

# **Yeast Microflora and Halitosis in Oral Squamous Cell Carcinoma- Two Microbiological Aspects of a Disease**

PhD Thesis



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## List of Abbreviations

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**ASR-W** – age-weighted standardized incidence rate

**IACR** – International Association of Cancer Registries

**ICD** – International Classification of Diseases

**IL-8** – Interleukin 8

**MALDI-TOF**- matrix assisted laser desorption ionization-time of flight mass spectrometry

**OSCC** – oral squamous cell carcinoma

**TNF- $\alpha$**  – Tumornecrosis factor alpha

**VOC**- volatile organic compound

**VSC**- volatile sulfur compound

**WHO-IARC** – World Health Organization, International Agency for Research on Cancer

**YCB-BSA**- yeast carbon base- bovine serum albumin

**ppbv**- parts per billion by volume

## **List of publications providing the basis and related to the topic of the thesis**

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### **Publications providing the basis of the thesis**

I. **Berkovits C**, Toth A, Szenzenstein J, et al. Analysis of oral yeast microflora in patients with oral squamous cell carcinoma. *Springerplus*. 2016;5(1):1257.

Q1 IF: 0.982 (2015)

Number of independent citations: 1

II. Szabo A, Tarnai Z, **Berkovits C**, et al. Volatile sulphur compound measurement with OralChroma(TM): a methodological improvement. *J Breath Res*. Jan 05 2015;9(1):016001.

Q1 IF: 4.177 (2015)

Cumulative impact factor: 5.159

Number of independent citations: 1

### **Related publications**

Nagy J, Braunitzer G, Antal M, Berkovits C, Novak P, Nagy K. Quality of life in head and neck cancer patients after tumor therapy and subsequent rehabilitation: an exploratory study. *Qual Life Res*. Feb 2014;23(1):135-143.

Q1 IF: 2.429 (2015)

Number of independent citations: 7

## I. Introduction

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Cancers of the oral cavity (ICD C00-C14) belong to the tumors of the highest morbidity and mortality. Their epidemiological characteristics are determined by geographical factors: they are more frequent in the developed countries than in the developing ones. In the Southern part of Mid-Asia, cancers of the oral cavity are among the three most frequent tumor types. In India, the age-standardized incidence rate (ASR-W) of oral cancers is 7.2<sup>1</sup> [WHO, 2012].

It is to be noted that the rise in the incidence of oropharyngeal cancers has been reported to be extreme in several countries of Eastern Europe, as compared to the more moderate changes in Australia, Japan, New Zealand and the USA [Petersen, 2003; Stewart & Kleihues, 2003].

Cancers of the oral cavity affect especially men, among whom this is the eighth most frequent type of cancer worldwide [WHO, 2004].

Histologically, 90% of the cancers of the oral cavity can be identified as squamous cell carcinoma [Bagan, Sarrion, & Jimenez, 2010; Pires et al., 2013].

In Europe, the highest rise in oral cancer-associated mortality was registered in Hungary between 1990 and 1992 (+119% for males and +294% for females) [La Vecchia, Levi, & Franceschi, 2000; La Vecchia, Lucchini, Negri, & Levi, 2004; La Vecchia et al., 1997; Levi, La Vecchia, Lucchini, & Negri, 1995]. Unfortunately, Hungary appears to be in a leading position regarding both the morbidity and mortality of oral cancer. This is well reflected in the age-standardized incidence rates of GLOBOCAN 2012 [WHO, 2012]: the ASR-W of Hungary in 2012 was 9.7<sup>2</sup>, while of the neighboring countries, Slovakia scored 6.5, Romania 5.4, and Austria only 4.2. In Europe, Greece scored the lowest (1.6). With a score of 9.7, Hungary is the first not only in Central Europe, but in the entire European region. Data of the International Association of Cancer Registries (IACR) and data from the national cancer registry of Hungary (Nemzeti Rákregiszter) show the same.

The causes of this unfortunate situation have not been clarified, and it probably cannot be explained by a few simple and well-known factors, such as smoking or excessive alcohol consumption (or the combination of these). These factors play a

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<sup>1</sup> This value expresses the incidence of a given disease as compared to a standard world population, for a population of 100,000, considering that the disease does not occur at the same frequency in all cohorts.

<sup>2</sup> Higher than that of India, and only one tenth less than that of Pakistan, known as the country most affected by oral cancer in the world.

significant role in the pathogenesis of the oral cancers of the Hungarian population beyond doubt. However, the increasing representation of non-smoking and non-drinking elderly women and young adults among the Hungarian oral cancer patients is definitely against such a simplifying explanation [Suba, Mihalyi, Takacs, & Gyulai-Gaal, 2009].

Certainly, this sadly notable position of Hungary on the international map of oral cancer is an incentive to the Hungarian researcher of oral health to focus on the causes, consequences and potential cures for oral cancer, especially squamous cell carcinoma (OSCC). This, however, is a vast field of study. In the present thesis I will narrow my focus down to two oral cancer-related questions, both rooted in the microbiology of the oral cavity. One is considered by many as a cause or promoter of OSCC, the other is obviously a consequence. Yeasts (especially *Candida spp.*) have long been suspected as causative agents of oral malignant and premalignant states [Cernea et al., 1965; A. Jepsen & Winther, 1965]. The thesis discusses the yeast microflora of the oral cavity in OSCC based on our own research, considering also the specific question of the role of lipase/protease activity in epithelial colonization. It is also known that the microbial composition of the oral cavity in OSCC differs from that of the healthy oral cavity both in qualitative and quantitative respects [Nagy, Sonkodi, Szoke, Nagy, & Newman, 1998]. This also means that the composition of the exhaled air of OSCC patients is different from that of healthy subjects [Schmutzhard et al., 2008], which can lead to oral malodor (halitosis) [Scully & Felix, 2005]. This, of course, can lead to serious psychosocial consequences, which makes halitosis in oral cancer (and otherwise) an important problem to be addressed. At the same time, the altered composition of the exhaled air could offer a new diagnostic possibility, even if this topic is scarcely researched in oral cancer [Gruber et al., 2014; Hakim, Billan, & Tisch, 2011; Hakim, Billan, Tisch, et al., 2011]. One of the reasons for the relative lack of research in this area is that the available methodologies are not precise and reliable enough. In the present thesis I describe a methodological improvement developed by our team and tested with OSCC patients.

