MOLECULAR EVIDENCE OF EPITHELIAL CELL DAMAGE CAUSED BY IQMIK,

AN ALASKA SMOKELESS TOBACCO MIXTURE

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MOLECULAR EVIDENCE OF EPITHELIAL CELL DAMAGE CAUSED BY IQMIK, AN ALASKA SMOKELESS TOBACCO MIXTURE

A

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Abstract

The percentage of Alaska Natives who use smokeless tobacco (SLT) is 4 times that of non-Native Alaskans and 45 times higher for Alaska Native women than non-Native women. The use of SLT is concentrated in Southwest Alaska where 32% of all adult Alaska Natives use SLT. Out of those users, 35% use only iqmik, a unique form of SLT in Alaska, which is a combination of tobacco leaf mixed with *Phellinus igniarius* (punk fungus). There is little evidence of the pathological effects of iqmik to assist in the development of an evidenced-based intervention regarding the harmful effects of iqmik. The current lack of evidence reinforces a belief that iqmik is less harmful than other tobacco alternatives. The overall objective of this thesis is to elucidate the effect of iqmik and iqmik-mediated metal exposure on oxidative stress and nuclear factor- κB (NF-κB) induced inflammation in human gingival epithelial cells. The central hypothesis of this thesis is that cadmium (Cd), cobalt (Co) and nickel (Ni) accumulate in human gingival epithelial cells from iqmik treatments, inducing oxidative stress and promoting an intracellular environment that alters NF-κB proinflammatory signaling targets. Our findings indicate that iqmik is a greater source of heavy metals, such as Cd, Co and Ni, than air-cured tobacco leaf. Human gingival epithelial cells accumulate more Cd, Co and Ni from the punk ash component of iqmik than from the air-cured tobacco. These metals have the capacity to accumulate in cells from iqmik treatments and generate and propagate the production of endogenous reactive oxygen species (ROS), which activates NF-κB significantly altering its signaling targets, more so than tobacco alone. The results of this thesis identify iqmik as a unique health hazard compared to other tobacco products and enhances our understanding of how iqmik may contribute to oral pathologies.

Table of Contents

Page

Page

List of Figures

Page

List of Tables

Page

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Introduction

Cancer of the oral cavity is the sixth most common cancer in the world and it is especially prevalent in Alaska (1-3). Squamous cell carcinoma accounts for 90% of all oral cancers (4). Head and neck squamous cell carcinoma (HNSCC) is a significant problem because it has a poor 5-year survival rate, which has not improved significantly over the last 30 years, and treatments can result in functional defects (5). Oral cancer is an aggressive cancer that commonly metastasizes to other organs. Early detection of oral lesions has a significant effect on survival rates. However, nearly half of patients diagnosed with oral cancer have already developed metastases prior to their diagnosis. Therefore, a new method for diagnosis is necessary to detect oral cancers in an earlier stage (6).

In the United States, 80-90% of head and neck cancers are attributed to tobacco and alcohol abuse (7). Tobacco and alcohol are important etiological agents in the development of HNSCC, and the International Agency for Research on Cancer (IARC) has concluded that smokeless tobacco (SLT) is carcinogenic and causes oral cancer (8-11). SLT users have a 4 - 6 times higher risk for developing oral cancer than non-users and risk of oral cancer increases with years of use in a dose-dependent manner (12).

Tobacco use is a significant risk factor for oral mucosal lesions, as well. Oral mucosal lesions, including leukoplakia, submucous fibrosis and oral lichen planus are common in SLT users and can develop with limited use (13). Although only a small percent of oral mucosal lesions transform into cancer with a malignant transformation rate of 12.1% (14), oral lesions precede the majority of oral cancers, with only approximately 30% of cases appearing *de novo* from histologically normal oral epithelium (15,16).

In India, tobacco chewing is the strongest risk factor for oral cancer. The most common form of chewing tobacco in India is betel quid, a mixture of areca nut, slaked lime and tobacco in a betel leaf. This form of tobacco is a major cause of oral submucous fibrosis and oral leukoplakia (17). In India, oral leukoplakia was found to precede the development of oral cancer in approximately 70% of the cases (18,19).

Erythroplakia, a red lesion, leukoplakia, a white lesion which is the most common, and erythroleukoplakia are premalignant chronic non-healing epithelial ulcers. The extent of epithelial dysplasia in a lesion is classified as low or high-grade. Dysplasia and carcinoma is much more likely with erythroplakia than leukoplakia (9,20). Lesions with granular or verrucous appearance and located along the floor of the mouth or border of tongue are associated with high risk of malignant transformation (4).

Oral damage from SLT use, including premalignant lesions and malignant tumors, can be seen during a clinical examination, and visual inspection is the most common diagnostic tool for detecting oral cancers. However, this is not a sufficient method for detecting oral cancers as there is little evidence that this form of screening reduces the incidence or mortality from oral cancers (12,15). Therefore, molecular and biochemical techniques can provide more accurate information about the preneoplastic changes occurring in the oral cavity. A practical application of these markers needs to be implemented in the clinic so that a more accurate characterization of oral lesions can be performed to improve detection of those lesions that will go on to malignant transformation (15).

The incidence of oral cancer is estimated to be rising annually by 2.6% in Alaska and by 6.5% specifically for Alaska Natives. Alaska has an oral cancer, age-adjusted, incidence rate of 10.8 cases per 100,000 per year, with an average of 71 cases per year. Alaska Natives have an oral

cancer, age-adjusted, incidence rate of 16.0 cases per 100,000 per year. Oral cancer has a mortality rate of 2.8 cases per 100,000 per year in Alaska and Alaska Natives have a mortality rate of 6.1 cases per 100,000 per year (SEERS).

SLT is used by 6% of all adult Alaskans compared to the national average of only 5% of all adults. The percentage of Alaska Natives who use SLT is 4 times that of non-Native Alaskans and 45 times higher for Alaska Native women then non-Native women. The use of SLT is concentrated in Southwest Alaska where 32% of all adult Alaska Natives use SLT. Out of all adult Alaska Natives who use SLT, 35% use only iqmik, a unique form of SLT in Alaska (21). In Southwest Alaska up to 60% of pregnant women self-report using iqmik during pregnancy (22). Alaska Native women have been reported to switch from other tobacco products to iqmik during pregnancy because it is perceived that iqmik is a safer alternative (23). However, very little is known about the health effects of iqmik exposure and prevalence of HNSCC correlated to the specific use of iqmik is undefined.

Iqmik is a combination of tobacco leaf mixed with *Phellinus igniarius* (punk fungus). The fungus is collected, air-dried and then burned to fine white ash. The ash is then mixed with air-cured leaf tobacco by mastication (24,25). Punk fungus has a higher concentration of alkali and alkaline earth metals compared to tobacco leaf, increasing the alkaline pH of iqmik, most likely accounting for the higher nicotine levels seen is users (25,26). Iqmik users have a nicotine intake two times that of cigarette smokers and greater than commercial SLT users (27). An alkaline pH converts nicotine, an alkaloid, from an ionized form to an un-ionized form that easily passes through biological membranes (28). This increases both the absorption and addictiveness of the nicotine (25,26,29).

The addition of the slaked lime to betel quid in India also increases the mean pH of the mixture to pH10, inducing the generation of reactive oxygen species (ROS) from the areca nut. The slaked lime also causes ulceration of the oral mucosa. The combination of open wounds and ROS may provide a mechanism for the development of leukoplakia that is more prone to malignant transformation (17,18,30). Whether this occurs with iqmik use is unknown.

The ash in iqmik is also a source of higher levels of pro-inflammatory toxic metals than cured leaf tobacco. The levels of copper (Cu), iron (Fe), manganese (Mn), cadmium (Cd), chromium (Cr), nickel (Ni) and lead (Pb) are 2 - 80 times higher in the ash compared to leaf tobacco (25). In addition, more cobalt (Co), Ni and Cd can be extracted from mixtures of ash and leaf tobacco than from leaf tobacco alone when extracted with artificial human saliva (31).

