

WHITE PAPER

THE SCIENCE OF EARLIER™: IMPROVING EARLY DETECTION OF ORAL AND OROPHARYNGEAL CANCER

Background of Oral Cancer

When it does not otherwise result in death, head and neck squamous cell carcinoma (HNSCC), defined here as cancers of the oral cavity and oropharynx (collectively referred to herein as “oral cancer”, oral squamous cell carcinoma (OSCC), or HNSCC), is a debilitating and deadly disease where both the treatment and the disease itself can often result in disfigurement and the impairment of speech and eating function. HNSCC is an aggressive tumor with poor response to chemotherapy and basic resistance to most standard of care anticancer therapies (1). Five-year survival rates are 63% for the United States (1), approximately 50% for Europe, and lower in developing countries (2). This devastating type of cancer arises from the mucosal lining of the oral cavity and oropharynx. HNSCC is the most frequent malignant tumor of the head and neck region accounting for 90% of malignancies in the oral cavity and oropharynx (1).

The American Cancer Society anticipates that nearly 50,000 individuals will be diagnosed with oral cavity and pharyngeal cancer in the United States in 2017 (3). (Pharyngeal cancer includes nasopharyngeal and hypopharyngeal, in addition to oropharyngeal cancer, however oropharyngeal cancer is the most common of these subsites (2).) The World Health Organization recently reported over 529,000 new cases of oral cavity and pharyngeal cancers diagnosed worldwide on an annual basis (2,4), a figure which is predicted to rise by 62% to 856,000 cases by 2035 (2). The incidence of HNSCC is approximately 2.5 times higher in men than women (2,4).

Traditionally, tobacco products are known to be a primary cause of this disease with predilection for the floor of the mouth, the lateroventral tongue, the tonsils and base of tongue (1). Excessive alcohol use is also considered a high risk factor, especially when combined with tobacco products since the two act synergistically (1). Betel quid (paan) chewing is a common practice in many parts of Asia and in migrant Asian communities around the world, with 600 to 1200 million users estimated globally. In populations with this habit, the tongue and buccal mucosa are the most common

sites (1). The human papilloma virus (HPV) infection, associated with sexual activity, is also an important risk factor independent of race, gender, tobacco use, alcohol consumption, or other risk factors (5).

For oropharyngeal cancer, prevalence of HPV related disease is highest (approximately 60%) in North America; intermediate (approximately 36% to 45%) in Asia, Oceania, and Europe; and low (approximately 15%) in South and Central America (1). The recent dramatic rise in HPV-related oropharyngeal cancer incidence points to a potential emerging cancer epidemic (1). In the early 1980s, HPV accounted for only 16% of oropharyngeal cancers in the United States. Now over 60% of oropharyngeal cancers in the United States are caused by HPV with increases in Europe mirroring this trend (1). While there are many oncogenic forms, HPV-16 is responsible for 90% of HPV-related oropharyngeal cancer (1).

In addition to the risk factors mentioned, others include gastroesophageal reflux disease (GERD) (6), passive smoking (7), and viruses such as Epstein-Barr virus (EBV) (8). Further reported risk factors include microbes, potentially through production and metabolism of carcinogens or induction of chronic inflammation; immunosuppression such as occurs with human immunodeficiency virus (HIV) and transplant patients; environmental and occupational exposures such as hot oil fumes (7), heavy metals, solvents and wood dust; dietary factors and deficiencies including low fruit and vegetable intake and vitamin deficiency; and heritable conditions such as Fanconi anemia, and dyskeratosis congenita, and Bloom syndrome (1).

The Oral Cancer Problem

Among the 292,000 people that die from oral cancer worldwide each year, the disease kills more than one person per hour each day in the United States (4). Survival rates have improved minimally over several decades (9). Oral cancers tend to recur locally but

also can metastasize. Second primary cancers are also common due to the effects of carcinogens on other parts of the upper aerodigestive tract (9,10).

Although the current 63% survival rate in the United States is a slight improvement over the last ten years, this improvement is due to the increase of HPV-16 associated cancers which are more vulnerable to existing treatment modalities that confer a significant survival advantage (11). Therefore, it is a change in etiology and not early discovery or treatments that are responsible for improvement. While prognosis is better with HPV-associated disease, this cancer occurs more frequently in younger patients (1). These younger patients must live a large percentage of their life with the long-term effects of treatment including swallowing and speech problems. Oncologists are working to identify de-intensified treatment strategies for these patients; however, those HPV patients diagnosed in late stage will still need intensive therapy (12).

