Head and Neck

Exhaled Breath Analysis in the Diagnosis of Head and Neck Cancer

Journal:	*Head & Neck*
Manuscript ID	HED-19-1009.R1
Wiley - Manuscript type:	Clinical Review
Date Submitted by the Author:	n/a
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Key Words:	Head and neck cancer, Exhaled breath analysis, Diagnosis, volatile organic compounds, Exhaled breath condensate

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Conflict of interest: The authors declare no conflict of interest

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Abstract

Head and Neck cancer (HNC) comprises a heterogeneous group of upper aerodigestive tract malignant neoplasms the most frequent of which is squamous cell carcinoma. HNC forms the eighth most common cancer type and the incidence is increasing. However, survival has improved only moderately during the past decades. Currently, early diagnosis remains the mainstay for improving treatment outcomes in this patient population. Unfortunately, screening methods to allow early detection of HNC are not yet established. Therefore, many cases are still diagnosed at advanced stage, compromising outcomes. Exhaled breath analysis (EBA) is a diagnostic tool that has been recently introduced for many cancers. Breath analysis is non-invasive, cost-effective, time saving, and can potentially be applied for cancer screening. Here, we provide a summary of the accumulated evidence on the feasibility of EBA in ~~exhaled breath analysis~~ the diagnosis of HNC.

Keywords: Head and neck cancer; Exhaled breath analysis; Diagnosis.

Introduction

Head and Neck Cancers (HNCs) constitute a heterogenous group of neoplasms. A recent report based on GLOBOCAN database estimated the diagnosis of more than 500 000 new cases of HNC during 2018 [1], and incidence is expected to continue increasing according to estimates for 2030 [2]. HNC is associated with high mortality [1], and one of the main reasons for the low survival can be explained by late stage of diagnosis. As a result, the development of effective early detection strategies is seen as an important approach to improve curative treatment outcomes [3,4].

Current methods to detect HNCs include visual inspection of the upper aerodigestive mucous membranes and pathological examination of tissue biopsy of suspicious lesions. Serological and salivary biomarkers are under evaluation to aid in the early primary diagnosis or follow-up surveillance of this patient population, but none has yet been proven useful in clinical practice [5,6]. Therefore, more reliable and non-invasive methods are warranted for early diagnosis of HNC. The purpose of this review is to provide a summary of the existing evidence on the feasibility of exhaled breath analysis (EBA) in the diagnosis of HNC.

Exhaled breath analysis

Non-invasive approaches to the early diagnosis of cancer are crucial topic of research. Available methods include, for example, surface brushing, saliva and blood sampling. The aim of these approaches is to identify cancer biomarkers or signatures that would allow early detection of cancer before any clinical symptoms or signs develop. Such early diagnosis could lead to identification, treatment planning and intervention of the disease at an early stage and that, indeed, could improve clinical outcome [7]. However, routine diagnosis of many cancers (including those of head and neck) is still based on evaluation of changes in macroscopic appearance, radiological features and

histopathological characteristics. The tools that are used in this traditional evaluation are not easily applicable in population screening as these diagnostic examinations are invasive, expensive, and/or time-consuming, and may involve exposure to radiation.

In contrast, analysis of exhaled breath may represent a non-invasive, rapid and inexpensive screening tool that has shown promising results in many cancers [8-14]. Easy access to sample supply is one of main advantages of breath analysis. The main gas phase constituents of exhaled breath are carbon dioxide, nitrogen, oxygen, water vapor and argon. In addition to these compounds, many volatile organic compounds (VOCs) are present at trace amounts, from part-per-million (ppm, 10^{-6}) to down to part-per-trillion (ppt, 10^{-12}) levels. VOCs can be transferred to blood either in the lungs via gas exchange between alveoli and blood or they can be released in the lower or upper airways (including the oral and nasal cavity) [15]. These volatile species can be analyzed in the gas phase using various mass spectrometry techniques, optical spectroscopy [16] or sensors based on, for example, nanoparticles or semiconductive metal oxides [17]. In addition to sampling the exhaled gas, breath samples can also be collected by condensing the breath on a cooled surface. The so-called exhaled breath condensate (EBC) is composed mostly of condensed water vapor but will also include small amounts of respiratory droplets containing nonvolatile molecules and water-soluble volatile compounds [18] as well as bacteria and viruses [19]. The content of the EBC can be analyzed, for example, using enzyme immunoassay kits or chromatography – mass spectrometry techniques. The theory of ~~exhaled breath analysis~~EBA for cancer diagnosis is based on the concept that cancer initiation is associated with increase in oxidative stress that can cause alteration in the profile of deoxyribonucleic acid (DNA), proteins and other components [20]. Such alterations can be detected in exhaled breath as increased or decreased concentrations of biomarkers connected with a specific disease that is associated with specific pathologic changes

[21,22]. The history of research on breath analysis is strongly associated with Linus Pauling, the Nobel Prize winner in 1971, who explained that human breath is a complex gas mixture containing more than 200 VOCs [23]. To date, almost 900 different VOCs have been detected in exhaled breath [24].