The IARC classifies Cd and Ni as group 1 human carcinogens and Co as a group 2B carcinogen (32-34). Cd, Ni and Co exist as divalent cations in aqueous solution with similar ionic radii to Calcium (Ca), Magnesium (Mg) and Zinc (Zn) ions, respectively. Cd, Ni and Co can pass through cell membranes by utilizing cation transporters, and interfere with the structure and function of proteins and enzymes when they substitute for Ca, Mg and Zn ions, respectively (35).

Oxidative stress, induced by endogenous or exogenous insults, such as iqmik, is the cumulative result of an imbalance between ROS production and antioxidant defense. Metal-toxicity can cause oxidative stress, which is one of the mechanisms for the carcinogenic effects of metals (35-38). Co and Ni are involved in redox reactions that produce ROS. Cd inhibits antioxidant enzymes, catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase, by binding to their thiol groups, leading to elevated ROS which is involved in oxidative damage to DNA, lipids and proteins (39). ROS can damage DNA through base

modification leading to a mutagenic event, and subsequent tumor formation (35,37,40). Oxidative stress can also cause up-regulation of signaling cascades that activate redox-regulated transcription factors, such as, AP-1, NF-κB, p53, NFAT and HIF-1. The genes regulated by these transcription factors are involved in DNA repair, inflammation, cell cycle arrest and apoptosis, processes involved in malignant transformation (35,37,40).

Redox-regulated NF-κB is the major inflammatory transcription factor that has a central role in many stress responses, regulating the transcription of various genes involved in inflammation, proliferation and cell survival (35,37). Overexpression of NF-κB may be involved in the development and progression of malignancies in multiple sites, including the oral cavity (5). NF-κB can be activated in oral epithelial cells exposed to khaini, a product similar to betel quid that is a tobacco and slaked lime mixture, with a very alkaline pH, used in India (41-43). The activation of NF-κB and the increased expression of NF-κB - regulated genes by iqmik are unknown.

The NF-κB regulated gene *PTGS2*, which encodes cyclooxygenase-2 (COX2) shows increased expression in oral epithelial cells exposed to an extract of khaini (42-45). COX2, a crucial enzyme in the biosynthesis of prostaglandins, is a key mediator of inflammation and is expressed by epithelial cells upon injury (42,46,47). Aberrant expression and activity of COX2 are causally involved in tumor promotion and progression (48). COX2 is expressed at high levels in many carcinomas, including HNSCC, as are other targets of NF-κB, such as interleukin (IL) -6 and IL-8 (49). NF-κB dependent cytokines, IL-1, IL-6, IL-8 and TNFα are elevated in tissue samples from oral squamous cell carcinoma and in saliva samples from patients with either HNSCC or oral premalignant lesions (50-52). Establishing a set of biomarkers, such as these NF-κB regulated targets, that is consistent and biologically relevant for a population would more easily

and accurately identify patients within that population that will go on to develop oral cancer (15,53).

The lack of information on the effects of iqmik on oral epithelial cells either before or after transformation prevents the establishment of an Alaska relevant set of biomarkers that could be used as a screening tool for the early identification of a premalignant state in SLT users. This gap in our knowledge raises the question, does an SLT like iqmik, which contains both punk ash and tobacco leaf, promote an environment of oxidative stress and inflammation that could favor the development of oral cancer? Specifically, does iqmik exposure produce endogenous ROS and trigger inflammatory signaling networks through the upregulation of NF-κB regulated gene products? Finally, could those responses be used to identify an early detection method for reducing oral cancers mortality rates in the Alaska Native population, the primary users of iqmik?

There is little evidence of the pathological effects of iqmik to assist in the development of an evidenced-based intervention regarding the harmful effects of iqmik. The current lack of evidence reinforces a belief that iqmik is less harmful than other tobacco alternatives (54-56). The results of this thesis will add to the understanding of the biological basis for the health conditions associated with the use of iqmik, principally, defining the changes in cellular environment induced by iqmik exposure and enhancing our understanding of how iqmik may contribute to oral pathologies.

The overall objective of this thesis was to elucidate the effect of iqmik and iqmik-mediated metal exposure on oxidative stress and NF-κB induced inflammation in human gingival epithelial cells. The central hypothesis of this thesis was that Cd, Co and Ni would accumulate in human gingival epithelial cells from iqmik treatments, inducing oxidative stress and promoting

an intracellular environment that alters NF-κB proinflammatory signaling targets. The following objectives were conducted to test this hypothesis.

The first objective of this thesis was to characterize the bioavailable concentrations of Cd, Co and Ni in iqmik and its components punk ash and tobacco leaf, and determine the relative contribution of these components and the metals within them to ROS generation in exposed human oral epithelial cells. We hypothesized that exposure of oral epithelial cells to artificial saliva extracts of iqmik would lead to absorption of cadmium, cobalt and nickel and would cause elevated ROS. The second objective of this thesis was to investigate the impact of tobacco and iqmik exposure on the expression of NF-κB signaling target genes in differentiated human oral epithelial cells. We hypothesized that iqmik would differentially alter the expression profile of NF-κB regulated genes compared to tobacco leaf alone. The final objective of this thesis was to propose a study to identify proinflammatory biomarkers for oral cancer in tobacco users in Alaska, which could be used to develop an accurate method for earlier detection of oral cancer in iqmik users. We hypothesize there will be a difference in proinflammatory cytokines expressed in whole unstimulated saliva among users of tobacco types in Alaska and those with oral cancer.

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Chapter 1. Exposure to smokeless tobacco mixture iqmik leads to transition metals accumulation and reactive oxygen species production in human gingival epithelial cells[1]

Abstract

Smokeless tobacco (SLT) users have 4-6 times higher risk for developing oral cancer than non-users. The percentage of Alaska Native peoples who use SLT is nearly four times that of other Alaskans. Of all adult Alaska Native peoples who use SLT, 35% use only iqmik, an Alaska-specific SLT variant of tobacco leaf mixed with punk fungus ash. A previous analysis of punk ash and tobacco leaf suggested that ash contains higher levels of Cd, Co and Ni. However, the bioavailability of these metals and the impact tobacco leaf, punk ash or iqmik may have on the intracellular redox response in primary human gingival epithelial cells is unknown. We therefore determined the bioavailability of these metals in air-cured tobacco leaf, punk ash and iqmik, as well as the level of reactive oxygen species (ROS) produced in primary human gingival epithelial cells exposed to these compounds. Significantly higher concentrations of Cd, Co and Ni were isolated in punk ash and iqmik than in tobacco leaf. Artificial saliva extracts of iqmik produced a greater than additive ROS response compared to extracts of the individual components of iqmik, tobacco leaf and punk ash. Exposure to Co alone produced significantly more ROS than either Cd or Ni alone, suggesting that Co within the ash component of iqmik may have a greater contribution to the significantly higher ROS seen following iqmik exposure.

[1] Dwyer, G.K., Hagedorn, B., Knall, C. (2015). Exposure to smokeless tobacco mixture iqmik leads to transition metals accumulation and reactive oxygen species production in human gingival epithelial cells. Submitted to the journal Carcinogenesis.

1.1 Summary

Iqmik, an Alaska specific smokeless tobacco product, is a greater source of carcinogenic heavy metals, such as Cd, Co and Ni, which have the capacity to accumulate in oral epithelial cells inducing the accumulation of endogenous ROS.