Certain ethnic groups suffer disproportionately with poorer survival. For example, blacks have mortality rates from oral cancer that are nearly twice as high as whites (13). Black males tend to have more advanced, aggressive forms of disease and a lower percentage of HPV-positive disease compared to their white counterparts (14). Diagnosis in early stage (I, II) rather than late or advanced stage (III, IV) would improve survival. Since many factors impact prognosis an accurate staging system is important to direct appropriate personalized treatment strategies.

Oral Cavity and Oropharyngeal Cancer Staging

Cancer staging systems are designed to group patients into categories that are associated with unique, prognostic gradations. In general, patients with HPV-associated oropharyngeal cancer have a much better prognosis than patients with HPV-negative oropharyngeal cancer or oral cavity cancer. Addressing this difference in prognoses, the recently released eighth edition of the American Joint Committee on Cancer (AJCC) Staging Manual, Head and Neck Section, introduced significant modifications from the prior seventh edition (15). The changes are very likely to improve stratification of patients into prognostically meaningful categories. The details of the new staging process are beyond the scope of this white paper, but major changes are noted. In the prior seventh edition, oral cavity and oropharyngeal cancer (whether HPV positive or negative) were staged in a very similar manner for the tumor (T), regional lymph nodes (N) and distant metastases (M). The stage progressed each 2 cm of tumor size from T1 to T3 with extent

into adjacent structures resulting in T4 and Stage IV designation. Similarly, nodal status was also treated the same regardless of HPV status. For example, a single positive node 3 cm or less resulted in N1 and Stage III designation. Additionally, any node greater than 3 cm resulted in N2 and Stage IV status irrespective of HPV. In the new system set forth in the eighth edition, there are separate stages for HPV-positive and HPV-negative tumors of the oropharynx. For example, in the new staging system, HPV-positive oropharyngeal cancer, a patient with T2, N1 disease is still stage I where in the prior version that patient would be stage III. In the new system, stage IV designation for HPV-positive tumors is reserved for patients with distant metastases. Other changes in the new system include addition of extracapsular spread (tumor outside the confines of the lymph node capsule) in nodal staging of HPV-negative tumors and depth of invasion (depth from basement membrane to deepest extent of tumor infiltration on pathology) in oral cavity tumor staging. Both of these changes have been shown to improve prognostic stratification in these scenarios (15).

A risk-based informative staging system is critical because oral cancer, when identified in stage I or II, carries an overall five-year survival rate over 80% (15). All too often, however, the manifestations of this invasive and devastating disease are found later, during either stage III or IV, where the five-year survival rate falls to less than 40% (15). Unfortunately, over 60% of oral cancer patients in the United States are identified with the advanced stage of disease (16). Clearly, a better method for identifying early oral cancer is needed.

The Current Standard for Oral Cancer Screening

Cancer screening by definition is the process by which a healthcare provider evaluates an asymptomatic patient to detect malignancy (17). In certain scenarios, cancer screening has been shown to decrease the risk of premature death and also reduce cancer-related morbidity since detection in early stage requires less aggressive treatment. The benefits of screening have to be weighed against the risks including physical injury (e.g. colon perforation for colon cancer screening, radiation for lung cancer screening); emotional and physical risks to the patient should there be a false-positive diagnosis and unnecessary intervention; overdiagnosis of cancers that would not be ever have been clinically important; and false-negative screening resulting in delayed diagnosis (17).

The current method used to diagnose oral cancer and oral potentially malignant lesions (also referred to as oral potentially malignant disorders) is history and

physical examination followed by biopsy (18). This is most commonly done on oral lesions self-identified by patients or detected during routine examinations by dental professionals and otolaryngologists. (18-20). Head and neck surgeons and oral and maxillofacial surgeons usually assist with biopsy and then direct treatment of these patients (21,22).

