation from those with wild type lung cancer.[18] Table 2 provides an overview of the aforementioned articles.

Table 2. Summary of previous studies

Author	Method	Patients	Validation set	Precautions	Results
Gasparri et al. [27]	e-nose Tedlar	70 LC 76 controls	Yes	Yes	Sensitivity 81% Specificity 91% Blinded set similar results.
D'amico et al. [14]	GS-MS Tedlar	28 LC 36 controls	No	Yes	Sensitivity 85% Specificity 100%
Rocco et al. [28]	e-nose Cartridge	23 LC 77 controls	No	Yes	Sensitivity 86% Specificity 95%
Phillips et al. [12]	GS-MS Tedlar	95 LC 82 controls	Yes	Yes	Sensitivity 74% Specificity 71% Blinded set similar results
Shlomi et al. [18]	e-nose Cartridge	16 LC 30 controls	No	Yes	Sensitivity 75% Specificity 93%

LC= lung cancer patient
Precautions= patients and controls needed to follow a special hygiene protocol prior to the measurement

E-nose technology has demonstrated its potential as an additional screening tool to use in existing screening programs for lung cancer. Out of the total number of

patients included in the NLST screening group who had positive outcomes, 96.4% of the results were false positives. In the NLST the overall positive predictive value for positive screening results was 3.8%.[4] Furthermore, 24% of the surgical interventions were performed for benign diseases. The risk of death from radiation-induced lung cancer was estimated at 1-3 per 10,000 people screened. For females the authors estimated 0.3 new deaths from breast cancer per 10,000.[4] In the future, a prospective trial combining low-dose CT imaging and e-nose technology could lead to a major reduction of false positive results and thus reduction of over-diagnosis.

This feasibility study, investigated whether five portable handheld e-noses could distinguish between breath samples of lung-cancer patients and breath samples of healthy controls. A single blinded set consisting of both healthy controls and lung-cancer patients was added for the validation of the ANN model. In this study, we found a high sensitivity and specificity when comparing healthy controls with lung-cancer patients using e-nose technology. The results in the training and blinded set were comparable, confirming the reliability of the model described in this article. The high negative predictive value (92% in the blinded set) suggests the added value of embedding e-nose to the armamentarium as an additional screening tool for lung cancer detection.

Limitations

This study compared patients with histologically confirmed lung cancers with healthy controls who had been selected for benign elective ENT surgery. These healthy controls did not receive special check-ups to exclude a possible lung malignancy.

Our patients group consisted mainly of patients with a stage III or IV tumor, which for screening purposes is not desirable. However, as Phillips et al. found using GS-MS, tumor mass affected serum levels of VOC markers but did not affect the abundance of VOC biomarkers.[29]

A follow-up study enrolling more stage I and stage II patients is needed to investigate if these early-stage tumors can be detected indeed.

Conclusion

E-nose technology is a promising tool for detecting lung cancer quickly in a non-invasive way. This study used a control group for validation of the models created by the ANN. A sensitivity of 83% and a specificity of 84% was found with 52 lung-cancer patients and 93 healthy controls. The blinded validation set showed comparable results.

Other applications should be explored to improve the diagnostic value of e-nose technology, such as combination with low-dose CT with the aim of reducing the false positive rates of CT and lowering over diagnosing and overtreatment.

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CHAPTER 7

General discussion, Summary and Impact

General discussion

Prior to planning and conducting this investigation, we had not found any studies on the use of a portable electronic nose (e-nose) for the detection of cancer. There were reports that different tumors expressed different volatile organic compounds (VOCs), but these were findings from fundamental research that was not aimed at the diagnostic use of VOCs. The main technique applied in previous clinical studies was gas chromatography with mass spectrometry (GC-MS), which requires personnel with specialized training, is time-consuming, and detects individual VOCs based on their molecular weight. The e-nose, in contrast, is a fast, cheap, and non-invasive technique for the diagnosis of cancer and is better suited for clinical use. Due to strict temperature control of the metal-oxide sensors, and data processing (see introduction figure 1.4 step 2), models created by the artificial neural network (ANN) can be transferred to numerous other e-nose devices without requiring new calibration.[1] This might facilitate clinical implementation of the technique for diagnosing disease.

Since the start of the investigation for this thesis, other groups have published on the diagnostic opportunities of portable e-nose technology for the detection of lung, colon, and prostate cancer.[2-4] This growing body of literature implies not only the acceptance but also the applicability of e-nose technology as a diagnostic tool.