1.2 Introduction

Tobacco use is a significant risk factor for oral diseases, specifically oral mucosal lesions, periodontal disease and oral cancers. The International Agency for Research on Cancer (IARC) recently concluded that there is a causal association between smokeless tobacco (SLT) and oral cancer (1). SLT users have a 4-6 times higher risk for developing oral cancer than non-users and risk of oral cancer increases with years of use. Head and neck squamous cell carcinoma is the sixth most common cause of death from cancer in the world, and it is especially prevalent in Alaska (2-5). In the United States 80-90% of head and neck cancers are attributed to tobacco and alcohol abuse (6). Tobacco and alcohol are identified as important etiological agents in the development of head and neck squamous cell carcinoma (7-9).

SLT is used by 6% of all adult Alaskans compared to the national average of only 5% of all adults. The percentage of Alaska Native peoples who use SLT is four times that of non-Native Alaskans. The use of SLT is concentrated in Southwest Alaska where 32% of all adult Alaska Native persons use SLT. Out of all adult Alaska Native persons who use SLT, 35% use only iqmik, a unique form of SLT used in Alaska (10). Iqmik is a combination of tobacco leaf mixed with *Phellinus igniarius* (punk fungus). The fungus is collected, air-dried and then burned to fine white ash. The ash is then mixed with the air-cured leaf tobacco by mastication (11,12).

Punk fungus has a higher concentration of alkali and alkaline earth metals compared to tobacco leaf, increasing the alkaline pH of iqmik (12,13). The ash is also a source of higher

levels of pro-inflammatory toxic metals than cured leaf tobacco. The levels of copper (Cu), iron (Fe), manganese (Mn), cadmium (Cd), chromium (Cr), nickel (Ni) and lead (Pb) are 2-80 times higher in the ash compared to leaf tobacco (12). In addition, more cobalt (Co), Ni and Cd can be extracted from mixtures of ash and leaf tobacco than from leaf tobacco alone when extracted with artificial human saliva (14). The International Agency for Research on Cancer classifies Cd and Ni as group 1 human carcinogens and Co as a group 2B carcinogen (15-17). Cd, Ni and Co exist as divalent cations in aqueous solution with similar ionic radii to Calcium (Ca), Magnesium (Mg) and Zinc (Zn) ions, respectively. Cd, Ni and Co can pass through cell membranes by utilizing cation transporters, and interfere with the structure and function of proteins and enzymes when they substitute for Ca, Mg and Zn ions, respectively (18).

Oxidative stress, induced by endogenous or exogenous insults, such as iqmik, is the cumulative result of an imbalance between reactive oxygen species (ROS) production and antioxidant defense. Metal toxicity can cause oxidative stress, which is one of the mechanisms for the carcinogenic effects of metals (18-21). Co and Ni are involved in redox reactions that produce ROS. Cd inhibits antioxidant enzymes, catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase, by binding to their thiol groups, leading to elevated ROS which is involved in oxidative damage to DNA, lipids and proteins (22). ROS can damage DNA through base modification leading to a mutagenic event, and subsequent tumor formation (18,20,23). These changes in turn cause up-regulation of signalling cascades activating redox-regulated transcription factors, such as, AP-1, NF-κB, p53, NFAT and HIF-1. The genes regulated by these transcription factors are involved in DNA repair, inflammation, cell cycle arrest and apoptosis (18,20,23). This study was designed to characterize the bioavailable concentrations of Cd, Co and Ni in iqmik and its components punk ash and tobacco leaf, and

determine the relative contribution of these components and the metals within them to ROS generation in exposed human oral epithelial cells.

1.3 Materials and methods

1.3.1 Iqmik, punk ash and air-cured tobacco preparation, extraction and pH determination

Following community practice for iqmik preparation without mastication (Renner, Personal Communication), air-cured whole leaf tobacco was cut into small homogeneous pieces using a coffee bean grinder. Punk fungus was sawed into pieces weighing 300g or less, placed into a metal coffee can and ashed on a hot plate set on high. Punk ash was weighed out to 1g for all punk ash extracts; air-cured tobacco leaf was weighed out to 1g for all tobacco extracts. Iqmik extracts were prepared by combining 0.5g punk ash and 0.5g tobacco. Artificial human saliva was prepared with 5.3mM KSCN, 15mM $NaHCO_3$, 20mM KCl, 1.4mM NaH_2PO_4, 0.1g/L CH_4N_2O (urea), and 0.1mg/L α-amylase. Each sample (1g) was extracted in 25mL artificial human saliva. Each sample was inverted 7 times and incubated for 30 minutes at 37°C and 5% CO_2. Samples were centrifuged for 10 minutes at 2000 x *g*, the supernatant was removed from the solid phase, and filter sterilized (14). For pH determination, prepared extracts in triplicate were either undiluted or diluted 1:1 with CnT-32 Oral Epithelium Medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) containing $CaCl_2$ (1μM) and measured with an Oakton pH meter (Oakton Instruments, Vernon Hills, IL) equipped with a Orion probe (Thermo Scientific, Waltham, MA) at room temperature,

1.3.2 Cell culture and differentiation

Primary human gingival epithelial cells (HGEP) (CELLnTEC) were cultured for use in the experiments. Cultures were maintained at a seeding density of 4.0×10^3 cells per cm^2 in CnT-24 PCT Oral Epithelium Medium (CELLnTEC). For cultures to be exposed, cells were grown for

approximately 6-8 days in CnT-24 PCT Oral Epithelium Medium until one day prior to confluency at which time the medium were changed to CnT-32 Oral Epithelium Medium (CELLnTEC). Cultures were grown for an additional 24 hours and then induced to differentiate in CnT-32 containing $CaCl_2$ (1μM) (differentiation media) per manufactures directions. Cells to be exposed were cultured in differentiation media for 96hrs before all experimental procedures.

1.3.3 Cell viability

The Live/Dead Cell Assay (Molecular Probes, Life Technologies, Carlsbad, CA) was used to determine viability of exposed differentiated HGEP. Differentiated HGEP were exposed to artificial saliva, artificial saliva extracts of iqmik, air-cured tobacco or punk ash and extracts diluted 1:1 in differentiation media for 24 hours. Treated differentiated HGEP were then incubated with live dye, calcein AM (1μM), and dead dye, ethidium homodimer-1 (2μM) for 30 minutes. Control differentiated HGEP were incubated in differentiation media (live control), or methanol (70%) (dead control) for 45 minutes. Cell viability was quantified as fluorescence intensity using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) (Data not shown).

1.3.4 Metals quantification

Metals quantification was accomplished using the Environmental Protection Agency (EPA) method 3051A (24) for sample digestion followed by inductively coupled plasma mass spectrometry (ICPMS) analysis.

1.3.4.1 Sample preparation. Triplicate samples of 1g compounds iqmik, air-cured tobacco and punk ash were dried and weighed to 1mg accuracy prior to digestion. Artificial saliva extracts of air-cured leaf tobacco, punk ash and iqmik were prepared as described above and aliquoted into 2ml for digestion. To measure metals accumulation in cells, differentiated HGEP, plated on

100mm plates, were washed with phosphate buffered saline (PBS) and then exposed to artificial saliva, or artificial saliva extracts of iqmik, tobacco, or punk ash diluted 1:1 with differentiation media for 24 hours prior to digestion. Following exposure, cells were washed with PBS and lysed with 1mL Mg lysing buffer (25mM Hepes pH 7.5, 150mM NaCl, 1% Igepal CA-630, 0.25% NaDOC, 10% glycerol, 25mM NaF, 10mM $MgCl_2$, 1mM EDTA, 1mM Na_3VO_4, 10μg/mL lupeptin, 10mg/mL aprotinin). The majority of the lysate (975μL) was used for digestion. The remaining portion of the lysate (25μL) was used in a RC DC Protein Assay (BioRad Laboratories, Hercules, CA) to standardize intracellular metals concentration to total protein concentration.