The US Preventative Services Task Force (USPSTF), sponsored by the US Department of Health and Human Services Agency for Healthcare Research and Quality, has been tasked to rigorously evaluate benefits of preventative services such as cancer screening, provide recommendations about which preventative measures to implement in clinical practice, and identify areas for future research (23). Based on the data available up until 2011, the last US Preventative Services Task Force review on screening for oral cancer in 2013 concluded that “the evidence is insufficient to determine the balance of benefits and harms of screening for oral cancer in asymptomatic adults by primary care providers” (24). Importantly, however, they also state that this recommendation/conclusion “does not pertain to dental providers or otolaryngologists. Dental care providers and otolaryngologists may conduct a comprehensive examination of the oral cavity and pharynx during the clinical encounter” (24). The American Dental Association recommends that clinicians remain alert for oral cancer while performing routine visual and tactile examinations in all patients, but particularly in those who use tobacco or consume alcohol heavily (25). The American Head and Neck Society also advocates for the performance of a comprehensive oral head and neck exam, particularly in symptomatic or at risk individuals since this is the best known method of detecting oral cancer (26).

Conventional Oral Exam and its Limitations

The oral cancer screening exam consists of visual and tactile palpation during an extra and intra oral inspection by the healthcare professional. This head and neck examination entails bimanual palpation of various areas of: 1) the external head and neck including the lower jaw, neck, glands and lymph nodes of this area, 2) the oral cavity including the oral tongue, cheeks, floor and roof of the mouth and lips, and 3) the back of the throat including tonsils and tongue base (27).

During this examination, the clinician traditionally looks for clinical features of oral lesions that might raise suspicion of potential malignancy including sharp or distinct margins, a red component (color variation), a non-homogenous white component (surface irregularity), persistent ulceration and large size (greater

than 200mm²)(1). The clinician also should view with suspicion any persistent or progressive lesion of the ventrolateral tongue or the floor of the mouth (both of which are high-risk sites for oral squamous cell carcinoma) (1). If these types of areas are present, head and neck surgeons and oral and maxillofacial surgeons usually perform the biopsy and then direct treatment of these patients if malignancy or premalignancy is found (21,22).

Recent evidence suggests that 28% of patients presenting to the dentist have some type of oral mucosal abnormality (28). With over 60 percent of adults visiting the dentist every year (29) and increasing concern regarding HPV-related oral cancer, the numbers of patients needing follow-up for concerning findings is staggering. Furthermore, missed cancer diagnosis is becoming the leading malpractice claim for dentists (30). This situation leaves clinicians in a conundrum where referring these patients for biopsy risks flooding the healthcare system with benign conditions, while not referring risks missing a potentially lethal diagnosis. Only about 30% of mucosal abnormalities that are biopsied are premalignant or malignant (31). Better methods for determining when to biopsy, including repeat biopsy, are needed.

In addition to the challenge of culling patients who have potentially malignant abnormalities from the vast number of individuals with abnormal oral mucosa, successful early detection is also thwarted because the disease can be invisible to the naked eye until it reaches advanced stage. Identifying this disease at a reversible stage could prevent the devastating effects of oral cancer. Although the visible tissue may appear normal, the truth hides within the cells below the surface of the mucosa. Certainly by this stage, the opportunity for early intervention is lost. The examination is subjective and not very accurate (sensitivity 64%, specificity 31-76%) (31,32). Furthermore, since premalignancy is a reversible state (33) and premalignant lesions often regress with tobacco cessation (34), this lead time offers the opportunity to administer smoking intervention. However, if the lesions are not visible, the opportunity to detect the disease in this reversible state is lost.

Adjuncts to Physical Exam

A number of technologies are used in clinical practice that attempt to augment physical exam and early detection (35).

Autofluorescence is based on altered interactions of light with epithelium and stroma based on changes in the structure (e.g., hyperkeratosis, hyperchromatin and increased cellular/nuclear pleomorphism) and metabolism (e.g. concentration of flavin adenine dinucleotide [FAD] and nicotinamide adenine

dinucleotide [NADH]) that occur in oncogenesis. Specifically, these epithelial and stromal changes can alter the distribution of tissue fluorophores and as a consequence the way they emit fluorescence after stimulation with intense blue excitation light (35).

Chemiluminescence technology involves the use of an oral rinse with a 1% acetic acid solution followed by the examination of the oral mucosa under diffuse chemiluminescent, blue-white light. Theoretically, the acetic acid removes the glycoprotein barrier and slightly desiccates the oral mucosa. As a result, abnormal cells of the mucosa absorb and reflect the blue-white light in a different way with respect to normal cells (35).