Methodological aspects in exhaled breath analysis

Collection procedure: The collection procedure for a breath sample depends on whether the aim is to collect the gas phase portion of breath or the EBC. For gas phase samples, breath is either collected to sorbent materials or sample bags (offline analysis) or it can be analyzed immediately after the sample collection (online analysis). Storage in sorbent tubes or bags introduces risks related to sample stability during handling and storage [25]. Exhalations can be divided into three phases. The brief initial phase contains gas from the oral cavity and trachea, this is often called "dead space phase". The next phase is the mixed expiratory phase in which gas from the lungs and lower airways mixes with the dead space sample. The final phase is the end-tidal, which most closely reflects alveolar gas in the lungs. In most applications of breath analysis, the focus is on collecting the end-tidal phase and the first two phases are discarded. This can be done by real-time monitoring of the carbon dioxide content during sampling. Carbon dioxide originates fully from the alveolar gas-exchange and can be used as a marker for the end-tidal phase [25]. However, in the case of HNC, the first two phases should not be discarded as they should contain the most relevant biomarkers for cancers in the upper aerodigestive tract.

Exhaled breath is saturated with water at the temperature of the airways. If the temperature of the breath sample is lowered, condensation occurs. The EBC is normally collected at temperatures below 4 °C. Collecting devices for EBC use several different types of cooling maneuvers such as dry ice, wet ice with salt, and liquid nitrogen, and accordingly the condensate

can be either liquid, solid or mix of both [26,27]. EBC analysis is always performed offline. Importantly, it has been reported that the condensation temperature could affect different biomarkers' concentration and therefore, reporting the condensation temperature is essential for process standardization and registration [27]. There is a wide variety of commercial EBC collection devices such as EcoScreen Turbo [28], TURBO-DECCS [29], RTube™ [30], and devices for specific patient groups e.g. infants and children [31-33], and mechanically ventilated patients [34].

Duration of sample collection: Duration of sample collection is dependent on the used technique. For gas phase sampling, the sampling time can be as short as the duration of a single exhalation (seconds). When collecting the breath on a sorbent material, the concentrations of the analyte gases can be enriched by using longer sampling times (minutes). In bag or online sampling, several samples can be acquired in succession to reduce the inter-individual variation of breath levels. Various physiological factors, such as breath holding, increased tidal volume and hyperventilation can have an influence on the retrieved breath concentrations but no universal breath gas sampling procedure currently exists. Some specific breath tests, which are already in clinical use have been standardized, an example being the fractional nitric oxide (FeNO) test for measuring airway inflammation [35].

Although the majority of published studies recommend EBC sample collection of 10 to 30 minutes, some studies have reported short collection times of 3 minutes and others prolong the time up to 60 minutes [36]. The approximate volume of ~~exhaled breath condensate~~EBC after 10 to 15 minutes of breathing is 1-3 ml [37]. Clearly, the length of sample collection time has a direct effect on the final volume [36]. Increased tidal volume and/or minute ventilation, hyperventilation

and mechanical ventilation are all associated with an increase in the volume of ~~exhaled breath condensate~~EBC [38-40].

Sample storage: When using offline gas phase samples, the storage temperature and time depend on whether the sample is stored in a bag or a sorbent material. The optimal temperature for bag storage is 37 °C to avoid water condensation on bag surfaces. Storage losses depend heavily on the specific biomarkers and the collection bag material. Bags manufactured from Tedlar have been shown to be stable for many compounds for up to 6 hours of storage [41]. For samples collected onto a sorbent tube, a stable storage time of 1.5 months was achieved at – 80 °C. However, of almost 600 compounds that were studied, a significant amount showed discernible levels of change in concentration after six months of storage [42].

The optimum temperature for an EBC sample storage is below -70°C. However, some biomarkers require assessment of their stability at the storage temperature. Furthermore, division of the exhaled breath samples into aliquots is recommended as repetitive freezing and thawing for analysis may result in breakdown of certain compounds such as nucleic acids and prostaglandins [43]. These parameters do vary between the investigated individual markers.

Potential contamination by saliva and oral bacterial activity: Salivary contamination can occur during collection of EBC and it is an important confounding factor, especially when analyzing volatile biomarkers. A clear example of salivary contamination is that exhaled breath nitrite levels are mostly attributable to oropharyngeal bacterial flora, as their levels decrease drastically following rinsing of the mouth with a chlorhexidine solution [44]. Salivary contamination can be prevented or minimized by using a saliva trap and by placing the exhaled breath condenser at a

higher level than mouth and thus making it unlikely for saliva to enter the collecting device [18]. Exclusion of saliva contamination can be done by testing salivary amylase in the EBC sample [45,46]. Additionally, the ATS/ERS Task Force on EBC recommend using a nasal clip to minimize the entry of nasal airway lining fluid into the sampled air and to ensure that all exhaled air will pass through the mouth [18]. Oral bacteria and oral enzymatic activity can also influence the exhaled gas concentrations. For example, exhaled ammonia is mainly produced by enzymatic hydrolysis of urea in the oral cavity [47]. The contribution of the oral cavity to the exhaled gas concentrations can be minimized by the use of an antiseptic [48].