### **I.1. Oral cancer and the oral microbiota**

It is known that the oral cavity is inhabited by a wide variety of microorganisms, including more than 750 bacterial species [Jenkinson & Lamont, 2005]. These

organisms are typically commensalists, that is, their presence does not have any effect on the health of the oral cavity under physiological conditions. Furthermore, they appear in the oral cavity in a location-dependent manner [Mager, Ximenez-Fyvie, Haffajee, & Socransky, 2003]. Given their commensalism, the idea that the intraoral microorganisms might play a role in oral cancer appeared relatively late, and for long it had been an idea of underestimated importance [Hooper, Wilson, & Crean, 2009]. Lately, however, intensive research has been aimed at the connection between oral cancer and the oral microbiota, especially as the traditionally recognized risk factors (such as excessive alcohol consumption and smoking) explain only part of the total incidence [Morse et al., 2007], and their explanatory power just weakens if one considers how rapidly the incidence is increasing [S. A. Jepsen & Closmann, 2008; Llewellyn, Linklater, Bell, Johnson, & Warnakulasuriya, 2003].

In 1994, WHO-IARC officially recognized *Helicobacter pylori* as a carcinogenic organism of the gastrointestinal system, which, at the same time was an official recognition of the fact that cancer can be of microbial origin [Bjorkholm, Falk, Engstrand, & Nyren, 2003; Correa, 1995; Peek & Blaser, 2002]. This, of course, boosted research in this field, which resulted in several other bacterial species' being identified as human carcinogens. *Chlamydia trachomatis* became associated with invasive cervical cancer [Wallin et al., 2002] *Chlamydophila pneumoniae* with malignant lymphomas and male lung cancer [Kocazeybek, 2003], while *Salmonella typhi* is suspected to play a role in the cancer of the gall bladder [Dutta, Garg, Kumar, & Tandon, 2000].

It is becoming clear that such a link exists also in the case of oral cancer, even if we are far from the identification of one definite bacterial species as the causative agent [Dimitroulis & Avery, 1998]. Of the viruses, however, human papillomavirus (HPV) has a well-documented role in OSCC, beyond cervical and tonsillar cancer [Pagano et al., 2004; Parkin, 2006; Ryerson et al., 2008].

There is an exciting line of research that focuses on how the well-known risk factors (drinking, smoking, bad oral hygiene) may lead to cancer through interactions with the microenvironment. The evidence is the most convincing regarding alcoholism and smoking [Brook, 2011; Kurkivuori et al., 2007; Muto et al., 2000; Salaspuro, 2003], which are, unfortunately, typical habits of the average Hungarian OSCC patient. As for alcoholism, the literature underlines the role of the dehydrogenase activity of the oral microflora in the production of acetaldehyde, a known carcinogen [Kurkivuori et al.,



2007; Muto et al., 2000; Salaspuro, 2003; Vakevainen et al., 2002]. Smoking increases the risk of oral cancer probably in synergy with excessive alcohol consumption [Pelucchi, Gallus, Garavello, Bosetti, & La Vecchia, 2006, 2008]. The gist of this synergy seems to be the permissive effect of ethanol on the penetration of tobacco-specific nitrosamines, the most well-documented carcinogenic agents in tobacco smoke, into the epithelium [Senel, Kremer, Nagy, & Squier, 2001].

Below I give a bit more detailed summary on the role of *Candida spp.* in OSCC, so as to provide a background to our own research.

### **I.1.1. Candidiasis and oral cancer**

The role of *Candida spp.* in various types of cancer is widely recognized today, but it has not always been so. Even though the link was suggested already from the 1960s [Banoczy & Csiba, 1976; Banoczy & Sugar, 1972; Cernea et al., 1965; A. Jepsen & Winther, 1965; Roed-Petersen, Renstrup, & Pindborg, 1970], research in this direction gained a new momentum only in the last few decades. *Candida spp.* appear to be carcinogenic primarily through promoting the malignant transformation of premalignant lesions [Mohd Bakri, Mohd Hussaini, Holmes, Cannon, & Rich, 2010].

Similarly to several other species mentioned before, most of these species are commensalists, and they become pathogenic only through opportunism [Marol & Yucesoy, 2008]. *Candida albicans* appears to have a distinguished role in human pathogenesis [Lipperheide et al., 1996]. What follows is a brief summary of the most accepted hypotheses regarding the carcinogenic effect of *C. albicans*.

The first question is how the fungus colonizes the epithelium. The literature offers two main hypotheses in this respect. The first one of these is that *C. albicans* produces enzymes (especially aspartate proteases), by which it degrades the surface of the epithelial cells and opens up the way for the hyphae towards the inside of the cells and between them [Mohd Bakri et al., 2010]. This hypothesis we also tested in the first study for this thesis (below). It was also observed that *C. albicans* can stimulate keratinocytes by Als3 invasin in a way that they emit pseudopodium-like structures, which, in turn, pull the fungus inside the cell [Phan et al., 2007]. Colonization, however, does not depend solely on *C. albicans*. It was shown that the type and level of differentiation of the keratocyte are also influencing factors [Dalle et al., 2010].

As for the carcinogenic effect itself, the same mechanisms are assumed as with other microbial factors, that is: the direct production of carcinogenic compounds, turning procarcinogens into carcinogens, or the induction of chronic inflammation. Naturally, these do not exclude one another.

*C. albicans* can cause OSCC by the direct production of nitrosamines [Hooper et al., 2009] or benzyl methyl nitrosamine [Krogh, Hald, & Holmstrup, 1987]. Regarding the transformation of procarcinogens, the available evidence suggests that *C. albicans* transforms ethanol into acetaldehyde [Alnuaimi et al., 2016; Mohd Bakri et al., 2010].

Chronic inflammation can come about as a result of *C. albicans* infection through the enzymatic degradation of keratocytes (see earlier), which provides entry for other microorganisms as well. This way, the surrounding tissues can become chronically inflamed, which leads to the upregulation of cytokines and growth factors, and this can promote malignant transformation [Fantini & Pallone, 2008; Lax & Thomas, 2002]. It is also documented that *C. albicans* stimulates the TNF- $\alpha$  production of epithelial cells. This, in turn, leads to IL-8 expression [Orozco, Zhou, & Filler, 2000]. IL-8 is a known antiapoptotic cytokine [Osawa et al., 2002], therefore, the ultimate effect of candidiasis is a more favorable environment for cancer expansion.

It is, however, not decided if candidiasis is a cause or consequence of OSCC, or both. Nagy and co-workers failed to detect *C. albicans* on the healthy mucosa of OSCC patients, while the fungus was present on the lesioned side [Nagy et al., 1998]. This suggests that *C. albicans* acts as an opportunist, and its appearance is a consequence. However, when one considers its ability to turn alcohol into acetaldehyde and to induce chronic inflammation, it is easy to see that candidiasis might be a cause as well. The clarification of this situation definitely requires further research [Sanjaya, Gokul, Gururaj Patil, & Raju, 2011].

### **I.1.2. Halitosis and oral cancer**

Halitosis is defined as an unpleasant odor that emanates from the oral cavity with intraoral and/or extraoral origin [Armstrong, Sensat, & Stoltenberg, 2010]. Halitosis can be physiologic (putrefaction on the dorsoposterior region of the tongue without disease) or pathologic (oral or extraoral). Further classes include pseudohalitosis (a false belief that one has oral malodor) and halitophobia (a false belief after halitosis treatment that halitosis still persists)[Murata, Yamaga, Iida, Miyazaki, &

Yaegaki, 2002; K. Yaegaki & Coil, 2000]. Intraoral contributing factors are responsible for halitosis in 90% of the cases ["Oral malodor," 2003].