Oral brush cytology involves the use of a specialized brush that samples the superficial, intermediate and parabasal/basal layers of the epithelium. This test was designed to investigate low-risk mucosal abnormalities that would otherwise not be biopsied (35).

Toluidine blue also known as tolonium chloride, is a vital dye that stains nucleic acids and is used as an aid to the identification of clinically occult mucosal abnormalities (35).

None of the technologies have been shown to decrease the morbidity and mortality of oral cancer, and therefore, have not become widely accepted (36). These adjuncts have a wide range of sensitivities and specificities (30-90%) and have been unable to significantly improve upon early detection rates (37-42). Furthermore, approaches that are limited to the visualization of the oral cavity may miss potential dysplastic regions in the oropharynx.

Salivary Testing

Using saliva for disease diagnostics and health surveillance is a promising approach given the specimen can be obtained at the site of the potential lesion, more inexpensively and less invasively than blood (which requires a detectable systemic change), and collected without special training (43). Over the past two decades, using salivary biomarkers specifically for early cancer detection attracted much research interest, especially for cancers occurring in the oral cavity and oropharynx for which the 5-year survival rate is still one of the lowest among all major human cancers (44,45). Saliva is a complex mixture which includes components of salivary gland secretions, sloughed mucosal cells, microbial flora, byproducts (e.g. food debris), nasal secretions, and a variety of macromolecules and proteins. In general, there are three methods for collecting saliva which include the following: a) salivary rinse, b) whole, unstimulated saliva, and c) whole stimulated saliva (43). One of the main advantages for using an oral (salivary) rinse, as opposed to the whole saliva or stimulated

salivary collection processes, is that the specimen is not restricted to the anterior oral cavity and allows a more complete sampling of the entire oropharynx, including parts of the larynx and hypopharynx.

Investigations on salivary biomarkers for oral cancer detection have been extensively reviewed by Cheng et al. (43). These and other studies have identified a series of salivary proteins (e.g. CD44, IL-8, cyclin D), auto-antibodies against aberrantly expressed TP53, and melanoma-associated antigen proteins (e.g. MAGE) (46-51). In addition to proteins there are a variety of salivary molecules including TP53 DNA variants, total DNA, evidence of loss of heterozygosity (LOH) or microsatellite instability, telomerase activity, mRNA's, metabolites, and epigenetic markers (52-58). Thus far, more than 100 potential salivary biomarkers have been reported in the literature relying mainly on a comparison of patients with oral cancer vs. controls to assess relevance in disease assessment.

CD44 and Total Protein

CD44, is a cell surface transmembrane glycoprotein involved in cell proliferation, cell migration, and tumor initiation (59-62). Premalignant lesions also show overexpression of CD44 (63-65). Thus, CD44 is involved in the very earliest stages of carcinogenesis and with aggressive disease. This is further exemplified by an enrichment of cells with CD44 on the surface in relapsed tumors when compared with the primary tumor (66). Soluble CD44 (solCD44), released by proteinases, is detectable in body fluids including saliva captured in an oral rinse (67-70). CD44 can be measured with simple, inexpensive assays, and is overexpressed in oral cavity and oropharyngeal cancers, and appears to be useful in both HPV positive and negative disease (47, 68-70). Early studies demonstrated that solCD44 is able to distinguish early stage and advanced HNSCC patients from benign disease controls with 62% sensitivity and 88% specificity (47).

In these earlier studies, total protein levels were utilized as a potential normalizer for solCD44 levels. However, the studies identified that total protein levels were elevated in oral rinses from cancer patients verses. controls (47). A follow-up clinical study confirmed a role for combining both solCD44 and total protein in the prediction of HNSCC (69). In fact, subjects that had high levels of both CD44 and total protein were 25 times more likely to have cancer (69). More recent case-control analyses have confirmed that the combined concentration of solCD44 and total protein measured in an oral saline rinse saliva specimen was significantly associated with oral cancer with an AUC of 0.77 independent of tobacco or alcohol use, age, oral

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health, gender, race, and other factors (71). The study showed that the markers could detect both early and late stage disease and that higher levels of solCD44 were associated with worse prognosis (71) consistent with prior publications on salivary CD44 levels by this and other groups (72).

PATIENTS WITH ELEVATED SOLCD44 AND PROTEIN LEVELS MAY BE 25X MORE LIKELY TO HAVE ORAL CANCER THAN THOSE WITHOUT.