1.3.4.2 Digestion. Five mL of trace metal grade concentrated HNO3 (J.T. Baker - Advantor, Center Valley, PA) was added to each sample and kept at room temperature overnight. Five mL of trace metals grade H2O (> 18 MΩ-cm) was added to each sample, followed by microwave assisted pressure digestion at 180 in a Milestone Ethos model microwave system (Shelton, CT) with a 10 minute hold at 180°C. Following digestion, 1mL of Tamapure AA-10 H2O2 (35%, Moses Lake Industries, Moses Lake, WA) was added to each sample while being heated at 180°C. The samples were then evaporated to near dryness over 3 hours and rehydrated in 10mL of 1% HNO3 for the iqmik and components digest and in 2mL 1% HNO3 for artificial saliva extracts and cell digest, and filtered (0.45μm) before analysis. Blanks were prepared in triplicate as described, but without the addition of experimental samples.

1.3.4.3 *ICP-MS analysis.* Analysis of metals in iqmik, air-cured tobacco and ash compounds was performed in the Applied Science, Engineering, and Technology (ASET) Laboratory at University of Alaska Anchorage using an Agilent 7500c ICP-MS with collision cell and AX 500 CETAC Technologies autosampler. Measurements were carried out at 1480 with Argon plasma

flow at 15L min^{-1}, carrier gas flow of 1.2mL min^{-1} and makeup gas flow at 0.2mL min^{-1}. Cd, Co, and Ni were analyzed in normal mode (no reaction gas). External seven level calibration at 0.5, 1, 5, 10, 50, 100µg L^{-1} was prepared from Agilent standard I stock solution (Agilent ®). To correct for instrument drift internal standard composed of Li(7), Sc, Y, In, and Bi was used at 10ug L^{-1} concentration (Agilent ®) was used for quantification. Calibration verification was performed every ten samples and international standard NIST SRM 1643e was used to verify calibration solutions; all values above 90% recovery were accepted. Limit of detection was calculated from regression analysis of calibration as outlined by Harris (25). Analysis of metals in the saliva extracts and HGEP samples was performed by the Elemental Analysis Core at Oregon Health Science University (OHSU) using an Agilent 7700x equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550W, an argon (Ar) plasma gas flow rate of 15L/min, and Ar carrier gas flow rate of 1.08L/min. Co and Ni were measured in kinetic energy discrimination (KED) mode using He gas (4.2mL/min) and Cd was measured in no-gas mode. For measurement, 2x dilutions of samples were prepared in 1% HNO_3 (trace metal grade, Fisher Scientific) in acid-treated 15mL conical tubes, via incubation in 1% HNO_3 for at least 24 hours. Data were quantified using a 10-point calibration curve (0, 0.25, 0.5, 1, 2, 5, 10, 50, 100, 1000 ppb (ng/g)) with external standards for Co, Ni, and Cd (Common Elements Mix 1 Multi-Element Aqueous Standard, VHG Labs) in 1% HNO_3. For each sample, data were acquired in triplicate and averaged. An internal standard (Internal Standard Multi-Element Mix 3, VHG Labs) introduced with the sample was used to correct for plasma instabilities, and frequent measurements of a 5 ppb all-analyte solution as well as a blank (containing 1% HNO_3 only) were used as quality control and to determine the coefficient of variance. To access recovery rates of elements and probe background contamination from

containers, a certified NIST standard reference material control was treated, prepared, and analyzed by the same method as the samples (trace metals in water, SRM 1643e).

1.3.5 ROS measurement

ROS was assessed by a procedure modified by Mitchell et al. (26). Briefly, total ROS was measured with membrane permeable oxidation-sensitive fluorescent dye H_2DCFDA (Invitrogen, Life Technologies) in differentiated HGEP following manufacturer's directions. Differentiated HGEP in 96-well plates were washed with PBS and incubated for 1 hour with H_2DCFDA (15μM), or PBS only (no dye control), washed with PBS and incubated for an additional 30 min in differentiation media alone. Following a PBS wash, labeled and control differentiated HGEP were exposed to artificial saliva, artificial saliva extracts of iqmik, air-cured tobacco or punk ash, or hydrogen peroxide (100μM) (ROS positive control, data not shown) for 3 hours at 37°C and 5% CO_2 followed by quantification of DCF-fluorescence intensity by fluorescence microplate reader (Molecular Devices) (26). To determine the extent of ROS production by Co, Ni or Cd alone, labeled and control differentiated HGEP were exposed to $CoCl_2$ (1mg/L), $NiCl_2$ (1mg/L) or $CdCl_2$ (1mg/L) in artificial saliva diluted 1:1 with differentiation media. Each treatment was tested in triplicate.

1.3.6 Statistical analysis

All reported results represent the mean ± SEM of data obtained in three independent experiments. Statistical analysis was performed with the one-way ANOVA and Tukey's HSD post hoc tests. $P \leq 0.05$ was considered statistically significant.

1.4 Results

1.4.1 Trace metals concentration in air-cured tobacco leaf, punk ash and iqmik

Total concentrations of Cd, Co and Ni in air-cured tobacco, punk ash and iqmik are reported in Figure 1.1. All samples reported above the limit of detection of the Agilent 7500c ICP-MS. Punk ash samples had significantly higher concentration of Cd ($P \leq 0.0001$), Ni ($P \leq 0.0001$) and Co ($P \leq 0.0055$) than air-cured leaf tobacco. Iqmik samples also had significantly higher concentration of Cd ($P \leq 0.0002$) and Ni ($P \leq 0.002$) than air-cured leaf tobacco. Punk ash samples had significantly higher concentrations of Cd ($P \leq 0.0006$) and Ni ($P \leq 0.0011$) than iqmik. It should be noted that iqmik was made of equal weights air-cured tobacco and ash, and thus contained only half the quantity of punk ash and leaf tobacco that was used to determine the concentration of metals (μg/g total weight) in each sample of the constituent compounds.

1.4.2 pH of,artificial saliva extracts

Because of the previously reported (12,13) alkaline nature of punk ash and iqmik, we determined the pH for the artificial saliva extracts of punk ash, tobacco and iqmik (Table 1.1). The pH of artificial saliva extracts of punk ash undiluted and diluted ($P \leq 0.0001$, $P \leq 0.0001$ respectively) was significantly more alkaline than the pH of the artificial saliva extracts of air-cured tobacco leaf. Therefore, the pH of the artificial saliva extracts of iqmik was primarily influenced by the punk ash component.

1.4.3 Trace metals concentration in artificial saliva extracts of,air-cured tobacco leaf, punk ash and iqmik

To determine the extent to which trace metals from the tobacco leaf, punk ash and iqmik would be extracted by the artificial saliva, ICP-MS analysis was performed on each of the artificial saliva extracts (Figure 1.2). Co and Ni were extracted from air-cured tobacco leaf, punk ash and

iqmik by the artificial saliva. Cd was extracted from air-cured tobacco leaf and iqmik, but not punk ash by the artificial saliva. Air-cured tobacco saliva extracts had significantly more Cd ($P \leq 0.0001$) and Co ($P \leq 0.0002$) than punk ash saliva extracts. Iqmik saliva extracts had significantly more Cd ($P \leq 0.001$), Ni ($P \leq 0.0001$) and Co ($P \leq 0.0002$) than punk ash saliva extracts. Punk ash saliva extracts had significantly more Ni ($P \leq 0.0001$) than air-cured tobacco saliva extracts. The extraction efficiency for Cd, Ni and Co in the artificial saliva extracts was determined as a percentage of the metals in the extracts compared to the metals available in the corresponding digest of whole air-cured tobacco, punk ash and iqmik (Table 1.2). Artificial saliva extracts of air-cured tobacco leaf had the highest extraction efficiency for Co and Cd, whereas, iqmik had the highest extraction efficiency for Ni. Punk ash had the lowest extraction efficiency for all three metals tested.