From a chemical point of view, malodor comes about as a result of the microbial degradation of organic substrates present in the saliva, in the crevicular fluid exudate, oral tissues and retained debris. During this process, volatile sulphur compounds (VSCs;  $H_2S$ ,  $CH_3SH$ ,  $(CH_3)_2S$ ), diamines (e.g. cadaverine, putrescine) and phenyl compounds (indole, skatole) are formed [Hughes & McNab, 2008; Thorn & Greenman, 2012; van den Broek, Feenstra, & de Baat, 2007]. It is these substances, and especially VSCs that are responsible for the malodor, and while a recent study [Bouza, Gonzalez-Soto, Pereiro, de Vicente, & Sanz-Medel, 2017] suggested some other VOCs as potential OSCC biomarkers, the measurement of these main VSCs remains the mainstay of breath assessment. It is not negligible that, as Ken Yaegaki points out in his review, that these malodor-causing compounds are also periodontopathogenic and carcinogenic, which suggests the existence of a kind of vicious circle [K Yaegaki, 2008]. The most common bacteria to produce these compounds are gram negative anaerobes, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Bacteroides forsythus* and *Treponema denticola* [Scully & Felix, 2005]. As pointed out by Nagy and colleagues, these species are indeed over-represented on the surface of OSCC lesions [Nagy et al., 1998], which, in itself suggests that OSCC patients will develop halitosis. In their study, they also showed that the oral microflora in OSCC differs from the healthy microflora not only in a quantitative sense, but also in its composition, which has been corroborated by other studies [Hooper et al., 2006; Pushalkar et al., 2012]. Furthermore, xerostomia, a frequent complication of irradiation therapy for OSCC, aggravates halitosis [Lee, Zhang, & Li, 2007]. All in all, halitosis is a problem in OSCC, but a problem that may bear the promise of a new method for early detection, especially considering the specific qualitative and quantitative characteristics of OSCC microflora. The problem is that the proper methodology is still missing.

Today, there are three primary methods to assess halitosis [van den Broek et al., 2007; van den Broek, Feenstra, & de Baat, 2008; K. Yaegaki & Coil, 2000]: the organoleptic method, gas chromatography and sulfide monitoring. The organoleptic method depends entirely on the subjective olfactory sensation of the clinician or a trained judge. By definition, this is a subjective method. Sulfide monitoring (Halimeter, Interscan Corporation, Chatsworth, CA) is affordable and the device is portable, but it is weak at distinguishing between individual VSCs, instead, it allows the measurement of

the total level of VSCs. In fact, Halimeter is the most sensitive to methyl mercaptan, and almost insensitive to dimethyl sulfide [Furne et al., 2002; Rosenberg, Kulkarni, Bony, & McCulloch, 1991]. Gas chromatography has the ability to distinguish between VSCs, and this method is objective enough to be fit for scientific purposes; however, it used to be way too expensive for scientific purposes. A change was brought about by Hanada and co-workers, who combined a semiconductor gas sensor and a compact gas chromatograph system, which became known as OralChroma, a portable, commercially available gas chromatograph [Murata et al., 2006]. While this device is quite reliable, it still has its weaknesses. Tangerman and Winkel pointed out that while the hardware meets the requirements of an accurate gas chromatograph, the software often assigns VSC peaks erroneously, and therefore yields false results [Tangerman & Winkel, 2008]. Still, the authors conclude that OralChroma is the most promising tool for the differential diagnosis of halitosis. In our study presented in this thesis, we sought to address the software problem, and to enable OralChroma to identify further components as well. The improved system was tested in OSCC patients and healthy controls.

## II. Aims and Hypotheses

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A. Regarding our study on candidiasis and OSCC, our aims were the following:

1. To describe the differences in oral yeast carriage between the OSCC-affected and healthy epithelium, with special attention to *Candida* strains.
2. To determine if the lipase/protease producing activity of the *Candida* strains is associated with the colonization rate.

We hypothesized that a bigger variety of yeast genera would be found on the neoplastic surface, and in higher numbers, as compared to the healthy surface, and we also hypothesized that the lipase/protease producing activity of the *Candida* strains would be associated with their ability to colonize the epithelium.

B. As for the halitosis study, our aims were:

1. To test an improved software version for OralChroma in a population of OSCC patients as compared to healthy controls, and to check the reliability of the new software version by comparing the measurements to Halimeter measurements.
2. To enable OralChroma to identify and measure isoprene and acetaldehyde too, so as to enhance diagnostics based on this device. We set this goal considering that these compounds are present in the breath of healthy controls too [Diskin, Spanel, & Smith, 2003; Jones, 1995; Tardif, 2007].
3. To determine the exhaled air composition of OSCC patients and to compare it against that of healthy controls of excellent oral hygiene.

We hypothesized that our enhanced software would allow a more precise determination of exhaled air components than the default software and that the measurement of isoprene and acetaldehyde would also become possible. Regarding the exhaled air of OSCC patients, we hypothesized that our results would corroborate the results of previous studies, especially concerning elevated VOC concentrations. As for acetaldehyde and isoprene, we expected different levels between controls and OSCC patients.

### III. The Oral Yeast Microflora of Patients with Oral Squamous Cell Carcinoma

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#### III.1. Methods

##### III.1.1. Subjects

Sixty subjects [20 OSCC patients (14 males, 6 females, median age: 62 (61.95), range: 44–86) and 40 controls (22 males, 18 females, median age: 67 (67.62), range: 49–82)] were enrolled in this study. The patients and the controls were recruited from among the patients of the Departments of Dentoalveolar Surgery and Maxillofacial Surgery at the Faculties of Dentistry and Medicine at the University of Szeged. Patient eligibility criteria were a histologically confirmed diagnosis and no prior treatment for OSCC. Controls were recruited from outpatients free of oral mucosal pathology who arrived for routine procedures (e.g. tooth extraction). The frequency and locations of cancer sites are given in Table 1.

**Table 1.** Frequency and locations of cancer sites in the OSCC patients

Location/diagnosis	Frequency	Percentage in total
carcinoma linguae	5	25%
carcinoma fundi oris	4	20%
carcinoma hypopharyngis	3	15%
carcinoma radice linguae	2	10%
carcinoma labii inferioris vestibularis	2	10%
carcinoma labii superioris vestibularis	1	5%
carcinoma gingivae	1	5%
carcinoma gingivae mandibulae	1	5%
carcinoma fundi oris et gingivae	1	5%

The study design complied with the tenets of the Declaration of Helsinki in all respects, and it was approved by the Research Ethics Committee for Human Medical Biology at the University of Szeged.

