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**Exhaled Breath Analysis in the Diagnosis of Head and Neck Cancer**

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**Abstract**

Head and Neck cancer (HNC) comprises a heterogeneous group of upper aerodigestive tract malignant neoplasms and the most frequently accounted histological type is squamous cell carcinoma. They form the eight most common cancer type and its incidence is increasing. However, survival results have improved only moderately during the past decades. Currently, early diagnosis remains the mainstay for improving treatment outcome in this patient population. Further, screening methods to allow early detection of HNC are not yet established. Therefore, many cases are still diagnosed at advanced stage, which results in impaired survival rates. Exhaled breath analysis 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 exhaled breath analysis in the diagnosis of HNC

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

**Introduction**

HNCs constitute a heterogenous group of neoplasms. A recent report based on GLOBOCAN database estimated diagnosis of more than half million new cases of HNC during 2018 45, and the incidence is expected to continue increasing according to estimate for 2030 46. HNC is associated with high mortality 45, and one of the main reasons for the low survival can be explained by late diagnosis. For example, human papilloma virus associated oropharyngeal squamous cell cancer has one of the most rapidly increasing incidences among all cancer types in high-income countries and supporting the development of effective early detection strategies is seen as an important approach to improve curative treatment (Lechner M, Breeze CE, O'Mahony JF, Masterson L. Early detection of HPV-associated oropharyngeal cancer. Lancet. 2019;393:2123.) (Timbang MR, Sim MW, Bewley AF, Farwell DG, Mantravadi A, Moore MG. HPV-related oropharyngeal cancer: a review on burden of the disease and opportunities for prevention and early detection. Hum Vaccin Immunother. 2019 May 3:1-9. doi: 10.1080/21645515.2019.1600985. [Epub ahead of print])

The current methods to detect HNCs include visual inspection of the upper aerodigestive mucous membranes and pathological examination of tissue biopsy of a suspicious lesion. Serological and salivary biomarkers are under evaluation to aid in the early primary diagnosis or follow-up surveillance of this patient population but none of them has yet been proved useful in clinical practice (refs). Therefore, more reliable and noninvasive methods are warranted for early diagnosis of HNC.

**Exhaled breath analysis**

Non-invasive approaches to the early diagnosis of cancer is a crucial topic of research. Available methods include, for example, surface brushing and blood sampling. The main idea of these non-invasive 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 will lead to treatment planning at an early-stage and that, indeed, could improve the clinical outcome 1. However, daily 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 either are invasive, expensive, and/or time-consuming, and involve exposure to radiation. On the other hand, analysis of exhaled breath would represent a non-invasive, fast and inexpensive screening tool that has shown promising results in many cancers 2-8. 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 vapour and argon. In addition to these compounds, many volatile organic compounds (VOCs) are present at trace amounts, from part-per-million (ppm, 10-6) 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) 9. These volatile species can be analyzed in the gas phase using various mass spectrometry techniques, optical spectroscopy or sensors based on, for example, nanoparticles or semiconductive metal oxides. 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 vapour but will also include small amounts of respiratory droplets containing nonvolatile molecules and water-soluble volatile compounds 10. 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 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 DNA, proteins and other components 11. 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 12. 13. 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 containing more than 200 VOCs 14. To date, almost 900 different VOCs have been detected in exhaled breath 15.

**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 16. Exhalations can be divided into three phases. The initial brief 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 16. 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.

Condensation of exhaled breath occurs below the temperature of 4oC. 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 17,18. 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 18. There is a wide variety of exhaled breath collection devices such as EcoScreen Turbo 19, TURBO-DECCS 20, RTube TM 21, and devices for specific patient groups e.g. infants and children 22-24, and mechanically ventilated patients 25.

**Duration of sample collection:** For gas phase sampling, the sampling time can be as short as the duration of a single exhalation (few seconds). When collecting the breath on a sorbent material, the concentrations of the analyte gases can be enriched by using longer sampling times (few 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 is the fractional nitric oxide (FeNO) test for measuring airway inflammation 26.