1.4.4 Trace metal concentration in exposed HGEP

Differentiated HGEP exposed for 24 hours to artificial saliva extracts of air-cured tobacco, punk ash or iqmik diluted 1:1 in differentiation media showed metals absorption for Co, Ni and Cd in cultures exposed to either punk ash or iqmik (Figure 1.3). Cd was the only metal that accumulated in HGEP cells exposed to artificial saliva extracts of air-cured tobacco leaf, and this accumulation was not statistically different ($P \leq 0.88$) from that in HGEP exposed to artificial saliva. HGEP exposed to artificial saliva extracts of punk ash showed significantly more Cd ($P \leq 0.03$, iqmik, $P \leq 0.0068$, tobacco leaf, $P \leq 0.0028$, saliva), Ni ($P \leq 0.0001$, all) and Co ($P \leq 0.0001$, all) accumulation than HGEP treated with either artificial saliva extracts of iqmik or air-cured tobacco. HGEP exposed to artificial saliva extracts of iqmik did show a significant Cd ($P \leq 0.3$), Ni ($P \leq 0.81$) or Co ($P \leq 0.63$) accumulation than HGEP treated with artificial saliva.

1.4.5 Cell viability of exposed differentiated HGEP

To determine the effect on cell viability of the artificial saliva, artificial saliva extracts of tobacco, punk ash or iqmik and the artificial saliva and artificial saliva extracts diluted 1:1 in differentiation media, differentiated HGEP cells were exposed for 24 hours to these various samples. The cells exhibited less cytotoxic response in the iqmik ($P \leq 0.2$) and artificial saliva ($P \leq 0.0001$) samples treated with extracts diluted 1:1 in differentiation media compared to the samples treated with pure extract. HGEP cells treated with artificial saliva extracts of iqmik diluted 1:1 in differentiation media exhibited significantly less cytotoxic response ($P \leq 0.0001$) than the control dead samples (Data not shown).

1.4.6 ROS production in exposed differentiated HGEP

As a measure of oxidative stress in differentiated HGEP exposed to artificial saliva extracts of iqmik, tobacco leaf or punk ash, ROS was measured (Figure 1.4A). Artificial saliva extracts of iqmik ($P \leq 0.0001$) or air-cured tobacco leaf ($P \leq 0.0092$) produced significantly more ROS in exposed cells than artificial saliva extracts of punk ash. Artificial saliva extracts of punk ash produced back ground levels similar to artificial saliva alone ($P \leq 0.52$). Artificial saliva extracts of air-cured tobacco leaf ($P \leq 0.0058$) and iqmik ($P \leq 0.0001$) produced significantly more ROS in exposed HGEP than artificial saliva alone.

The ability of Cd, Co or Ni alone to produce ROS in exposed differentiated HGEP was determined by exposing cells to 1mg/L of $CoCl_2$, $NiCl_2$ or $CdCl_2$ in artificial saliva diluted 1:1 in differentiation media (Figure 1.4B). Co exposure produced a significantly higher ROS response than the artificial saliva control ($P \leq 0.0001$), Cd ($P \leq 0.0001$) or Ni ($P \leq 0.0001$) exposures. Cd ($P \leq 0.97$) and Ni ($P \leq 0.74$) treatments produced similar levels of ROS compared to the artificial saliva control.

1.5 Discussion

As previously identified by Pappas et al., (14) punk ash has significantly more Cd, Co and Ni than the air-cured tobacco leaf, accounting for the elevated levels of carcinogenic metals in iqmik. The extraction efficiency of Cd, Co and Ni is pH dependent. In an alkaline solution, Cd, Co and Ni form insoluble hydroxide salts (27). This trend is evident when comparing the concentrations of metals in artificial saliva extracts of iqmik, air-cured tobacco and punk ash. Studies of other alkaline ash extracts have shown similar results. Cd is minimally soluble between pH ranges 10.5 and 13 in fly ash (28). The pH of the punk ash extracts and iqmik extracts are just outside the range of minimal solubility for Cd. Based on the similarity of solubility constants for the metal hydroxides, Co and Ni are likely to have similar pH ranges for solubility. This is further confirmed by the fact that punk ash has a higher concentration of metals than air-cured tobacco alone (Figure 1.1), but the alkaline artificial saliva extracts of punk ash have lower levels of Cd and Co than artificial saliva extracts of air-cured tobacco (Figure 1.2). The minimal risk levels for Cd, Co and Ni published by the Agency for Toxic Substances and Disease Registry are 0.0005 mg/kg/day intermediate oral exposure and 0.0001 mg/kg/day chronic oral exposure for Cd, 0.01 mg/kg/day intermediate oral exposure for Co, 0.0002 mg/m^3 intermediate inhaled exposure and 0.00009 mg/m^3 chronic inhaled exposure for Ni. Intermediate exposure is 15-364 days; chronic exposure is greater than 1 year. The minimal risk level for Ni from oral exposure has not been determined, but water sources have a median Ni range of 0.5 to 6 μg/L. The US Environmental Protection Agency requires drinking water to have less than 5 μg/L of Cd, and the average reported level of Co in drinking water is 2 μg/L. A 30 min artificial saliva extract of iqmik contains 1/3 of the maximum recommended amount of Cd in drinking

water, and exceeds the normal amount of Co and Ni in drinking water by 5 – 10 fold, respectively (29-31).

The accumulation of metals by the cells is a very different trend than the saliva extraction efficiencies. HGEP exposed to punk ash extracts have the greatest metal absorption for Cd, Co and Ni. Active transport of the metals into the cells occurs primarily in the punk ash and iqmik saliva extract treated HGEP. The pH of the punk ash and iqmik saliva extracts are both basic, to compensate for the alkalinity of the external environment the HGEP would try to maintain an internal environment that is close to physiological pH. To maintain a more neutral internal environment a net internal accumulation of H^+ must occur (32,33). Na^+/H^+ antiporters are actively involved in intracellular homeostasis, but in an alkaline environment the antiporter must be electrogenic rather than electroneutral. The internal transmembrane electrical gradient must be negative to support a larger influx of H^+ to a small efflux of Na^+ (33). The negative internal environment created within the cell may be influencing the transport of divalent transition metals across the cell membrane. Divalent metals can move across the membrane by a group of transporters that make up the SLC1 family. The SLC1 family uses the H^+ electrochemical gradient to transport a broad range of divalent metal ions including Fe^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Pb^{2+}; many of the transporters in the family function as cotransporters (34-36). It is possible that the alkaline external environment created by the exposure to punk ash or iqmik saliva extracts promotes the accumulation of Cd, Co and Ni in HGEP.

Exposure of HGEP to air-cured tobacco or iqmik artificial saliva extracts results in significantly more ROS generation than artificial saliva alone. The air cured tobacco extracts are similar to hydrogen peroxide, but iqmik extracts produce significantly more ROS than hydrogen peroxide. Exposure to punk ash alone does not significantly increase ROS over artificial saliva.

Levels of ROS seen in cells exposed to iqmik artificial saliva extracts are greater than the sum of ROS produced upon exposure with punk ash and air-cured tobacco artificial saliva extracts when corrected for artificial saliva alone, suggesting a synergistic effect. This is even more compelling because iqmik is only half the total punk ash and air-cured tobacco that elicits the ROS response seen in Figure 1.4. In regards to the relative contribution of each metal, Cd, Co, and Ni, in ROS formation in differentiated HGEP, Co exposure produces significant levels of ROS similar to the level in iqmik, and 10 times that seen with saliva alone. The concentration of Co that differentiated HGEP are exposed to in artificial saliva extracts of iqmik is likely contributing to the high levels of ROS produced.

Heavy metals, such as Cd, Co and Ni, are carcinogenic and have many mechanisms for participating in carcinogenesis, including oxidative damage to cellular proteins and DNA, as well as inhibiting the repair of damaged DNA. Cd, Co and Ni all contribute to the production of free radicals in cells. Many divalent metals participate in a Fenton-like reaction to produce free radicals in cell (37,38). Cd also binds to sulfhydryls like glutathione thus depleting glutathione stores, contributing to its toxicity and allowing ROS to propagate in the cell (20). It has been demonstrated in cell cultures that Cd, Ni and Co compete with zinc in zinc finger motifs, some of which are integral to the DNA repair process (39,40).