The Science of Earlier™

In 2011, Vigilant Biosciences, Inc. (“Vigilant”) licensed the intellectual property based on this research from University of Miami and commercialized the technology for clinical assay development. Vigilant leveraged this technology to develop its initial, pioneering products for oral cancer using the CD44 and total protein biomarkers. The initial products under the OncAlert® brand include: the OncAlert® Oral Cancer RAPID Test (a rapid, point-of-care qualitative test) kit and the




OncAlert® Oral Cancer LAB Test (a pair of quantitative, laboratory tests). Both products are CE Marked.

The Vigilant OncAlert product line is designed to address a clinical unmet need by providing simple, accurate, and cost-effective early detection assays. The OncAlert RAPID Test is an easy to use lateral flow device with results reported in 20’ without expensive equipment while the OncAlert LAB test is a quantitative algorithmic assay providing probability risk scores for predicting head and neck cancer.

NO SPECIAL TRAINING IS REQUIRED WITH THE ONCALERT® ORAL CANCER TESTS.

No special training is required for implementing either of the OncAlert tests into general clinical practice. The OncAlert Oral Cancer product line provides the treating physician with useful tools to assist in identification HNSCC possibly in its earliest stages for optimal health outcomes associated with earlier detection and intervention.

Table 1.

Detection Technique	Tumor Initiating and Stem Cell Associated Biomarker	Noninvasive	Sensitivity **	Specificity **
OncAlert Oral Cancer Product Line			0.84 RAPID Test (0.71 – 0.85)	0.93 LAB Test (0.30 - 0.93)
Current Standard of Care: 1st Step - Visual; 2nd Step: Biopsy of suspicious lesions (Conventional Oral Exam – COE)			0.64	0.31-.76
Vital Staining (toluidine blue, tolonium chloride)*			0.84 (0.56-1.00)	0.70 (0.0-0.93)
Oral Cytology			0.91 (0.38-1.00)	0.91 (0.25-1.00)
Light-Based Detection (fluorescence, chemiluminescence)			0.91 (0.42-1.00)	0.58 (0.0-0.98)

*Macey R, Walsh RT, Brocklehurst P, et al. Diagnostic tests for oral cancer and potentially malignant disorders in patients presenting with clinically evident lesions. (Review). Cochrane Database of Systematic Reviews. 2015, Issue 5. The Cochrane Collaboration.

**Challenges when comparing testing approaches include overall cohort composition and heterogeneity of disease under investigation. In the Macey et al. 2015 review, prevalence ranged from 4-97% across all studies (median range: 20-50%) and there was additional heterogeneity in the eligibility criteria used, which makes a direct comparison more problematic.

Summary Note: None of the adjunctive tests can be recommended as a replacement for the currently used standard of a scalpel biopsy and histological assessment.

OncAlert Specimen Collection and Analysis Process:

1. Swish and gargle 5 ml saline in the mouth for 10 seconds.
2. Spit into specimen cup. The same specimen can be used for either the OncAlert Oral Cancer RAPID or LAB tests.
3. For the OncAlert Oral Cancer RAPID Test - Insert OncAlert Oral Cancer RAPID test cassette into the specimen cup.
4. Wait 20 minutes for a colorimetric result indicating presence of CD44 and/or total protein based on predetermined threshold(s). See Figure 1.
5. For the OncAlert Oral Cancer LAB Test, the entire sample will be sealed and stored at -20C and then shipped to an authorized reference laboratory for the OncAlert Oral Cancer LAB Test.
6. Once the patient has provided the sample, the health care provider is advised to perform an internal and external visual and bimanual palpation head and neck examination to determine if there are any visible or palpated abnormalities, as follows:
 - » Lips
 - » Floor of mouth
 - » Roof of mouth (hard and soft Palate)
 - » Buccal mucosa
 - » Tongue (dorsal and ventral surfaces and lateral borders)
 - » Tonsil areas
 - » Palpate base of tongue, floor of mouth
 - » Palpate neck

The results of this examination are noted in the patient record.