### **III.1.2. Oral sample collection**

Oral swabs were taken from a 1 cm<sup>2</sup> area from two different locations in the oral cavity (in the case of OSCC patients, both from the surface of neoplastic and healthy epithelium). Samples were inoculated on Sabouraud dextrose agar plates and Sabouraud broth, both incubated for 7 days at room atmosphere, at 32 °C. Direct samples that tested negative for yeast growth on agar plates after the first 3 days were subcultured from the liquid medium and incubated again for 7 days (henceforth “subculture”). Basic identification of the isolated yeasts was carried out based on macro- and microscopic morphology, the catalase test and CHROMagar Candida plates (Becton–Dickinson, UK). This was followed by a MALDI-TOF analysis (Bruker Daltonics, Bremen, Germany) of all the isolates.

### **III.1.3. MALDI-TOF MS**

Sample preparation was carried out according to the Bruker protocols using three methods: (1) direct transfer (DT), (2) extended direct transfer (eDT), and (3) ethanol (EtOH)–formic acid (FA) extraction.<sup>3</sup> For all methods, each tested strain was spotted in duplicate. For this study, the standard, commercially available Bruker Daltonics database (BDAL) was used. The MALDI-TOF MS identification results were automatically classified using the log-score values generated by the MALDI Biotyper software (Bruker Daltonics, Germany), performed according to the manufacturer’s instructions. Scores higher than 1.7 indicated genus level identification.

### **III.1.4. Analysis of enzymatic activity**

Extracellular lipase activity of *Candida* strains was examined on YNB-rhodamine B plates, according to the method of Nemeth and colleagues [Nemeth et al., 2013]. To summarize: strains were inoculated onto YNB-rhodamine B (yeast nitrogen base) plates (Sigma-Aldrich) and incubated at 30 °C for 7 days. Lipase positivity was determined by the presence of pink halo around the colonies. For the detection of proteolytic activity, *Candida* strains were cultured on YCB-BSA (Sigma-Aldrich) agar plates at 30 °C for 7 days, and proteolysis around the colonies was visualized by amido black (Sigma-Aldrich) staining. For the analysis, we chose 40 isolates from healthy controls and 140 isolates from OSCC patients.

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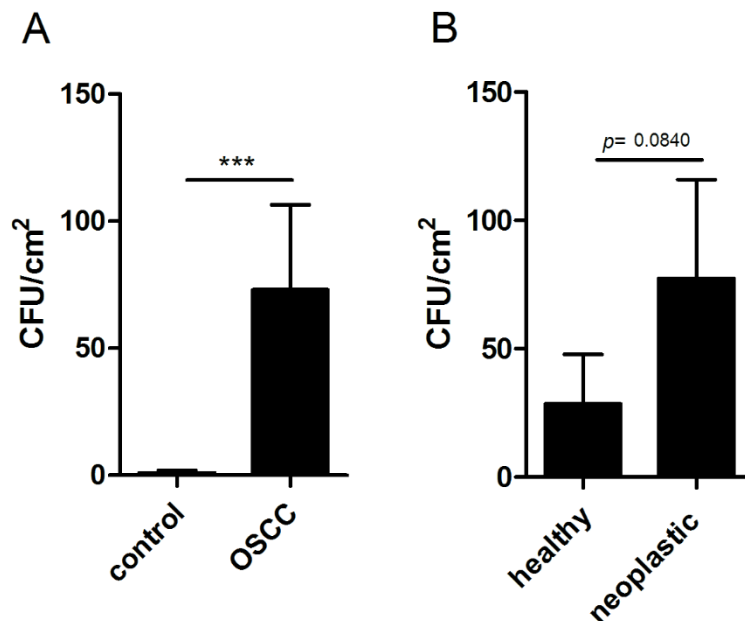
<sup>3</sup> See the attached article in the Appendix for details.

### III.1.5. Statistical analysis

All statistical analyses were performed in GraphPad Prism 5.0 (GraphPad, CA, USA). The Fisher's exact test, the Mann–Whitney U test or the Wilcoxon signed-rank test was used, as appropriate. Differences between the groups were considered significant at  $p < 0.05$ .

### III.2. Results

Eighteen (90 %) of the 20 OSCC patients and 12 (30 %) of the healthy controls had yeast isolated from their oral cavity, indicating significantly higher colonization in OSCC patients compared to the controls (Fisher's exact,  $p < 0.0001$ ). OSCC patients also had a significantly higher average fungal burden ( $73.08 \pm 33.39$  CFU/cm<sup>2</sup>) in their oral cavity compared to healthy individuals ( $1.10 \pm 0.78$  CFU/cm<sup>2</sup>, Fig. 1a), and samples taken from the neoplastic surface contained more yeast cells ( $77.38 \pm 38.53$  CFU/cm<sup>2</sup>) compared to the swabs taken from the healthy epithelium of the same individual ( $28.58 \pm 19.18$  CFU/cm<sup>2</sup>, Fig. 1b).



**Figure 1.** Fungal burdens in the oral cavity of patients and controls. A: Average fungal burdens (mean  $\pm$  SEM) in the oral cavity of control (n = 40) and OSCC patients (n = 20). In the case of OSCC patients, the average fungal burden of the healthy and neoplastic epithelial surfaces are shown. \*\*\* $p < 0.001$  (Mann–Whitney U). B: Average fungal burdens on the healthy and neoplastic epithelial surfaces in the oral cavity of OSCC patients (n = 20). The level of significance was determined by the Wilcoxon signed-rank test.



The results of the MALDI-TOF-MS are summarized in Table 2. *Candida* was the dominant genus in both groups beyond doubt, but it is to be seen that the yeast microflora of the OSCC patients was more diverse.

**Table 2.** Results of the MALDI-TOF-MS analysis

Genus	Number of isolates (%)	
	control (n=12)	patient (n=22)
<i>Candida</i>	10 (83.3)	15 (68.2)
<i>Rhodotorula</i>	-	2 (9.1)
<i>Saccharomyces</i>	-	2 (9.1)
<i>Kloeckera</i>	-	1 (4.5)
other (not identified)	2 (16.7)	2 (9.1)

The analysis of enzymatic activity indicated no significant difference between the protease/lipase producing activity of *Candidae* isolated from the healthy and OSCC samples (Table 3)

**Table 3.** Extracellular enzymatic activity of the isolates

		lip+	lip-	prot+	prot-	lip+/prot+	lip+/prot-	lip-/prot+	lip-/prot-
Control (N=40)	N	13	27	15	25	6	7	9	18
	%	32.5	67.5	37.5	62.5	15	17.5	22.5	45
OSCC (N=140)	N	53	87	66	74	37	16	29	58
	%	37.86	62.14	47.14	52.86	26.43	11.43	20.71	41.43

### III.3. Interpretation of the results

In this study we sought to characterize the oral yeast microflora of OSCC patients, with special attention to protease/lipase production as a proposed differentia specifica of carcinogenic yeasts [Mohd Bakri et al., 2010].