The main events leading to cancer are genetic alteration, chronic inflammation and malignant transformation and ROS generation is associated with all these conditions (38). Oral squamous cell carcinoma (OSCC) patients have raised levels of ROS and reactive nitrogen species (RNS) in their saliva and the salivary antioxidant content is significantly reduced compared to healthy controls. The salivary DNA and proteins are more oxidized in OSCC patients then healthy controls and the combination of ROS, RNS and depleted antioxidants promotes OSCC. (41-43).

In summary, iqmik is a greater source of heavy metals, such as Cd, Co and Ni, than air-cured tobacco leaf. HGEP accumulate more Cd, Co and Ni from the punk ash component of iqmik than from the air-cured tobacco. These metals are identified carcinogens that have the capacity to accumulate in cells and generate and propagate the production of endogenous ROS. The results of this study help elucidate the unique properties of iqmik and stress the necessity to understand the biological response to iqmik use through future studies.

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1.8 Conflict of interest statement

None declared.

1.9 References

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Table 1.1 The pH for artificial saliva extracts of iqmik, air-cured tobacco, punk ash and saliva extracts diluted 1:1 in cell culture media.

Saliva Extract	pH Undiluted	pH Diluted
Punk Ash	10.59 ± 0.01	9.59 ± 0.08
Tobacco	6.32 ± 0.02	6.86 ± 0.09
Iqmik	10.02 ± 0.05	9.03 ± 0.08

Table 1.2 Artificial saliva extraction efficiency.

	Co	**Cd**	**Ni**
Punk Ash	1.7%	0.0%	1.7%
Tobacco	17.3%	26.0%	2.4%
Iqmik	10.3%	3.6%	5.9%

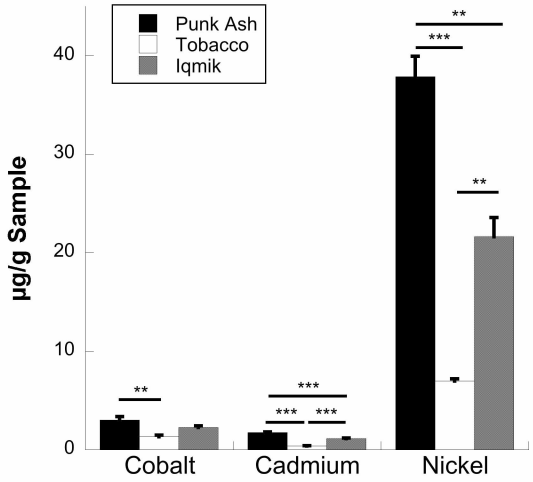

Figure 1.1 The concentration of Co, Cd and Ni in iqmik and its components. Iqmik, air-cured tobacco and punk ash (1g) samples were processed and digested as detailed in the 'Material and Methods' to determine the concentration of Co, Cd and Ni in each sample. Each bar is representative of mean ± SEM of triplicate samples. Statistical significance is indicated between means by horizontal line and *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

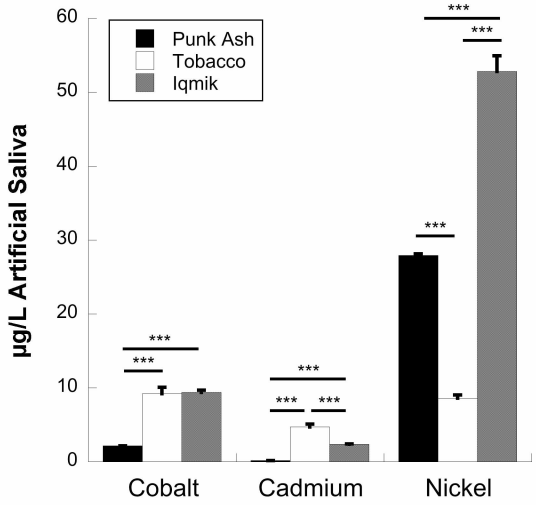

Figure 1.2 The concentration of Co, Cd and Ni in iqmik, air-cured tobacco and punk ash artificial saliva extracts. Iqmik, air-cured tobacco and punk ash (1g) samples were extracted in 25mL artificial human saliva as detailed in the 'Material and Methods' to determine the concentration of Co, Cd and Ni in each artificial saliva sample. Each bar is representative of mean ± SEM of triplicate samples. Statistical significance is indicated between means by horizontal line and *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

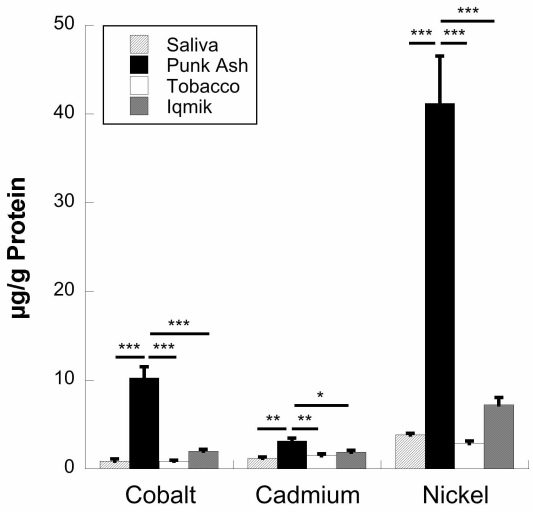

Figure 1.3 The concentration of Co, Cd and Ni accumulation in HGEP. Differentiated HGEP were exposed to artificial saliva, or artificial saliva extracts of iqmik, tobacco, or punk ash diluted 1:1 with differentiation media for 24 hours prior to digestion as detailed in the 'Material and Methods' to determine the concentration of Co, Cd and Ni accumulation in HGEP. Each bar is representative of mean ± SEM of triplicate samples. Statistical significance is indicated between means by horizontal line and *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