7. OncAlert Oral Cancer RAPID Test results are observed at 20 minutes. Together with the physical examination, the healthcare provider would then discuss the results with the patient. If the test has a negative result and with no observable lesion, it is recommended to repeat the OncAlert Oral Cancer RAPID Test within 1 year and have a discussion with the patient surrounding modifications of any risk behavior. If a lesion is observed and the test is negative, the decision to biopsy remains at the sole discretion of the treating physician. At any point in this discussion the quantitative OncAlert Oral Cancer LAB test can be introduced to help refine the risk discrimination process.
8. If the OncAlert Oral Cancer RAPID or LAB Test(s) are positive and a suspicious lesion is identified, the clinician is recommended to proceed with appropriate action. If either test is positive without an observable lesion, the clinician will discuss several possible options with the patient including (without limitation):

- a. Lifestyle changes to help reduce and possibly reverse disease progression (tobacco cessation, alcohol reduction or elimination, practice good oral hygiene and improve nutrition).
 - b. Schedule a second OncAlert Oral Cancer RAPID Test in 1 to 3 months.
 - c. Schedule a more quantitative assay such as the OncAlert Oral Cancer LAB test.
9. If a second OncAlert Oral Cancer RAPID or LAB Test is negative, routine follow-up (per healthcare profession protocol) with repeat OncAlert Oral Cancer RAPID or LAB Test annually.
 10. If second OncAlert Oral Cancer RAPID or LAB Test is positive, refer to appropriate specialist (e.g. oral surgeon, otolaryngologist, head and neck surgeon). Specialist can perform additional tests available for further examination including the use of the quantitative OncAlert Oral Cancer LAB Test if only the OncAlert Oral Cancer RAPID Test has been used previously.

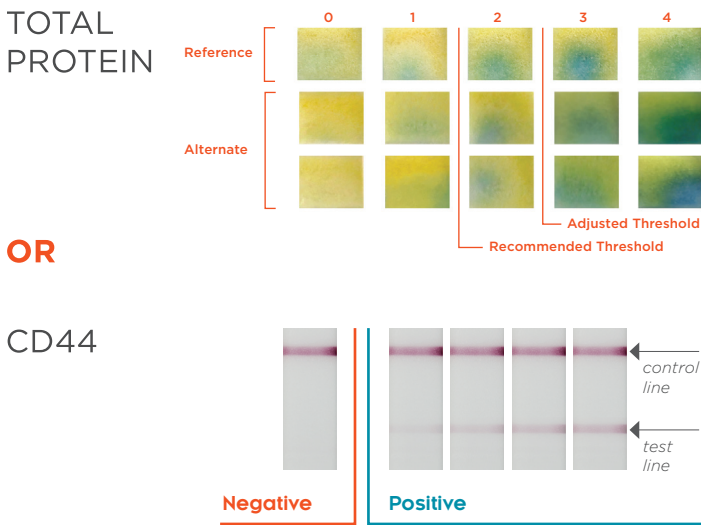
Anytime a worrisome lesion is identified by a clinician, a biopsy would be strongly recommended. If at biopsy the lesion is of uncertain significance, OncAlert™ testing may be performed to guide further decision-making including encouraging all patients to practice and maintain a healthy lifestyle.

ONCALERT SUPPORTIVE CLINICAL VALIDATION STUDIES

OncAlert Oral Cancer RAPID Test

In the OncAlert Oral Cancer RAPID Test (“RAPID”) validation studies, there were 38 oral cavity and pharyngeal cancer patients and 96 controls evaluated. Demographics for case-control patients comprised of the following: (i.) cases; mean age 60 years, 60% male, 97% white and 74% smokers with an even distribution of Stage I/II and III/IV HNSCC; and (ii.) controls; mean age 43 years, 28% male, 96% white and 0% smokers. The instructions for use included with the RAPID provide grading scales for CD44 and total protein are provided in the instructions for use document. A test was considered positive if the operator scored the CD44 reading as a ≥ 1 where the operator sees at least a faint line or the total protein reading as a ≥ 2 using the corresponding grading scales and representative images.

Figure 1. OncAlert Oral Cancer RAPID Test Gradient



Analysis of OncAlert Oral Cancer RAPID Test between operators: In addition to the results by the operator described previously, all 134 samples were also assessed by a second independent operator. Agreement between the 2 operators for CD44 score resulted in a weighted kappa of 74.63% (95% CI:65.91% -83.34%) and a McNemar’s p-value of 0.9704. Agreement between the 2 operators for total protein score resulted in a weighted kappa of 72.41% (95% CI:65.03% -79.79%) and a McNemar’s p-value of 0.9172. Since a kappa of 0.4 is indicative of moderate agreement, the results demonstrated adequate agreement between operators.