First of all, we found that the level of oral yeast carriage was significantly higher in OSCC patients. This came as no real surprise, as the association between oral yeast carriage and epithelial carcinoma had previously been pointed out by several authors [Alnuaimi, Wiesenfeld, O'Brien-Simpson, Reynolds, & McCullough, 2015; Barrett,

Kingsmill, & Speight, 1998; McCullough et al., 2002; Nagy et al., 1998]. In this sense, our results corroborate those of earlier studies.

Second, we tested the extracellular protease/lipase producing capacity of *Candida* strains to see if correlation can be found between the enzyme production and the colonization rates. We found no such correlation, which is against the role of fungal hydrolytic enzymes in the development of epithelial dysplasia.

Third, using MALDI-TOF-MS that represents a new and rapid method for the identification of yeasts in clinical samples [Bader et al., 2011; Pinto et al., 2011], we found that, in addition to higher fungal burdens, the spectrum of isolated yeast genera was wider in samples derived from OSCC patients compared to healthy controls. This finding provides further support to the assumption that the altered microenvironment associated with tumorigenesis leads to the development of a more diverse oral microflora.

What does this all tell us about the big question of yeasts and OSCC? Are yeasts a cause or a consequence? We suggest that the presented results support the latter. It has been recognized during the recent years that there is a delicate interplay between the innate immune system and commensal fungi that is responsible for maintaining the integrity of the mucosa. On the one hand, components of the innate immune system recognize the invading pathogens and limit the overgrowth of commensal fungi by intra- or extracellular killing. Furthermore, contact with commensal microbes induces tolerance that limits host damage caused by excessive inflammation [Romani, 2011]. Interleukin-10 (IL-10) is one of the most effective mediators of immune tolerance and it has been shown that patients with chronic candidal diseases often present high levels of this cytokine [Lilic et al., 2003]. It has been shown that patients with OSCC have increased levels of salivary IL-10, and high expression of this cytokine in tumor cells has been associated with poor prognosis [Aziz et al., 2015; Chen et al., 2013]. Therefore, the antiinflammatory environment of neoplastic epithelium might support the proliferation of commensal yeasts by suppressing the activity of innate immune cells that are responsible for the limitation of microbial overgrowth.

Therefore, from our results we draw the general conclusion that the altered immunological environment in OSCC opens up the way toward colonization by yeasts, even in the case of genera which would normally be suppressed. This also explains why extracellular hydrolytic enzyme production shows no correlation with colonization

rates: while enzyme production is indeed an important virulence factor, higher colonization rates in OSCC also require an altered immunological milieu.

## **IV. A methodological improvement for halitometry tested in OSCC patients**

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### **IV.1. Methods**

#### **IV.1.1. Subjects**

Thirty-five volunteers participated in the study. The volunteers were either healthy controls with excellent oral hygiene (11 females, 10 males, average age: 35.6 years), or OSCC patients (2 females, 12 males, average age: 59.8 years). Exclusion criteria included antibiotic treatment within four weeks prior to the measurements, and the consumption of onions, garlic or alcohol in two days prior to the measurements. All measurements were performed at least three hours after the last meal, drink or oral hygienic measure (e.g. toothbrushing, flossing, etc.). All measurements were carried out in triplicate, in each case between 8:30 AM and 12:30 PM.

The study protocol conformed to the tenets of the Declaration of Helsinki in all respects. All subjects gave their informed consent and the protocol was approved by the Research Ethics Committee for Human Medical Biology at the University of Szeged.

#### **IV.1.2. Hardware**

The two most common devices used in clinical breathanalysis - OralChroma (Abimedical Corporation, Japan) and Halimeter (Interscan Corporation, CA, USA) - were used (see also I.1.2). OralChroma is a portable gas chromatograph, which uses ambient air as a carrier gas and a semiconductor ( $\text{In}_2\text{O}_3$ ) gas sensor to detect the VSCs [Murata et al., 2006]. Halimeter is a portable sulphur monitor that uses an electrochemical sensor which generates a signal when exposed to sulphur-containing gases [Furne et al., 2002; Rosenberg et al., 1991]. Halimeter has a good time resolution, therefore it is capable of indicating changes within a narrow time window (5 min), which other techniques of measurement (including gas chromatography and gas chromatography-mass spectrography) would fail to detect. As the poor correlation between the various measurement systems is also a known problem, we sought to determine the correlation between the OralChroma and Halimeter measurements before and after the implementation of a newly written software for OralChroma (see below).

### **IV.1.3. Preparation of the Equipment**

First of all, the influence of the sampling time, sampled volume and syringe material on the reproducibility of the OralChroma chromatograms was determined.

Syringes with a rubber barrel seal (provided by the manufacturer) and all-plastic syringes (2 ml B Braun Inject Luer Solo, B. Braun Medical Inc., Germany) were tested. The reason for this was that in our experience all-plastic syringes are preferable, and a study by Tangerman and Winkel also came to this conclusion [Tangerman & Winkel, 2008].

Furthermore, our experience suggested that the 30 s sampling time (as recommended by the manufacturer) was too short in terms of reproducibility, wherefore we worked with a 2 min sampling time (i.e. the syringes were held in the oral cavity for 2 min).

OralChroma and Halimeter were calibrated with humidified (~2% water vapor) H<sub>2</sub>S mixed in synthetic air. Various H<sub>2</sub>S concentrations were prepared from certified cylinders (200 ppmv H<sub>2</sub>S in N<sub>2</sub> and synthetic air, Messer Hungarogas, Hungary) using mass flow controllers. The cross-sensitivity of OralChroma for volatile organic compounds (VOCs) was investigated quantitatively. Liquid standards (analytical grade) of isoprene and acetaldehyde (Sigma Aldrich, Schnellendorf, Germany) were used to prepare gases for the calibration of OralChroma. Gas samples with a known amount of isoprene were prepared by adding isoprene with Hamilton syringes (Hamilton Messtechnik GmbH, Germany) through a septum into a closed glass flask filled with air. Gas samples containing 46, 93, 185, 276, and 555 ppbv isoprene were prepared. The same procedure was used for acetaldehyde and samples containing 85, 250, 460, 505, 755, 925, 965, and 1385 ppbv acetaldehyde.