E-nose technology and HNSCC

During and after the period of our research, other investigators have also conducted breath analysis of VOCs, with promising results. For example, Dharmawardana et al. examined 50 patients and 50 controls that were suspected of having HNSCC. The study analyzed sampling bags in a lab using a selected ion flow-tube mass spectrometer. They found a sensitivity (SE) and specificity (SP) of 80% and 86%, respectively. However, their analysis was not fast, nor was it performed with a portable handheld device; therefore their approach was less suited than ours for clinical practice.[5] Chandran et al. studied breath samples taken from 23 HNSCC patients and 21 healthy volunteers and found similar results with GC-MS and sampling bags. These sampling bags limit the practical implementation of VOC analysis by requiring lab work, in contrast to the e-nose where the patient breathes into a handheld device and analysis occurs on the spot.[6] Overall, the SE, SP, and AUC scores are lower for studies that focus on HNSCC than for studies that examine other cancerous processes when VOC pattern analyses are used.[7] A systematic review by Hanna et al. identified 63 relevant publications on the diagnostic accuracy of exhaled VOC detection. Pooled analysis of outcomes showed a sensitivity of 79% and specificity of 89% for the detection of cancer using exhaled VOCs. Table 1 gives an overview of the pros (+) and cons (-) of the 2 main types of breathomics technology.

Table 1. Comparison of breathomics technology

Chemical analysis (such as GM-MS)	Pattern recognition (e-nose)
+ Identification and quantification of individual volatile compounds	+ Integrated breath profile
+ Very low detection limits	+ Fast
- Time consuming	+ Large-scale implementation
- Costly	- Black box
- Trained personnel	- Individual VOC's unknown

In chapter 2 we demonstrated an improved diagnostic accuracy of e-nose technology when analyzing separate subsites instead of analyzing HNSCC as one entity. This could be explained by the heterogeneity of HNSCC. A literature review by Konings et al. presents some possible biomarkers for HNSCC, describing a variety of microbiomes that are found in different subsites. For example, the fusobacterium is often found in oral squamous cell carcinomas (OSCCs) but not in laryngeal SCCs.[8] Form earlier studies it is known that bacterial cultures have a unique VOC pattern.[9] These subsite-specific microbiomes could possibly influence the general VOC pattern analysis when the HNSCC is studied in its entirety instead of in separate subsites. Besides describing different microbiomes, Konings et al. also described other differences between subsites, e.g., mi-RNA, interferons, cytokines, and HPV-related markers. At the time of writing, it remains unknown if and how each of these differences contributes to differences in VOC patterns. Nonetheless, by creating subsite-specific models, we have achieved better diagnostic results. Interestingly, GC-MS could differentiate between various genetic mutations of breast cancer by breath analysis. However, such a study has not yet been conducted for HNSCC.[10] Another noteworthy study was conducted by Pentred et al. by creating volatile profiles with a cross-reactive nanosensor array based on gold nano particles (GNP), they could correctly classify 19 cell lines according to EGFRmut, KRASmut, EML4-ALK fusion, or wild type for 3 mutations.[11] In our clinical study we did not perform comparable analyses. We tried to incorporate HPV status in our models, but that was not possible because of our limited sample size.

A critical aspect of any diagnostic tool is that the test has to be specific to a particular health problem. The test should measure the pathology of interest, which in our case is cancer. Crucially, the tool should differentiate between tumors of different types in different compartments of the human body. In Chapter 3 we showed that e-nose technology can distinguish between 3 types of cancer, and our results are in line with other studies that could discriminate between different cancer types using e-nose technology. [12-14] These findings support the hypothesis that e-nose technology is able to discriminate between different types of cancer, which is an important requirement for clinical implementation.

A clinical problem for Head and Neck oncologists is how to interpret PET/CT positive lesions after treatment. In the Netherlands, patients who are treated curatively for HNSCC receive a 3-month post-treatment PET/CT for determination of radiotherapy efficiency. The proportion of inconclusive results post-treatment after 3 months is 26% and declines to 8.4% after 6 months.[15] In light of the treatment dilemmas this causes for both patient and doctor, chapter 4 describes our feasibility study for detecting recurrent HNSCC. Although the diagnostic results were reasonable, there was only a limited spread between the e-nose values of each patient in our healthy control group and those in the group with a local/regional recurrence of disease. These individual values can range between -1 and 1. Eighty percent of the individual e-nose values were between -0.5 and 0.5. The limited spread between these groups could be explained by the group characteristics, which consist of both local and regional recurrent HNSCC. This suggests that a complex interplay of multiple factors contributes to VOC output. To our knowledge, no other studies have been published that discriminate between local and regional recurrence of HNSCC using VOC pattern recognition technology. Steenhuis et al. used the same e-nose (Aeonose™) for patients treated for colorectal carcinoma (CRC). Their device was able to identify extra luminal local recurrences or metastases of CRC with a sensitivity and specificity of 88% (CI 69%-97%) and 75% (CI 57%-87%), respectively, with an overall accuracy of 81%.[16] This suggests that e-nose technology combined with an artificial neural network (ANN) could be used for post-treatment follow-up of patients with cancer. Although our results are promising, they must be interpreted with caution. The current study was conducted in a heterogenous group of patients treated for HNSCC. Further modelling should be specific to local, regional, or second primary malignancies. The model presented here should be improved and then tested in larger subsite-specific populations, and subsequently validated by a blinded control group. After that it could be implemented in clinical practice with the aim of improving post-treatment follow-up for patients with HNSCC. Thereby, the e-nose could become a powerful decision tool to prevent invasive procedures in the follow-up of previously treated HNSCC patients.[17,18]