Sensitivity

Sensitivity of the RAPID utilizing either CD44 or total protein above the established cut-points was estimated to be 84.21% with a 95% confidence interval of 72.62% to 95.80% (Table 2). Adequate sensitivity of the test was demonstrated as the sensitivity was shown to be noninferior to 70% with a noninferiority margin of 10% because the lower 95% confidence limit (CL) of the sensitivity value for RAPID (72.62%) was greater than 60% (70% minus 10%). The specificity was 30% with a 95% CI: 21 - 40% and similarly represented non-inferiority as with the sensitivity.

Table 2

Sensitivity & Specificity of OncAlert Oral Cancer RAPID TEST		
Sensitivity	84.21%	95% CI: 72.62% -95.80%
Specificity	30.21 %	95% CI: 21.02 %-39.39%

Negative Predictive Value

The negative predictive value (NPV) of RAPID utilizing either CD44 or total protein above the established cut-points was estimated to be 94.93% using a prevalence estimate of 9.27% (Table 3) (37). The NPV showed greater than 90% and therefore appropriate for ruling out patients for oral cancer when their test is negative. The positive predictive value was 11%.

Table 3

Positive Predictive Value & Negative Predictive Value of OncAlert Oral Cancer RAPID Test Utilizing Prevalence 9.27% (31)		
Negative Predictive Value	94.93%	95% CI: 89.43% - 97.64%
Positive Predictive Value	10.97%	95% CI: 9.25% - 12.98%

OPTIONAL ASSESSMENT

A RAPID total protein score of 2 is recommended to be considered as a positive result for optimal sensitivity. However, it is important to note that a total protein score of 3 will improve the specificity from 30% to 50% (95%CI: 40.00%-60.00%) with a resultant decrease in sensitivity to 71.05% (95%CI: 56.63%-85.47%). Importantly, both the NPV and PPV remain similar at 92.46% (95% CI: 87.84-95.42%) and 10.37% (95%CI: 8.12-13.16%), respectively, for either total protein score reading.

The OncAlert Oral Cancer RAPID Test is a qualitative assay which has pre-specified thresholds for both CD44 and total protein. Based on the results of the current cohort analysis the assay has a sensitivity of 84% which equates to a false negative rate of 16% and a specificity

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of 30% with a false positive rate of 70%. Furthermore, utilizing a prevalence of 9.27%, the test has an NPV of >90%. In summary, the data suggests that a patient with a negative Rapid result, in conjunction with clinical judgment, has a lower likelihood of oral/oropharyngeal cancer. A positive test result would require further discrimination such as repeating the RAPID, utilizing the quantitative OncAlert™ Oral Cancer LAB Test, or both to improve specificity.

OncAlert Oral Cancer LAB Test

In the validation studies for the OncAlert Oral Cancer LAB Test (“LAB”), there were 310 patients were validated; 107 with oral/oropharyngeal cancer and 203 controls. A 50:50 train and test cohort analysis was implemented as part of the design. CD44 ELISA and total protein colorimetric assays were completed on all patients with multivariate logistic regression, sensitivity, specificity, AUC, NPV and PPV to assess performance. Demographics for case-control patients were as follows: (i.) cases; mean age 61 years, 72% male, 97% white and 65% smokers with an even distribution of Stage I/II and III/IV HNSCC; and (ii.) controls; mean age 34 years, 36% male, 60% white and 44% smokers.

Levels of CD44 and total protein combined with clinical features including age, sex, race and smoking history were combined to produce a score, range 0-100 and probability to determine the likelihood of having oral/oropharyngeal cancer. Although the pre-determined cut-points for both CD44 ≥ 1.68 , and total protein ≥ 0.32 were available, the model utilizes absolute levels of each of the protein biomarkers to construct the final score. Based on the cohort used to generate the clinical efficacy data, white, male smokers over the age of 40 appeared to be at the highest risk for head and neck squamous cell carcinoma. Furthermore, continuous levels for both CD44 and total protein provided a similar sensitivity and specificity as outlined below.