### **IV.1.4. Software**

A new software (written in LabVIEW, National Instruments, TX, USA) was developed to simplify and accelerate the re-evaluation of the OralChroma chromatograms. The code reads the files that are automatically generated by OralChroma when a measurement is saved, and detects local maxima in  $\pm 10$  s intervals of the expected peak locations (i.e. at 30, 60, 100, 150, 250, 350 s). Then it fits the sum of six Gaussian (18-parameter) functions using the Levenberg–Marquardt method [Levenberg, 1944; Marquardt, 1963]. As far as the initial parameters of the fitting

procedure are concerned, local maxima are used as initial peak heights, while initial full widths at half maxima and the peak centres are constant. The concentrations of hydrogen sulfide, isoprene, methyl mercaptan, dimethyl sulfide and acetaldehyde are calculated from the areas under the peaks at 60, 100, 150, 250 and 350 s, respectively. The sensitivity of OralChroma to methyl mercaptan and dimethyl sulfide was determined from chromatograms with regular peaks (without overlaps or retention time shift) at 150 and 250 s.

Statistical analysis was carried out in Statistica for Windows 11.0 (StatSoft, Inc., OK, USA).

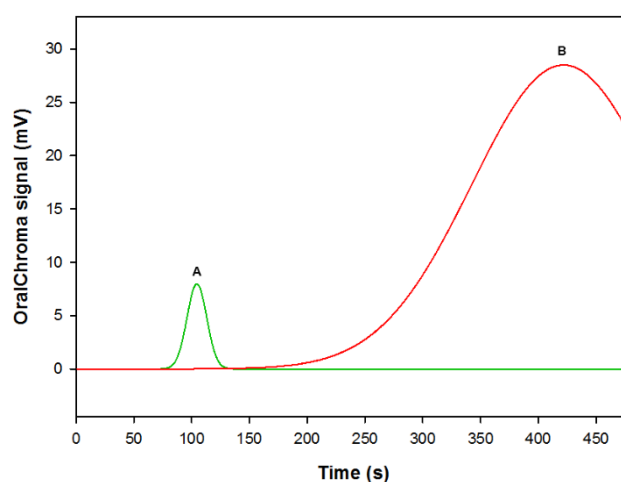
## IV.2. Results

### IV.2.1. Calibrations for isoprene and acetaldehyde

A distinct peak at 100 s was noticed on the chromatograms of 34 volunteers (97%) indicating the isoprene content of the sample. A linear relationship ( $R = 0.9980$ ) was found between the peak area and the concentration of isoprene. The sensitivity to isoprene was found to be  $4.32 \pm 0.07$  (mV·s)/ppbv.

In the majority of the cases (77%), the chromatograms showed a broad peak at 350-400 s, indicating the acetaldehyde content of the sample. A linear relationship ( $R = 0.9990$ ) was found between the peak area and the concentration of acetaldehyde. The sensitivity to acetaldehyde was found to be  $1.10 \pm 0.02$  (mV·s)/ppbv.

These peaks are shown in Figure 2.

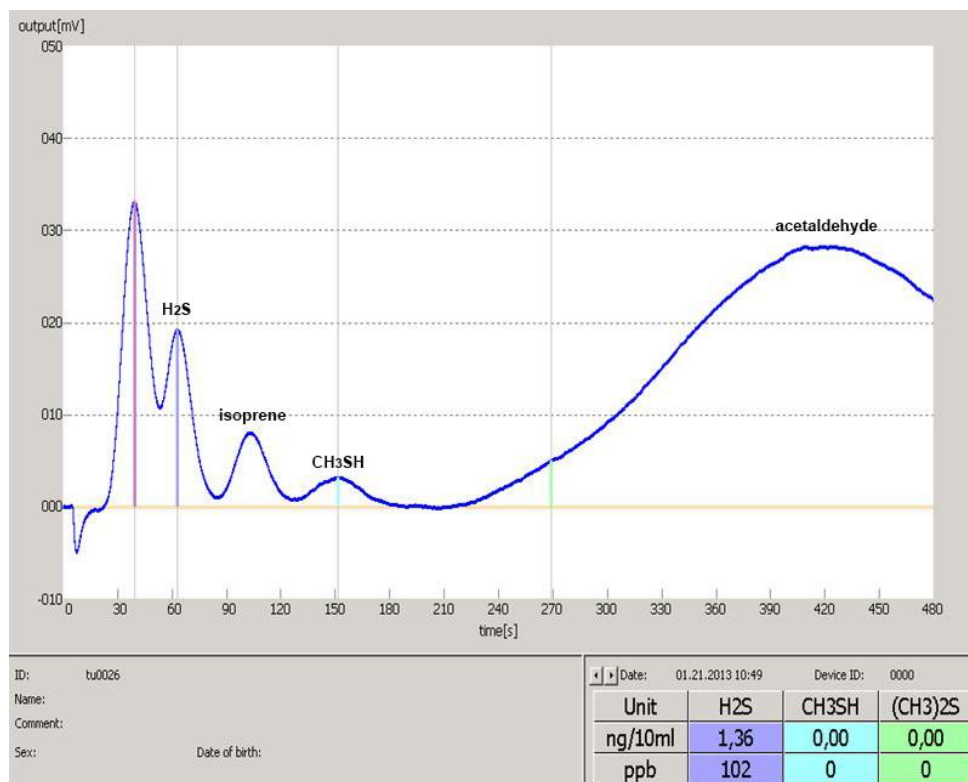


**Figure 2.** The peaks for isoprene (A) and acetaldehyde (B) as shown in the chromatogram (image generated in SigmaPlot from the data of OralChroma).

That is, we demonstrated that OralChroma is capable of measuring isoprene and acetaldehyde, even though its software was not designed to indicate the concentration of these substances in exhaled air.

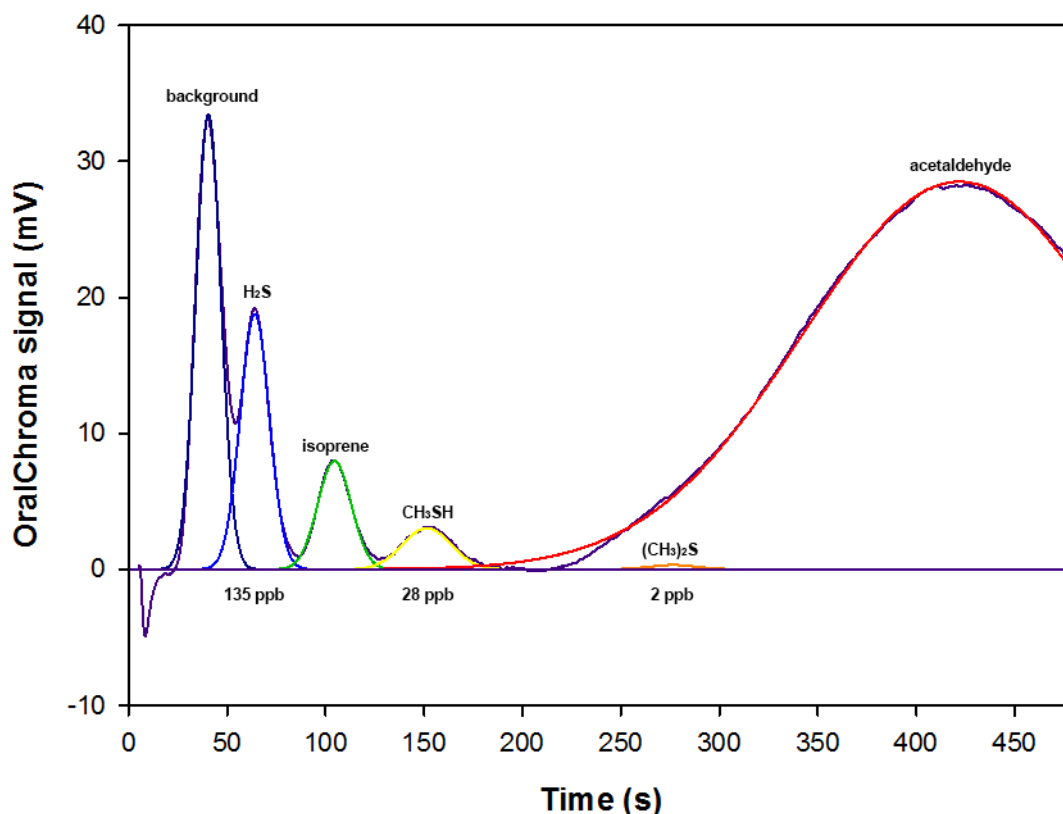
#### IV.2.2. The problem with the old software – and its solution