In chapter 5 we tested the feasibility of e-nose technology for detecting OSCC in the limited-resource setting of a developing country like Sudan. The subsite of OSCC was chosen because of its high incidence in this part of the world and the relatively easy access for histopathological confirmation. Similar studies testing the feasibility of the e-nose have been carried out in Western populations. However, there are significant differences between the Sudanese and Western populations, not only in terms of risk factors like the type of the tobacco consumed but also in the availability of resources and healthcare networks. This is the first study conducted in Sudan using e-nose technology. We think this step can help close the knowledge gap between the developed and developing world and that it is of great importance to engage these parts of the world in advancing e-health. There are several projects that pursue e-health in Africa, such as telemedicine and mobile pharmacy. The main objectives

of most of these projects are to improve access to healthcare for communities in developing countries and to enable healthcare workers to make data-driven decisions. If e-nose technology could be implemented for patients suspected of OSCC in rural areas of Sudan, they would be able to produce an e-nose breath sample near their home. Only when the test result is suspicious for OSCC would the need arise for travel to the nearest hospital for histopathological confirmation. Furthermore, in addition to our main objectives, we tested whether the e-nose can function in a hot and humid area such as Sudan, which proved to be the case. The most recent version of the Aeonose™ is controlled with an iPad which sends the data from the e-nose to the to the cloud, one only needs WIFI or 4g for near real-time results. Therefore, in the future, the e-nose might be used as a screening instrument in resource-limited areas where OSCC or perhaps other subsites of HNSCC form a major burden of disease. Newer devices can connect to the internet and run an unlimited number of validated models that had been created with e-nose technology. One might argue that our results would not contribute to better treatment of HNSCC in low-income countries because their resources are limited compared to high-income countries. Nonetheless, e-nose technology could be used there as a decision-making tool, taking into account the infrastructural challenges and cultural differences.

Analyzing exhaled VOCs for lung cancer has drawn huge interest over the past decade. The most recent studies show promising results, the most interesting of which is that the addition of clinical parameters to e-nose values improves the AUC from 0.76 to 0.86.[2] Our group was the first to study the diagnostic capabilities of the Aeonose™ in the diagnosis of lung cancer, and these results are presented in chapter 6. Placing our study alongside more recent ones implementing portable e-noses, we see comparable results.[19-21] Binson et al. found a diagnostic accuracy of 79%.[20] Tirzïte found both high sensitivity (95.8%) and high specificity (90%) in a group of 252 cancer patients and 223 controls. In a study using the same Aeonose™, Kort et al. found an AUC of 0.76 compared to an AUC of 0.84 in our study.[2] One explanation could be that our control group consisted of healthy ENT patients. Part of the control group in the study of Kort et al. consisted of patients suspected of having lung cancer by the general practitioner but were found negative in the diagnostic process. These patients most likely had underlying lung pathology that caused the general practitioner to refer the patients in the first place. In chapter 6 we discussed the possibility of using e-nose technology and low-dose computed tomography in a complementary setting for improved accuracy. Shaffie et al. combined VOC profiles with CT scans from 467 patients with 727 pulmonary nodules, and breath samples from 504 patients were analyzed. Their system achieved 97.8% accuracy, 97.3% sensitivity, 100% specificity, and 99.1% area under curve in classifying pulmonary nodules.[22] In the near future we hope that e-nose technology can be combined with CT scans for a more patient-specific policy.

General Conclusion

In this thesis we used an electronic, portable, and handheld device for the diagnosis of primary and recurrent HNSCC and primary lung cancer. We could classify these cancers with a promising diagnostic accuracy. E-nose technology showed good potential for use in outpatient care in a hospital setting and hopefully also in a primary care setting. More studies in larger populations must be performed to confirm our results and build the best models. Once this is done successfully, e-nose technology could be incorporated as a screening/diagnostic instrument in daily healthcare practice.

Summary

The main objective of this thesis was to study whether a portable e-nose (Aeonose™, The eNose Company, Zutphen, The Netherlands) is able to detect HNSCCs. Early detection of HNSCC is of interest, since there is no HNSCC screening method yet, whereby patients are often diagnosed with advanced disease and thus a poor prognosis. Therefore, we studied the applicability and reliability of the e-nose as a diagnostic tool in an outpatient clinical setting. Our secondary objectives were to test whether the e-nose is able to discriminate HNSCC from other malignancies and to detect recurrent HNSCCs or second primary HNSCC tumors as well as primary lung cancer.