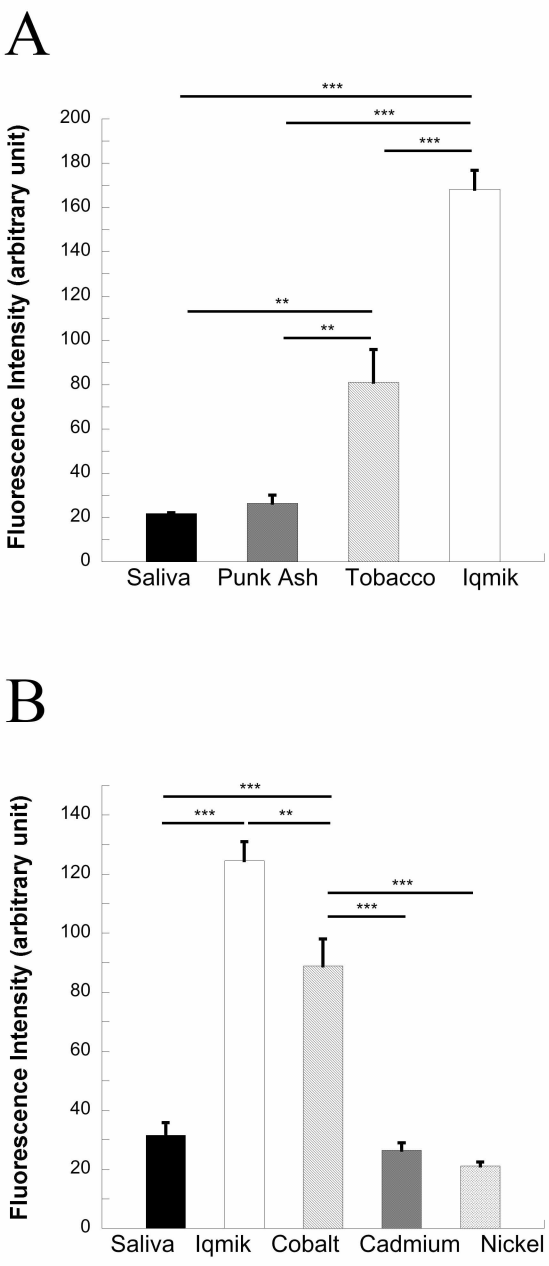

Figure 1.4 The effect of iqmik, Co, Cd and Ni on intracellular ROS levels. (A) Differentiated HGEP were treated H_2DCFDA (15μM) for 1 hour, or PBS. HGEP were exposed to artificial saliva, artificial saliva extracts of iqmik, air-cured tobacco or punk ash diluted 1:1 with differentiation media for 3 hours as detailed in the 'Material and Methods' to measure ROS levels. (B) Differentiated HGEP were treated H_2DCFDA (15μM) for 1 hour, or PBS. HGEP were exposed to $CoCl_2$ (1ppm), $NiCl_2$ (1ppm) or $CdCl_2$ (1ppm) in artificial saliva diluted 1:1 with differentiation media for 3 hours as detailed in the 'Material and methods' to measure ROS levels. Each bar is representative of mean ± SEM of triplicate samples. Statistical significance is indicated between means by horizontal line and *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Chapter 2. Iqmik, an Alaskan smokeless tobacco mixture, alters NF-κB mediated inflammatory and cellular stress response genes[2]

Abstract

Objectives: Smokeless tobacco (SLT) users have a 4 - 6 times higher risk for developing oral cancer than non-users and risk of oral cancer increases with years of use. The mechanism by which SLT, especially iqmik, triggers an inflammatory stress response is poorly defined. The purpose of this study was to establish an in vitro differentiated human gingival epithelial primary (HGEP) cell system, and to investigate the impact of tobacco and iqmik exposure on the expression of NF-κB signaling target genes in these differentiated human oral epithelial cells.

Material and methods: Differentiation was determined by protein and gene expression of keratin 10 and keratin 14. COX2 and NF-κB signaling target gene expression was measured a using qPCR and analyzed using RT^2 PCR Array Data.

Results: Iqmik induced 1.9 fold greater COX2 gene expression than tobacco exposure alone. Iqmik treatment altered gene expression of 41 of the NF-κB signaling target genes compared to the saliva treatment. Genes altered by iqmik treatment are involved in evading and inducing apoptosis, angiogenesis, blocking differentiation, proliferation, coagulation cascade and inflammation.

Conclusion: Iqmik induces a distinct profile of redox-regulated gene expression compared to tobacco leaf alone, including many of the key NF-κB dependent inflammatory and proangiogenic cytokines including IL-1, IL-6, IL-8, GM-CSF and members of the TNF family.

[2] Dwyer, G.K. and Knall, C. (2015). Iqmik, an Alaskan smokeless tobacco mixture, alters NF-κB mediated inflammatory and cellular stress response genes. Submitted to the journal Oral Oncology.

2.1 Introduction

Prolonged use of smokeless tobacco (SLT) has been reported to have significant adverse effects on human health. Tobacco use is a significant risk factor for oral disease specifically oral mucosal lesions, periodontal disease and oral cancers. Oral mucosal lesions, including leukoplakia, submucous fibrosis and oral lichen planus are common in SLT users and can develop with limited use [1]. Leukoplakia is the most prevalent epithelial precancerous lesion and has a malignant transformation rate of 12.1%, worldwide [2, 3]. Head and neck squamous cell carcinoma (HNSCC) is commonly preceded by the appearance of oral mucosal lesions. The International Agency for Research on Cancer (IARC) recently concluded that there is a causal association between SLT and oral cancer [4]. SLT users have a 4 - 6 times higher risk for developing oral cancer than non-users and risk of oral cancer increases with years of use. Tobacco and alcohol are identified as important etiological agents in the development of HNSCC. [5-7]. HNSCC is a significant problem because of the poor 5-year survival rate and functional defects associated with treatments [2]. HNSCC, the sixth most common cancer in the world, is especially prevalent in Alaska [8-10].

SLT is used by 6% of all adult Alaskans compared to the national average of only 5% of all adults. The percentage of Alaska Native people who use SLT is 4 times that of non-Native Alaskans and 45 times higher for Alaska Native women than non-Native women. The use of SLT is concentrated in Southwest Alaska where 32% of all adult Alaska Native people use SLT. Out of all adult Alaska Native people who use SLT, 35% use only iqmik, a unique Alaska-specific form [11]. Iqmik is a combination of tobacco leaves mixed with *Phellinus igniarius* (punk fungus). The fungus is collected, air-dried and then burned to fine white ash which is mixed with the air-cure leaf tobacco by mastication [12, 13]. However, very little is known about

the health effects of iqmik exposure and prevalence of HNSCC correlated to the specific use of iqmik.

The addition of ash not only increases the amount of bioavailable nicotine from the tobacco for absorption into the oral mucosa, but also is a source of higher levels of pro-inflammatory toxic metals than cured leaf tobacco. More cobalt (Co), nickel (Ni) and cadmium (Cd) could be extracted from mixtures of ash and leaf tobacco than from leaf tobacco alone when extracting with artificial human saliva [14]. Co and Ni engage in redox reactions, and induce the production of reactive oxygen species (ROS). Cd depletes antioxidant enzymes by binding their sulfhydryl groups, allowing ROS production to go unchecked. Exposure to saliva extracts of iqmik induces significantly more ROS than those of tobacco leaf (Dwyer and Knall, unpublished data). High levels of ROS activate redox-regulated transcription factors such as nuclear factor κB (NF-κB), which is the major inflammatory transcription factor, and plays a central role in many stress responses [15, 16].

NF-κB regulates the transcription of various genes involved in inflammation, proliferation and cell survival. Overexpression of NF-κB may be involved in the development and progression of malignant processes in multiple sites, including the oral cavity [2]. NF-κB can be activated in oral epithelial cells exposed to khaini, a tobacco and slaked lime mixture, with a very alkaline pH, used in India [17-19]. The NF-κB regulated gene cyclooxygenase-2 (COX2) shows increased expression in oral epithelial cells exposed to an extract of khaini [18-21]. COX2 is a crucial enzyme in the biosynthesis of prostaglandins and is expressed in high levels in many carcinomas including HNSCC [22]. Aberrant expression and activity of COX2 are causally involved in tumor promotion and progression [23]. The activation of NF-κB and the increased expression of NF-κB regulated genes such as COX2 by iqmik are unknown.

The epithelium of the oral cavity is composed of keratinocytes that are continuously renewed from the basal cell layer. The masticatory mucosa of the gingiva and hard palate undergo keratinization when differentiating [23, 24]. The intermediate filaments of the epithelial cell cytoskeleton are composed of keratin proteins whose specific isotype expression changes as a consequence of differentiation. Keratin 14 is expressed in cells of the basal layer, but upon differentiation, Keratin 10 is expressed in the gingiva [24]. Disruption of terminal keratinocyte differentiation seems to be a mechanism by which COX2 promotes epithelial tumor formation [23].

The purpose of this study was to establish an in vitro differentiated human gingival epithelial primary (HGEP) cell system, and to investigate the impact of tobacco and iqmik exposure on the expression of NF-κB signaling target genes in these differentiated human oral epithelial cells.