The most important problem with the default software of OralChrome is illustrated in Figure 3.



**Figure 3.** The output of OralChroma with the peaks and concentration data for the three most important VSCs (factory default software). The first peak is the background.

Two things are noticeable in this figure. First, while there is an obvious peak assigned to methyl mercaptan, its concentration is indicated as zero. Second, the system is completely insensitive to dimethyl sulfide – even though it is supposed to be able to detect it (no peak, zero concentration). This latter observation is especially interesting if one takes a look at the re-evaluated chromatogram (Fig.4.)



**Figure 4.** A re-evaluated chromatogram. The numbers under the baseline indicate the concentrations returned by the new software. Note that the chromatogram is very similar to the default one, but there are minute - still very important - differences, like the appearance of the dimethyl-sulfide peak.

The figure shows that the new software enabled OralChroma to estimate the concentration of methyl mercaptan (28 ppb), and a dimethyl sulfide peak also appeared (2 ppb). The latter had probably been masked by the wide acetaldehyde peak. It is to be noted that the software modification enabled the hardware to detect dimethyl sulfide at a rather small concentration. Comparative data on the accuracy of concentration estimation for the two software versions are given in Table 4. The table does not only show that the new software allowed a more precise estimation, but also that OSCC patients were characterized by significantly higher VSC concentrations (except for dimethyl sulfide).

Table 5 shows the isoprene and acetaldehyde concentration of the volunteers' samples assessed from the re-evaluation of the OralChroma chromatograms. Measurable isoprene was found in the breath samples of all healthy volunteers, and in those of 13 patients. As for acetaldehyde, this compound could be measured in the



samples of only 16 controls (of 21) and 11 patients (of 14). No significant difference between the patient and control group was found for isoprene and acetaldehyde concentrations. At the same time, acetaldehyde concentrations were significantly higher than isoprene concentrations in both groups.

**Table 4.** The accuracy of concentration estimation for the three main VSCs (and the summarized VSC concentration) for the factory default software and the new one. ppbv values are given as mean  $\pm$  SEM.

		factory default		new	
		ppbv	range	ppbv	range
<b>controls</b>	H <sub>2</sub> S	63 $\pm$ 16	0–264	66 $\pm$ 16	2–285
	CH <sub>3</sub> SH	28 $\pm$ 8	0–373	16 $\pm$ 5	0–67
	(CH <sub>3</sub> ) <sub>2</sub> S	29 $\pm$ 14	0–285	16 $\pm$ 8	0–165
	sumVSC	119 $\pm$ 43	0–906	98 $\pm$ 27	4–477
<b>patients</b>	H <sub>2</sub> S	274 $\pm$ 92	1–996	285 $\pm$ 91	2–1025
	CH <sub>3</sub> SH	122 $\pm$ 42	0–430	138 $\pm$ 38	0–401
	(CH <sub>3</sub> ) <sub>2</sub> S	32 $\pm$ 9	0–108	28 $\pm$ 9	0–113
	sumVSC	428 $\pm$ 132	4–1351	451 $\pm$ 131	3–1367

**Table 5.** Concentration of isoprene and acetaldehyde estimated from the re-evaluated chromatograms.

	isoprene (ppbv)		acetaldehyde (ppbv)	
	mean $\pm$ SEM	range	mean $\pm$ SEM	range
<b>controls</b>	70 $\pm$ 10	7–164	810 $\pm$ 220	0–4120
<b>patients</b>	36 $\pm$ 10	0–143	729 $\pm$ 490	0–7050

A further indicator of accuracy is the standard error of consecutive measurements (three in this study). We calculated this both for Halimeter and OralChroma. The results are summarized in Table 6.

**Table 6.** Relative standard errors from three consecutive measurements.  $\text{sumVSC} = [\text{H}_2\text{S}] + [\text{CH}_3\text{SH}] + [(\text{CH}_3)_2\text{S}]$ .

	Halimeter	OralChroma factory default		OralChroma new software	
		H <sub>2</sub> S	sumVSC	H <sub>2</sub> S	sumVSC
<b>controls</b>	0.131	0.455	0.463	0.289	0.285
<b>patients</b>	0.121	0.365	0.343	0.281	0.264

Indeed, it seems that Halimeter yields better reproducibility than OralChroma, but it is also clear that the software improvement improved the reproducibility of the OralChroma measurements.

#### IV. 2.2. Correlation between OralChroma and Halimeter

As the poor correlation between instrumental measurements in this field is a well-known problem, OralChroma measurements were compared to Halimeter measurements in order to find out if the new software can also address this problem. Measurements in two parameters were compared: the concentration of hydrogen sulfide and the summed VSC concentration. Summed VSC concentration was defined as the sum of the concentrations of the three main VSCs (H<sub>2</sub>S, CH<sub>3</sub>SH, (CH<sub>3</sub>)<sub>2</sub>S). The results are shown in Table 7. As the table shows, the correlations between the measurements were significant already with the default software, but with the new software, they became stronger (see next page).

**Table 7.** Correlation between the measurements of OralChroma and Halimeter before and after the software improvement. Asterisk denotes significance at  $p < 0.01$ ,  $\text{sumVSC} = [\text{H}_2\text{S}] + [\text{CH}_3\text{SH}] + [(\text{CH}_3)_2\text{S}]$ .