Odorous molecules provide detailed information about the environment. In nature, many animals trust their sense of smell when performing their most important tasks: finding prey, avoiding predators, identifying mates, marking territory, navigating their environment, and establishing social hierarchies. Although humans tend to rely more heavily on other senses such as vision and hearing to gather information about their surroundings, we retain the capacity to detect and discriminate a large number of odorous molecules.[23] The brain combines the signals received and determines what ensemble of molecules is detected and which odor "belongs" to it. The brain needs to be trained to correctly classify a combination of molecules as an odor. With the continuous improvement of processing capacities in computers and better sensors, it was possible to develop an artificial nose (e-nose). Breath is exhaled through the device and analyzed by an array of sensors interacting with volatile organic compounds in the air using a signal transduction mechanism and pattern recognition algorithm. In a healthcare setting, these recognition algorithms might be used for clinical diagnosis. Head and neck cancer is the sixth most common cancer in the world.[24] These malignant tumors are often squamous cell carcinomas and are characterized by a high mortality rate. The high mortality is attributed to advanced tumor stages at first presentation and high rates of recurrences and second primary malignancies.[25] Screening tools that could detect head and neck squamous cell carcinomas (HNSCCs) in a fast, reliable, and non-invasive way could lead to improved mortality and morbidity rates. Therefore, e-nose technology could play a role in the screening and/or diagnostics of HNSCCs.

Chapter 1 provides an introduction and the outline of this thesis.

Chapter 2 presents a study on the ability of the portable e-nose to discriminate between patients diagnosed with HNSCC and healthy controls. We showed that the e-nose is able to distinguish HNSCC patients from healthy controls with a diagnostic accuracy of 72%, sensitivity of 79%, specificity of 63%, and area under the curve (AUC) of 0.75 when all subsites are included. An analysis of the different subsites of HNSCC showed higher sensitivity and specificity for oral, oropharyngeal, and glottic HNSCC, probably because the separate groups are more homogeneous than the entire study population. This is in line with the finding that different VOCs occur at different subsites, even though these are all HNSCC tumors.

A tool that is used to screen for primary malignancies should be able to differentiate between tumors of different origin in different parts of the human body. Therefore, to further assess the usefulness of e-nose technology as a diagnostic and/or screening tool, samples of patients diagnosed with HNSCC were compared with samples of patients with cancer of other origin (colon or bladder cancer) in **chapter 3.** Our results show that VOC pattern analysis with the e-nose, using double cross-validation, is feasible. The results also show that differentiation between HNSCC and the various types of cancer is possible with a reasonable degree of sensitivity and specificity. Interestingly, e-nose technology is able to distinguish colon cancer from bladder cancer (sensitivity 88%, specificity 86%).

Survival rates of HNSCC patients have improved only marginally in the past decades, which may be partially attributed to high rates of locoregional recurrences and/or second primary HNSCCs. Between 10% and 50% of patients treated for HNSCC develop locoregional recurrences and approximately 20% a second primary tumor in the first years after initial diagnosis. Diagnosing these patients is challenging; even if a recurrence is suspected, biopsies often give false negative results. We studied the ability of the e-nose to discriminate between follow-up patients with locoregional recurrent or second (or third) primary HNSCC and follow-up patients without evidence of disease in **chapter 4**. All included patients had received curative treatment for HNSCC in the past. Figure 1 (chapter 4) displays a scatterplot of individual values for each e-nose measurement. To obtain the best possible diagnostic values, the threshold was set to -0.06 and the range was set between -1 and 1. Individual predicted values above this threshold were classified as positive and values below it as negative for recurrent or second (or third) primary HNSCC. Approximately 80% of the predictive values ranged between -0.5 and 0.5. This limited spread in individual values between the correct positive patients and correct negative patients indicates that, besides having (had) malignant disease and oncologic treatment, a complex interplay of multiple factors contributes to VOC output. Nonetheless, a sensitivity (SE), specificity (SP), and overall diagnostic accuracy (ACC) of respectively 85%, 80%, and 83% were achieved and a corresponding ROC curve was found, with an area under the curve (AUC) of 0.85. These results show high diagnostic accuracies in differentiating follow-up patients *with* from those *without* recurrent or second (or third) primary HNSCC. Our findings illustrate the feasibility of e-nose technology for diagnosing recurrent and second (or third) primary HNSCC after prior oncological treatment. In this feasibility study, patients with locoregional recurrent HNSCC as well as patients with second (or third) primary HNSCC were analyzed together. By merging these groups, the true diagnostic performance of an e-nose might be underrated for detecting either local or regional recurrence, or second (or third) primary HNSCC.