The sensitivity and specificity for the multivariate model is illustrated in Table 4. The sensitivity of LAB utilizing both CD44 and total protein above cut-point, together with clinical features, was estimated to be 80% with a 2-sided 95% confidence interval of 67% to 88% and a 1-sided lower 95% confidence limit of 69%. Adequate sensitivity of the test was demonstrated because the sensitivity was shown to be noninferior to 70% with a noninferiority margin of 10%. The lower 95% confidence limit (CL) of the sensitivity value for LAB (69%) was greater than 60% (70% minus 10%). The specificity of LAB utilizing both CD44 and total protein above cut-point, together with clinical features, was estimated to be 93% with a 2-sided 95% confidence interval of 86%

to 97% and a 1-sided lower 95% confidence limit of 88%. Adequate specificity of the test was demonstrated because the specificity was shown to be noninferior to 70% with a noninferiority margin of 10%. The lower 95% CL of the specificity value for LAB (88%) is greater than 60% (70% - 10%).

Table 4

Sensitivity & Specificity of OncAlert Oral Cancer LAB TEST		
Sensitivity	80.0%	95% CI: 67%-88%
Specificity	93%	95% CI: 86.4 %-96.6%

The positive predictive value (PPV) and negative predictive value (NPV) were calculated along with 95% confidence intervals using disease prevalence estimates from literature and from the study data only is in Table 5.

Table 5

Positive Predictive Value & Negative Predictive Value of OncAlert Oral Cancer LAB Test Utilizing Prevalence 9.27%*		
Negative Predictive Value	97.8%	95% CI: 96.3% - 98.7%
Positive Predictive Value	54.0%	95% CI: 36.2% - 70.8%

By incorporating the clinical variables (age, sex, race, and smoking status) the false positive rate is less than 10% and the false negative rate is 20%. These results demonstrate a maintained ability by the combined biomarker and clinical feature model to identify cancerous lesions while not over-calling more benign or non-malignant processes. This is further illustrated by Table 6 when the combined cut-points for both CD44 and total protein are incorporated into the multivariate model.

Table 6

OncAlert Oral Cancer LAB Test Result			
Combined CD44 + total protein (>= cut-points) with age, gender, race and smoking.			
	Test Result		
COHORT	Positive	Negative	Total
CANCER	43	11	54
NORMAL	7	94	101
TOTAL	50	105	155

Without incorporating clinical features to rely solely on either the CD44 or total protein levels above cut-point, the test yields a false negative rate of 15% and a false positive rate of 52%. The biomarker assay performs well in confirming when there is a reasonable suspicion of cancer but less effectively in discriminating more benign processes.

The outcome of the OncAlert™ LAB Test is the generation of a score ranging from 0 (lowest risk) to 100 (highest risk). This score is developed by combining the respective CD44 and total protein (TP) levels with clinical features. The score and associated probability is derived from an algorithm which assigns individual

statistical weights to each of the clinical features and the individual CD44 and total protein biomarker levels. Based on mathematical modeling results from the validation study, a cut-point score of 50 was developed to discriminate between patients with low risk (<=50) from patients with high risk (>50) of having HNSCC.

Analysis of the OncAlert Oral Cancer LAB Test

When a saliva sample is collected in 5 mL of sterile saline and tested using the methodologies enclosed, there is a likelihood of oral cancer if CD44 levels are above 1.68 ng/mL and the total protein in the sample is above 0.32 mg/mL. Patients with an elevated LAB score which incorporates the CD44 and total protein levels should be further evaluated to determine if there is an increased likelihood for oral cancer. Additional risk factors to consider may include heavy drinking, smoking, presence of HPV virus, and other clinical attributes.

When a suspicious lesion is present in the mouth and the LAB test is positive, the clinician is recommended to take appropriate action. With a negative test with an obvious lesion, the clinician is recommended to use best and appropriate judgment which may include observation and repeat visit or biopsy. For a positive test without an observable lesion, the clinician is recommended to consider performing additional studies including possibly repeating the LAB test. In contrast, routine follow-up would be recommended with a negative test result coupled without an observable lesion.

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CORPORATE HEADQUARTERS MAILING ADDRESS:

Vigilant Biosciences, Inc.
6301 NW 5th Way, Suite 1500
Ft. Lauderdale, FL 33309

+1 (954) 487-1740 | info@vigilantbiosciences.com

www.vigilantbiosciences.com

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