	Pearson's correlation coefficient between Halimeter and OralChroma			
	default software		new software	
	H <sub>2</sub> S	sumVSC	H <sub>2</sub> S	sumVSC
<b>controls</b>	0.788*	0.581*	0.819*	0.723*
<b>patients</b>	0.570*	0.571*	0.634*	0.689*

### IV.3. Interpretation of the results

OralChroma is a commonly used device that allows differentiation between the three major VSCs in breath air. While its hardware meets the requirements of the field of halitosis research, its software has several limitations that make it less suitable for routine use (see before). There were studies that suggested that OralChroma chromatograms should always be inspected visually to correct the erroneous VSC peak assignment of the default software [Tangerman & Winkel, 2008]. Other authors went so far as to recommending a manual calculation of the concentration of the individual VSCs [Laleman, Dadamio, De Geest, Dekeyser, & Quirynen, 2014; Snel et al., 2011; K. Yaegaki et al., 2012]. It is easy to see, though, that such an approach is especially time-consuming, and the uncertainty of the analysis is considerable.

With this study we sought to address this problem by improving the default evaluation software of OralChroma by a) making it more precise, b) enabling it to identify and measure isoprene and acetaldehyde. Involving OSCC patients in the study allowed us to a) test the new system also at higher VSC concentrations, and b) to determine if the concentration of isoprene and acetaldehyde are present in elevated concentrations in the exhaled air of OSCC patients.

As for the methodological goals of the study, we have succeeded in enabling OralChroma to assign peaks more correctly and precisely, and as a result of the improvement, the concentrations of both CH<sub>3</sub>SH and (CH<sub>3</sub>)<sub>2</sub>S could be determined. It is

notable that  $(\text{CH}_3)_2\text{S}$  was detected at a very small concentration, showing both the sensitivity of the hardware and the ability of the software to exploit the possibilities of the hardware.

As for the composition of exhaled air in controls and OSCC patients, the results were partially expectable: as demonstrated by several studies before [Aylikci & Colak, 2013; Hakim, Billan, Tisch, et al., 2011; Schmutzhard et al., 2008], the main VSCs were found to be elevated in OSCC. In this sense, our measurements corroborated the results of previous studies. The results regarding acetaldehyde, though, were somewhat surprising. Naturally, as acetaldehyde is an endogenous metabolite, which is removed mostly through the lungs, it is present in exhaled air even without drinking any alcohol. Jones adds that higher levels were observed in abstinent alcoholics and smokers [Jones, 1995]. The surprising element here is that, based on the above, in an OSCC sample, acetaldehyde should be elevated, smoking and alcoholism being the most important risk factors of the disease [Nemes, Redl, Boda, Kiss, & Marton, 2008], and also considering the practical observation that the patients often fail to quit either habit when they get to know about their condition. Unfortunately, we did not assess the smoking and alcohol consumption of our patients, but given that they were chosen in a way as to be representative of the Hungarian OSCC population, it is less than likely that they quit cigarettes and drinking all of a sudden when they learned about the diagnosis. One explanation for this counterintuitive result could be that the device is not sensitive enough in terms of difference, even with the new software. In other words, this would mean a poor resolution. A look at the dimethyl sulfide peak, though, proves this assumption wrong. The device was capable of detecting a tiny peak of 2 ppb concentration. There is no good reason to assume that it could be markedly less sensitive to acetaldehyde. Ultimately, this unlikely result is probably statistical in nature: Tables 4 and 5 clearly show that the variability of acetaldehyde (and isoprene) concentration is pretty high as compared to that of the main VSCs. This means that a significant difference was easily detectable between the groups regarding the main VSCs even with only 14 patients, but this number of patients was not enough in the case of acetaldehyde, given the high variability. Re-assessment with a higher number of patients is definitely necessary here. The lack of significant difference in the concentration of isoprene came less as a surprise. Breath isoprene accounts for most of the hydrocarbon removal via exhalation [King et al., 2010; King et al., 2012], and

altered levels are mostly associated with systemic conditions [Karl et al., 2001; Neupane et al., 2016]. This could be seen as evidence that isoprene concentration is not a marker of OSCC, but, once again, it must be emphasized that the number of observations was low. A glance at the means tells us that the concentration of isoprene in the control group was half of that of the study group, while the difference in acetaldehyde concentration was negligible as compared to that. This raises the possibility that a decrement in isoprene concentration (for reasons unknown at this point) could be a marker, but measurements with a larger study group are definitely necessary. The ratio of the two components (i.e. isoprene and acetaldehyde), though, was rather similar – and significantly different – in the two groups, which supports the similarity of the two groups. As can be seen, therefore, our results regarding isoprene concentrations in the exhaled air of OSCC patients and controls can be regarded as preliminary results, but they show a direction for further research.

## **V. Recapitulation of the results and conclusions**

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In this thesis I examined two problems related to (the microbiology of) oral squamous cell carcinoma: oral yeast carriage and the composition of the exhaled air. In connection with the latter, I also demonstrated a methodological improvement.

As for the first topic, the hypotheses were:

- a) a bigger variety of yeast genera would be found on the neoplastic epithelial surface, and in higher numbers, as compared to the healthy surface.

This hypothesis was correct, the yeast (especially *Candida*) burden in OSCC was higher than in healthy controls, corroborating the well-known link between OSCC and *Candida* spp. The finding regarding the variety of genera provides further support to the assumption that the altered microenvironment associated with tumorigenesis leads to the development of a more diverse oral microflora.

- b) the lipase/protease producing activity of the *Candida* strains would be associated with their ability to colonize the epithelium.

This hypothesis was proven wrong. There was no association between the extracellular hydrolytic enzyme production of the isolated strains and their colonization potential. Also considering the variety of colonizing species in OSCC, we conclude that the altered immunological environment in OSCC opens up the way toward colonization by yeasts, even in the case of genera which would normally be suppressed. The lack of correlation between extracellular enzyme production and colonization does not mean that the production of such enzymes is not an important virulence factor. It is, as it was shown by several studies. However, higher colonization rates in OSCC also require the altered immunological milieu described in this disease.

As for the second topic, the following hypotheses were set:

- a) our enhanced software would allow a more precise determination of exhaled air components than the default software and that the measurement of isoprene and acetaldehyde would also become possible.

This hypothesis was correct. The improvement of the default factory software of OralChroma made it possible for us to measure VSC concentrations more accurately. The new software also enabled the hardware to measure the concentration of isoprene and acetaldehyde.

- b) regarding the exhaled air of OSCC patients, we hypothesized that our results would corroborate the results of previous studies, especially concerning elevated VSC concentrations. As for acetaldehyde and isoprene, we expected different levels between controls and OSCC patients.

The first part of this hypothesis was correct: like in several previous studies, we found elevated VSC concentrations in OSCC. However, the concentrations of isoprene and acetaldehyde did not differ significantly between the two groups. This we put down to the small sample size, and we propose that research with larger sample sizes could show significant difference – especially in the case of isoprene, the concentration of which appears to decrease in OSCC. This latter observation is an especially intriguing one.

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## **APPENDIX**