Considering the possible high potential of the e-nose as a screening instrument, we used this technology in a low-income African country with scarce healthcare resources. In **chapter 5** we studied the performance of a portable e-nose to discriminate

between patients with an oral SCC (OSCC) and patients with other benign diseases in Sudan, a low-income country in Africa. A diagnostic accuracy of 81% was observed, with 88% sensitivity and 71% specificity. Although these results were promising, the main objective of the study was to evaluate whether e-nose technology can be used in a setting that differs fundamentally from European standards. Detection is just the first step in the treatment of patients with OSCC, but a crucial one. Since the e-nose used in this study is easily applicable and requires limited training, the instrument might play a pivotal role in the development of diagnostics for HNSCC but also for other cancers in large rural areas where resources are scarce.

Besides investigating the capability of detecting HNSCC with the e-nose technology, we also wanted to find out if it could detect carcinomas of the lower airways. **Chapter 6** describes a feasibility study in which we studied whether portable handheld e-noses could distinguish between breath samples of lung-cancer patients and those of healthy controls. Part of the data was pre-marked as either benign or malignant to train the artificial neural network (ANN). The results of the model showed a diagnostic accuracy of 83% with a sensitivity of 83%, specificity of 84%, and area under the curve of 0.84. After the training, the model created by the ANN was used to score the blinded set of patients with benign or malignant conditions. The blinded group showed comparable results with a sensitivity of 88%, specificity of 86%, and diagnostic accuracy of 86%. Therefore, we conclude that e-nose technology could have added value in the diagnosis of patients with lung cancer. How e-nose technology will be implemented in daily healthcare remains to be studied. But some possible applications are for screening in first-line healthcare, complementary to existing diagnostic instruments, and for follow-up of treated patients with cancer.

The General discussion above placed the results of our studies in the context of the international literature. The next section will consider the impact that this thesis could have on future developments in healthcare.

Impact

The aging of the Western population puts increasing pressure on the limited resources of our healthcare system. The most recent models predict that in 2040, 26% of the Dutch population will be older than 65 (CBS Statline). Introduction of e-nose technology could relieve some of that pressure. E-nose technology offers healthcare workers a non-invasive, easily applicable, diagnostic tool. Its implementation could help them determine which patient might benefit from further testing and which patient would not. The success of e-nose technology depends on the reliability of the models. This thesis studied the first step in the process of testing whether this device could actually detect cancer: the feasibility of detecting HNSCC and lung cancer with e-nose technology had not yet been studied. In the discussion above, we drew attention to the limited accuracy of detecting recurrent HNSCC with PET/CT. Against that background we asked if e-nose technology could aid as a follow-up tool for treated patients with HNSCC. We hypothesized that further e-nose studies could contribute to the development of a more reliable, cost-efficient diagnostic tool that limits the need for invasive panendoscopy.

The results of this thesis offer grounds on which to develop new diagnostic, non-invasive tools to be implemented in the follow-up of patients treated for HNSCC.

This thesis provides insights that can be relevant for patients as well as primary and secondary healthcare providers. We have shown that a portable version of an e-nose can detect head and neck cancer. The fact that the device is portable and easy to handle makes it applicable in the primary healthcare setting. Given the characteristic attributes of the e-nose, the device is usable wherever there is electricity and where internet is available. In the future, the quick availability of the measurements could offer the patients considerable advantages, such as fewer hospital visits, less-invasive tests, fewer false-positive test results, and shorter waiting times.

Future studies for HNSCC and other cancers of the upper airway and digestive tract should focus on different subsites and on linking models to specific complaints and clinical parameters. For example, e-nose technology could be used in patients with persisting dysphonia in order to differentiate between benign alterations of the vocal cords and laryngeal cancer in a first-line and/or outpatient setting. Or it could be used in cases with symptoms such as hemoptysis, which may originate from HNSCC, esophageal, or pulmonary malignancies. As soon as a model is validated for each of these cancer localizations, e-nose technology could be used to improve differential diagnostics. The first-line practitioner could run the test with a single breath analysis and refer the patient to the right specialist for the suspected diagnosis.

One of the innovative aspects of the e-nose technology used in this thesis is its practical usability compared to other e-nose devices, which need to be calibrated to adapt a specific model to specific hardware. This hinders the practical application

and clinical reproducibility for larger series of e-nose devices. The e-nose technology used in this thesis, in contrast, was designed for broad implementation. Due to the accurate temperature control of the sensors, these models can be easily transferred to an unlimited number of electronic noses.[1] This is a requirement for the widespread adoption of e-nose devices for screening.[8,25]

For the implementation of e-nose technology as a diagnostic instrument (or for screening) in everyday medicine, certain criteria need to be met (table 2).