Potential breath biomarkers of HNC

In many neoplasms, exhaled breath has been a rich source of potential cancers biomarkers [20,49,50]. These biomarkers can be divided into three categories. The first category includes the small volatile compounds. These can be measured in the gas phase and, in the case of water-soluble compounds also in the breath condensate (e.g. ammonia and formic acid). The second category includes biomolecules of low-molecular weight (e.g. isoprostanes, polypeptides and nucleic acids). The third category is miscellaneous including many compounds such as lipid mediators, chemokines and cytokines. The second and third category are mainly present in the breath condensate. DNA from ~~exhaled breath condensate~~EBC of healthy people has been used to identify mutations (e.g. *TP53* gene mutations) that are associated with early neoplastic changes [51]. A recent study recognized several volatile organic compounds as breath gas biomarkers of thyroid cancer [49]. Reported potential breath gas biomarkers for oral squamous cell carcinoma are alkanes, alkenes and aldehydes including undecane, dodecane, decanal, benzaldehyde, 3,7-dimethyl undecane, 4,5-dimethyl nonane, 1-octene, and hexadecane when analyzed by solid-phase microextraction with

gas chromatography-mass spectrometry [52]. By using linear discriminant analysis classification of these compounds, well-defined clusters for patients and controls have been revealed [52]. In addition, dimethyl disulfide, decamethylcyclopentasiloxane (D5) and p-xylene (PX) have been reported as gas phase biomarkers that decrease after surgery [53]. Ethanol, 2-propenenitrile and undecane have also been identified as exhaled gas biomarkers that could distinguish laryngeal and pharyngeal head and neck squamous cell carcinoma from benign tumors and from healthy subjects [54].

Exhaled breath analysis as a diagnostic tool in head and neck cancer

Recent research has analyzed exhaled breath to aid in early detection of HNC [22,52,55]. An early attempt to analyze exhaled breath for diagnosis of HNC was conducted by Hakim et al. (2011) [22] who used breath gas testing with an artificial nose based on gold nanoparticle sensors. They were able to recognize patients with HNC from healthy people and from patients with lung cancer [22]. The nanoparticle sensors did not provide identification of the biomarkers responsible for the sensor response. A few years later, Gruber et al. [54] in their analysis of exhaled breath from patients with laryngeal and pharyngeal cancers found that ethanol, 2-propenenitrile and undecane can be used as potential biomarkers for these two cancers. Using artificially intelligent nanoarrays, Nakhleh et al. [56] identified a set of many diseases including HNC from exhaled breath. In oral squamous cell carcinoma, a recent study used ~~exhaled breath analysis~~EBA and found three compounds (benzaldehyde, 3,7-dimethylundecane, and butyl acetate) that have a relationship with pathological parameters of these cancers [52]. The methods used for EBA of HNC patients so far include gas chromatography-mass spectrometry [51], various nanomaterial-based sensors [22,53,55] and chemical ionization mass spectrometry [54].

Exhaled breath analysis as a predictive tool to monitor the treatment outcome

Assessment of treatment outcome is of great importance during follow-up for early identification of patients with residual or recurrent disease. ~~Exhaled breath analysis~~EBA has been recently introduced as an effective tool for monitoring response to treatment in lung cancer [57]. In HNC, Hakim et al [22] suggested that breath analysis using an artificial nose could be utilized as a test to follow-up after treatment of HNC especially for those cases at high-risk of developing second primary tumors. Moreover, breath analysis from cured patients who underwent resection of HNC was similar to breath analysis of healthy control, which potentially indicates a successful surgery [58]. Interestingly, Hartwig et al. [53] collected breath samples before and after surgical treatment of oral squamous cell carcinoma and compared the breath analysis for each case. They reported disappearance of cancer-associated volatile organic compounds in the breath after treatment [53]. However, using breath analysis in monitoring the treatment of HNC is still a new field of research that requires more scientific efforts. Also, the protocols would need to define further steps in the management of the patient population with positive findings in their breath samples.

Conclusion and Future

In recent years, ~~exhaled breath analysis~~EBA has received increasing research interest in the early detection of many cancers. The currently available body of evidence refers to potential clinical use of exhaled breath in the early diagnosis of HNC. Such evidence requires further validation in large cohorts with comparison of different protocols that have been developed. That will allow standardization of the methods of breath sampling, including sample collection, storage, and analysis. It is noteworthy, that a recent review proposed a framework for conducting and reporting future studies investigating the role of VOCs in cancer diagnosis [59]. Translation of breath analysis

from lab into clinic, after generalizability of the currently identified biomarkers, could be a step toward early detection of HNC through screening of high-risk populations.

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