Although the majority of published studies recommend EBC sample collection of 10 to 30 minutes, some studies reported short collection time of 3 minutes and others prolonged the time up to 60 minutes 27. The approximate volume of exhaled breath condensate after 10 to 15 minutes breathing is 1-3 ml 28. The length of sample collection time has a direct effect on the final volume 27. Increased tidal volume and/or minute ventilation, hyperventilation and mechanical ventilation are all associated with an increase in the volume of exhaled breath condensate 29-31.

**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 used bag material. Bags manufactured from Tedlar have been shown to be stable for many compounds for up to 6 hours of storage 32. For samples that were collected onto a sorbent tube, a stable storage time of 1.5 months was achieved at – 80 °C. Out of the almost 600 compounds that were studied, a significant amount showed discernible levels of change in concentration after six months of storage 33.

The optimum temperature for an EBC sample storage is below -70oC. 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 may result in breakdown of certain compounds such as nucleic acids and prostaglandins 34.

**Potential contamination by saliva and oral bacterial activity:** Salivary contamination can occur during collection of EBC and it is 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 rinsing the mouth with a chlorhexidine solution 35. Exclusion of saliva contamination can be done by testing salivary amylase in the EBC sample 36,37. Additionally, the ATS/ERS Task Force on EBC recommend using nasal clip to minimise the entry of nasal airway lining fluid into the sampled air and to ensure that all exhaled air will pass through the mouth 10. Oral bacteria and oral enzymatic activity also influence the exhaled breath gas levels. For example, exhaled ammonia is mainly produced by enzymatic hydrolysis of urea in the oral cavity 38.

**Potential breath biomarkers of HNC:** In many neoplasms, exhaled breath has been a rich source of potential cancers biomarkers 11,39,40. 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 of healthy people has been used to identify mutations (e.g. *TP53* gene mutations) that are associated with early neoplastic changes 41. A recent study has recognized several volatile organic compounds as breath gas biomarkers of thyroid cancer 39. Reported potential breath gas biomarkers for oral squamous cell carcinoma are 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 42. By using linear discriminant analysis classification of these compounds, well-defined clusters for patients and controls were revealed 42. In addition, dimethyl disulfide, decamethylcyclopentasiloxane (D5) and p-xylene (PX) were reported as gas phase biomarkers that decrease after surgery 43. Ethanol, 2-propenenitrile and undecane were identified as exhaled gas biomarkers that could distinguish laryngeal and pharyngeal head and neck squamous cell carcinoma from benign tumors and from healthy subjects 44.

**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. An early attempt to analyze exhaled breath for diagnosis of HNC had been conducted by Hakim et al. (2011) 13 who used breath gas testing with nanoscale artificial nose. They were able to recognize patients with HNC from healthy people and from patients with lung cancer 13. The artificial nose based on nanoparticle sensors did not provide identification of the biomarkers responsible for the sensor response. A few years later, Gruber et al. 44 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. 47 have identified a set of many diseases including HNC from exhaled breath. In oral squamous cell carcinoma, a recent study has used exhaled breath analysis and found three compounds (benzaldehyde, 3,7-dimethylundecane, and butyl acetate) that have a relationship with pathological parameters of these cancers 42.

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

Assessment of treatment outcome is of great importance during the follow-up to ensure high survival rate and to early recognize any failure. Exhaled breath analysis has been recently introduced as an effective tool for monitoring response to treatment in lung cancer 48. In HNC, Hakim et al 13 suggested that breath analysis using artificial nose could be utilized as a test to follow-up after treatment of HNC especially for those cases at high-risk of developing a second primary tumor. Moreover, breath analysis from cured patients who underwent resection of HNC was similar to breath analysis of healthy control which indicates a successful surgery 49. Interestingly, Hartwig et al. 43 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 43. However, using breath analysis in monitoring the treatment of HNC is still a new field of research that requires more scientific efforts.

**Conclusion and Future**

In recent years, exhaled breath analysis 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 easily and safely. Such evidence requires further validation in large cohorts and comparing different protocols that have been introduced. That will allow standardisation of the methods of breath sampling, including sample collection and storage. Translation of breath analysis from lab into clinic, after generalizability of the currently identified biomarkers, could be a step forward toward early detection of HNC through screening of high-risk populations.

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