Table 2. Wilson & Jungner's principles of screening

1.	The condition should be an important health problem.
2.	There should be an accepted treatment for patients with recognized disease.
3.	Facilities for diagnosis and treatment should be available.
4.	There should be a recognizable latent or early symptomatic phase.
5.	There should be a suitable test or examination.
6.	The test should be acceptable to the population.
7.	The Natural history of the condition, including development from latent to declared disease, should be adequately understood.
8.	There should be an agreed policy on whom to treat as patients.
9.	The cost of case-finding (including a diagnosis and treatment of patients diagnosed) should be economical balanced in relation to possible expenditure on medical care as a whole.
10.	Case-finding should be a continuous process and not a ''once and for all'' project.

A recent guide published by the WHO (Screening programs: a short guide) emphasizes the importance of increased effectiveness, maximized benefits, and minimized harm.[26] Although HNSCC and the e-nose technology used in this thesis meet several of these criteria, more research is needed before the e-nose can be adopted as a screening tool. We do believe it could be implemented in the diagnostics of patients with primary HNSCC and recurrent HNSSC.

The cost of one e-nose test has not yet been determined, and a separate cost-effectiveness analysis still needs to be conducted. Since the sensors in Aeonose™ are mass produced, the cost of building one e-nose is relatively low. However, a market price is not yet available. For each test, one needs to replace the mouthpiece and the carbon filters, which are low-cost disposable parts. Furthermore, the e-nose is an easy-to-use portable device that requires limited training to operate. During our study in Sudan, we were able to train persons without any background knowledge of e-nose technology in one day. Finally, a reduction in costs is conceivable, since patients are less often referred to a medical specialist and could possibly receive reduced follow-up diagnostics, which would largely compensate for the production costs of the e-nose.

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CHAPTER 8

Nederlandse samenvatting

Curriculum Vitae

List of publications

Dankwoord / Acknowledgments

Nederlandse samenvatting

In dit proefschrift doen we verslag van ons onderzoek naar het gebruik van een draagbare elektronische neus (e-neus) in de detectie van hoofd-halskanker. Kanker in het hoofd-halsgebied is de zesde meest voorkomende tumor in de wereld. Vaak betreft het plaveiselcelcarcinomen die gekarakteriseerd worden door een hoge mortaliteit. Deze hoge mortaliteit wordt veroorzaakt door het gevorderde stadium waarmee de patiënt zich presenteert bij de specialist en de hoge kans op een recidief van de primaire tumor, als ook een tweede primaire tumor.

Screenings instrumenten die in staat zijn hoofd-halstumoren op een snelle, betrouwbare en niet invasieve manier te detecteren zouden de mortaliteit en morbiditeit kunnen reduceren. Op dit moment is er geen goede methode voor vroege detectie van hoofd-halskanker beschikbaar behoudens invasieve. Wij onderzochten de toepasbaarheid en de betrouwbaarheid van een e-neus als een niet invasief diagnostisch instrument voor hoofd-halskanker. Secundair onderzochten we of een e-neus kan discrimineren tussen hoofd-halskanker en andere maligne tumoren. Doordat recidieven van hoofd-halskanker vaak moeilijk te behandelen zijn, onderzochten we ook of e-neus technologie recidieven bij patiënten die reeds behandeld zijn voor hoofd-halskanker kan detecteren. Tot slot onderzochten we of deze technologie ook andere primaire tumoren kan detecteren, zoals longkanker.

De e-neus detecteert volatiele organische verbindingen (VOV's). Dit zijn gasvormige metabole afbraakproducten die het lichaam via de longen uitscheidt. Infecties en maligniteiten veranderen de samenstelling van VOV's, wat ze interessant maakt in de ontwikkeling van een diagnostische methode. Geuren kunnen gedetailleerde informatie geven over een bepaalde omgeving. In de natuur gebruiken veel dieren hun reukzintuig voor het voltooien van hun primaire levensbehoefte, denk aan het zoeken van prooi (of vermijden van roofdieren), vinden van een partner, markeren van hun territorium en bepalen van sociale hiërarchie. Alhoewel wij, mensen, meer gebruik maken van zicht en gehoor voor het verkrijgen van informatie over onze omgeving speelt ons reukorgaan wel degelijk een belangrijke rol en is het derhalve ook in staat om veel geuren te onderscheiden. Het brein combineert de ontvangen signalen en bepaalt welke combinatie van geurstoffen leidt tot welke geursensatie. Ons brein moet echter eerst getraind worden om deze geursensatie correct te classificeren.

Door de continue ontwikkeling van sensoren en de verbetering van proces snelheden van computers is het mogelijk een artificiële neus te creëren, de e-neus. Een ademanalyse wordt uitgevoerd met behulp van sensoren in de e-neus die een interactie aangaan met VOV's in de uitgeademde lucht van patiënten. Deze VOV's zorgen voor een verandering in signaaloverdracht op de sensoren die met een patroon herkenningsalgoritme (kunstmatig neuraal netwerk) aan een diagnose, zoals hoofd-halskanker, verbonden kunnen worden.

Hoofdstuk 1 geeft een overzicht over dit proefschrift alsmede een introductie.

Hoofstuk 2 beschrijft het discriminerend vermogen van de draagbare e-neus in een studie met patiënten met hoofd-halskanker en een gezonde controlegroep. Hierbij werd een diagnostische accuraatheid van 72%, een sensitiviteit van 79%, een specificiteit van 63% en oppervlakte onder de ROC-curve van 0,75 gevonden. Een separate analyse naar de verschillende subsites van hoofd-halskanker toonde een hogere sensitiviteit en specificiteit voor mondholte, keelholte en stemplooi kanker. Dit komt mogelijk omdat deze patiëntgroepen homogener zijn dan de volledige studie populatie. Dit komt overeen met de bevinding dat verschillende VOV's voorkomen in de verschillende gebieden van de mond-keelholte, ondanks dat het allemaal hoofd-halstumoren zijn.

Een instrument dat gebruikt wordt voor de diagnose van primaire tumoren zou ook in staat moeten zijn om onderscheid te maken tussen tumoren op verschillende locaties in het lichaam. Om te bepalen of de e-neus hieraan voldoet, hebben we in **hoofdstuk 3** monsters van patiënten met hoofd-halskanker vergeleken met die van patiënten met darm- of blaaskanker. Allereerst laat deze studie zien dat VOV-patroonanalyse door de e-neus technologie met gebruik van 'double cross-validatie' haalbaar is. Daarnaast laten we zien dat e-neus technologie in staat is om te differentiëren tussen hoofd-halskanker en andere kankertypes zoals die in de blaas en darm. Interessant was ook te zien dat de e-neus zelfs in staat is om onderscheid te maken tussen darm- en blaaskanker (sensitiviteit 88% en specificiteit 86%).

De laatste jaren is de overleving van patiënten met hoofd-halskanker nauwelijks verbeterd. Dit komt mede doordat er een grote kans is op recidivering van de tumor en ook op het ontwikkelen van een tweede primaire tumor. Tussen de 10% en 50% van de patiënten die behandeld worden voor een kanker in het hoofd-halsgebied ontwikkelt een locoregionaal recidief en ongeveer 20% een tweede primaire tumor in de eerste jaren na initiële diagnose. Het diagnosticeren van deze patiënten is uitdagend. Zelfs als er verdenking is op een recidief, geven biopten vaak een vals negatief resultaat. In **hoofdstuk 4** onderzochten we of e-neus technologie in staat is om onderscheid te maken tussen hoofd-halskanker patiënten met een recidief of een tweede (of derde) primaire tumor en patiënten die behandeld zijn voor hoofd-halskanker zonder recidief. Alle geïncludeerde patiënten waren eerder curatief behandelt. Uit de analyse bleek dat de individuele waardes van de patiënten met een recidief of een tweede (of derde) primaire tumor en behandelde patiënten zonder recidief erg dicht bij elkaar lagen. Dit suggereert dat de VOV-profielen van beide groepen overeenkomsten hebben. Mogelijk komt dit doordat de patiënt karakteristieken op veel vlakken overeenkomen. Ondanks de beperkte verschillen was het wel mogelijk onderscheid te maken tussen deze groepen met een sensitiviteit, specificiteit en diagnostische accuraatheid van respectievelijk 85%, 80% en 84% en een oppervlakte onder de ROC-curve van 0.85. Deze resultaten tonen

een hoge diagnostische accuraatheid in het onderscheiden van behandelde hoofd-halskanker patiënten met of zonder recidief, of met een tweede (of derde) primaire hoofd-halskanker. Onze bevindingen illustreren de haalbaarheid van het gebruik van e-neus technologie voor het diagnosticeren van recidiverende en tweede (of derde) primaire hoofd-halskanker na een eerdere oncologische behandeling. In deze studie worden patiënten met locoregionale recidieven en patiënten met tweede of derde tumoren samen geanalyseerd. Door het samenvoegen van deze groepen kan de ware diagnostische prestatie van de e-neus ondergewaardeerd zijn voor het detecteren van een locoregionaal recidief, een tweede of derde primaire hoofd-hals tumor.

Uit voorgaande hoofstukken is gebleken dat e-neus technologie veelbelovend kan zijn in de diagnostiek van hoofd-hals tumoren. We gebruikten deze technologie in een Afrikaans ontwikkelingsland met beperkte medische voorzieningen. In **hoofdstuk 5** onderzochten we, in Sudan, of de draagbare e-neus onderscheid kon maken tussen patiënten met mondholte kanker en patiënten met andere benigne aandoeningen. Een diagnostische accuraatheid van 81% werd gevonden met een sensitiviteit van 88% en specificiteit van 71%. Alhoewel deze resultaten veelbelovend zijn, was het hoofddoel van deze studie om te evalueren of e-neus technologie gebruikt kan worden onder voorwaarden die fundamenteel verschillen van die in Europa. Het detecteren van de kanker is de eerste, maar wel een cruciale, stap in de behandeling van patiënten met mondholte kanker. Aangezien de e-neus, makkelijk toepasbaar is en bediend kan worden met een minimale training zou deze een grote rol kunnen spelen in de diagnostiek van hoofd-halskanker maar ook voor andere type kankers in afgelegen gebieden in de wereld waar de toegang tot de gezondheidszorg beperkt is.

Naast het detecteren van hoofdhals kanker onderzochten we ook of e-neus technologie in staat is om kanker in de lage luchtwegen te detecteren. **Hoofdstuk 6** beschrijft een haalbaarheidsstudie waar we onderzochten of een draagbare e-neus onderscheid kon maken tussen ademanalyses van longkankerpatiënten en een gezonde controlegroep. Om het kunstmatige neurale netwerk te trainen, werd op voorhand aangegeven of het monster van een patiënt met longkanker of van een gezonde controle was. De resultaten in dit model lieten een diagnostische accuraatheid van 83% een sensitiviteit van 83% een specificiteit van 84% en een oppervlakte onder de ROC-curve van 0,84 zien. Na de training, werd het model dat gecreëerd was door het kunstmatige neurale netwerk gevalideerd met een blinde groep van patiënten met of zonder longkanker. We zagen hierbij vergelijkbare resultaten ten aanzien van de diagnostische accuraatheid (85%), de sensitiviteit (88%) en specificiteit (86%). Hieruit concludeerde we dat e-neus technologie een toegevoegde waarde kan hebben in de diagnostiek van patiënten met longkanker.

In dit proefschrift tonen we dat e-neus technologie in staat is om primaire hoofd-halskanker en longkanker te detecteren. Daarbij lijkt de e-neus in staat onderscheid te maken tussen verschillende primaire tumoren in het lichaam. Verder kan e-neus

technologie mogelijk een bijdrage leveren in het tijdig opsporen van tweede primaire hoofd-halstumoren of recidieven bij reeds behandelde patiënten met hoofd-halskanker. Het grote voordeel hierbij is dat de methode niet invasief is en niet tot opnames in het ziekenhuis leidt. Tot slot is de e-neus technologie goed inzetbaar in (ontwikkelings-) landen waar weinig middelen voorhanden zijn. Hoe deze technologie exact geïmplementeerd kan worden in de dagelijkse gezondheidszorg moet verder onderzocht worden. Maar mogelijke toepassingen zijn screening in de eerste lijn complementair aan bestaande diagnostische instrumenten of in de vervolg controles van patiënten die reeds zijn behandeld voor kanker.

Curriculum Vitae

Martinus Gerardus Eimbertus (Rens) van de Goor werd op 17 juni 1988 geboren in OSS. Hij behaalde in 2006 zijn vwo-diploma aan het Maasland college in Oss. Aansluitend startte hij zijn geneeskundestudie aan de Radboud Universiteit Nijmegen. In 2013 startte hij met zijn opleiding tot KNO-arts in het MUMC, bij de opleiders prof. dr. B. Kremer, prof. dr. R. Stokroos en later dr. J. Hof. Gedurende de opleiding doorliep hij twee perifere stages in respectievelijk het Elkerliek ziekenhuis in Helmond (opleider dr. P. Schuil) en het Zuyderland ziekenhuis in Heerlen (opleiders dr. T. Zijlker en dr. E. Bergshoeff). Tijdens zijn opleiding werd het fundament gelegd voor dit proefschrift, waarbij het onderzoek parallel gedaan werd aan de opleiding en later aan de werkzaamheden als medisch specialist.

Na het afronden van zijn specialisatie tot KNO-arts werkte Rens een jaar als chef de clinique in het Zuyderland ziekenhuis. Vervolgens heeft hij een half jaar als staflid in het Amsterdam Medisch Centrum gewerkt om zich verder te verdiepen in de bijholte chirurgie. Op 1 juli 2019 werd hij staflid-KNO in Bernhoven te Uden, waar hij sinds 1 januari 2022 de rol van vakgroepsleider KNO vervult. Nijmegen is zijn thuis, waar hij woont met zijn vrouw Marika en twee prachtige kinderen, Lotte en Siem. Samen hopen ze nog veel te genieten van elkaar, goed eten en reizen met de camper.

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