2.2 Materials and methods

2.2.1 Iqmik, punk ash and air-cured tobacco preparation, extraction and pH determination

Following community practice for iqmik preparation without mastication (Renner, Personal Communication), air-cured whole leaf tobacco was cut into small homogeneous pieces using a coffee bean grinder. Punk fungus was sawed into pieces weighing 300g or less, placed into a metal coffee can and ashed on a hot plate set on high. Punk ash and air-cured tobacco leaf were each weighed out to 1g for extracts. Iqmik extracts were prepared by combining 0.5g punk ash and 0.5g tobacco. Artificial human saliva was prepared with 5.3mM KSCN, 15mM $NaHCO_3$, 20mM KCl, 1.4mM NaH_2PO_4, 0.1g/L CH_4N_2O (urea), and 0.1mg/L α-amylase. Each sample (1g) was extracted in 25mL artificial human saliva. Each sample was inverted 7 times and incubated for 30 minutes at 37°C and 5% CO_2. Samples were centrifuged for 10 minutes at 2000 x *g*, the supernatant was removed from the solid phase, and filter sterilized [14]. For pH

determination, prepared extracts in triplicate were either undiluted or diluted 1:1 with CnT-32 Oral Epithelium Medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) containing $CaCl_2$ (1mM) and measured at room temperature with a Oakton pH meter (Oakton Instruments, Vernon Hills, IL) equipped with a Orion probe (Thermo Scientific, Waltham, MA).

2.2.2 Cell culture and differentiation

Primary human gingival epithelial cells (HGEP) (CELLnTEC) were cultured for use in the experiments. Cultures were maintained at a seeding density of 4.0×10^3 cells per cm^2 in CnT-24 PCT Oral Epithelium Medium (CELLnTEC). For cultures to be exposed, cells were grown for approximately 6-8 days in CnT-24 PCT Oral Epithelium Medium until one day prior to confluency at which time the medium were changed to CnT-32 Oral Epithelium Medium (CELLnTEC). Cultures were grown for an additional 24 hours and then induced to differentiate in CnT-32 containing $CaCl_2$ (1mM) (differentiation media) per manufactures directions. Cells to be exposed were cultured in differentiation media for 96hrs before all exposures.

2.2.3 Cell viability

The Live/Dead Cell Assay (Molecular Probes, Life Technologies, Carlsbad, CA) was used to determine viability of exposed differentiated HGEP. Differentiated HGEP were exposed to artificial saliva, artificial saliva extracts of iqmik, air-cured tobacco or punk ash and extracts diluted 1:1 in differentiation media for 24 hours. Treated differentiated HGEP were then incubated with, calcein AM (1μM), and ethidium homodimer-1 (2μM), live and dead dyes, respectively, for 30 minutes. Control differentiated HGEP were incubated in differentiation media (live control), or methanol (70%) (dead control) for 45 minutes. Cell viability was quantified as fluorescence intensity using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

2.2.4 Gene expression during differentiation

Keratin 10 (KRT10), Keratin 14 (KRT14) and COX2 (PTSG2) gene expression was assayed in HGEP during the differentiation process at 4 time points, just prior to switching from CnT-24 Oral Epithelium Medium to CnT-32 Oral Epithelium Medium (undifferentiated state), and then 24hrs, 48hrs, and 96hrs after the addition of differentiation media. RNA was isolated using the TRIzol protocol (Invitrogen-Life Technologies, Carlsbad, CA). RNA concentrations were determined using a spectrophotometer. Complimentary DNA was synthesized using the SuperScript II Reverse Transcriptase protocol (Invitrogen-Life Technologies). PTGS2, KRT10 and KRT14 primers (Qiagen, Valencia, CA) were used to analyze the gene expression of COX2, KRT10 and KRT14. β actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATA-binding protein (TBP) primers (Qiagen) were used to amplify these housekeeping genes to normalize gene expression. The gene expression was quantified with quantitative polymerase chain reaction (qPCR) following the Fast SYBR Green Master Mix protocol (Invitrogen-Life Technologies) using the StepOnePlus (Applied Biosystems, Grant Island, NY) real-time PCR instrument programmed as follows, 10 minute, 95°C cycle followed by 40 cycles of alternating 15 sec, 95°C denaturation and 1 minute, 60°C annealing/extension.

2.2.5 Preparation of cell lysates and protein assay

Cells were grown to confluence in 6 well tissue culture plates, washed with PBS and lysed with 100µL Mg lysis buffer (25mM Hepes pH 7.5, 150mM NaCl, 1% Igepal CA-630, 0.25% NaDOC, 10% glycerol, 25mM NaF, 10mM $MgCl_2$, 1mM EDTA, 1mM Na_3VO_4, 10µg/mL lupeptin, 10mg/mL aprotinin) on ice. A cleared lysate was collected following centrifugation at 4°C, 14,000Xg for 10 min. Protein concentrations in cleared cell lysates were determined using RC DC Protein Assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions, and the

absorbance determined using a DU 730 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Indianapolis, IN).

2.2.6 SDS-PAGE and western blot analysis

Whole cell lysates were diluted with 5X SDS sample buffer for 1 mg/mL total protein. Samples (1 mg/mL protein) were separated by SDS-PAGE and transferred on to Immobilon-FL PVDF membranes (EMD Millipore, Bellerica, MA). Membranes were blocked with 1X TBS, 0.1% Tween-20 (TBST) containing 5% nonfat dry milk (blocking buffer) for 1hr, and then incubated overnight at 4°C in TBST containing 5% BSA and antibody against keratin 14 (1:20,000, Abcam, Cambridge, MA), keratin 10 (1: 50,000, Abcam) or β-actin (1:1000, Thermo Scientific, Waltham, MA) as loading control. Membranes were washed 3 times for 5 min each in TBST and incubated with secondary anti-rabbit IgG HRP-linked antibody (1:2000, Cell Signaling Technology, Beverly, MA) in blocking buffer. Membranes were washed in TBST 3 times for 5 min each and protein bands visualized with SuperSignal West Femto maximum sensitivity substrate (Life Technologies) using FlourChem HD2 and AlphaView FlourChem HD2 software (Proteinsimple, San Jose, CA).

2.2.7 Gene expression following exposure

Differentiated HGEP were exposed for 4hr to artificial saliva, or saliva extracts of iqmik or air-cured tobacco (40 mg/ml w/v) diluted 1:1 in differentiation media. Following exposure, COX2 gene expression was assayed as described for gene expression during differentiation.

To determine alterations in NF-κB regulated gene expression in differentiated HGEP as a consequence of exposure to artificial saliva alone or artificial saliva extracts of iqmik or tobacco diluted 1:1 with differentiation media, NF-κB Signaling Targets RT^2 Profiler PCR Arrays (Qiagen) were used following manufacturer's instructions. Differentiated HGEP were exposed

for 4hr to artificial saliva or diluted saliva extracts of iqmik or tobacco with each exposure being performed in three independent experiments. Following the exposure, RNA was isolated using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions. RNA concentrations were determined using a spectrophotometer. The first strand cDNA was synthesized using the RT^2 First Strand Kit (Qiagen), which contains a proprietary buffer to eliminate any residual genomic DNA contamination in RNA samples. The cDNA was mixed with the RTC SYBR Green Mastermix and aliquoted into the 96-well plate of the RT^2 Profiler PCR Array, containing PCR primer pairs for a total of 84 genes to be screened. Amplification was performed using the StepOnePlus (Applied Biosystems) programmed for a 10 minute, 95°C cycle followed by 40 cycles of alternating 15 sec, 95°C denaturation and 1 minute, 60°C annealing/extension. The threshold cycle (CT) was calculated using the StepOnePlus software following RT^2 Profiler PCR Array guidelines. Dissociation curve analysis on each well was performed to verify PCR specificity.

2.2.8 RT^2 Profiler PCR Array data analysis

Data was analyzed using RT^2 Profiler PCR Array Data Analysis freeware (Qiagen). The housekeeping genes, ACTB, GAPDH and RPLP0 (Ribosomal protein, large, P0), included in the arrays, were selected for normalization of data. Genomic DNA controls were negative and controls for reverse-transcription efficiency and the efficiency of the PCR reaction were within manufacturer defined parameters (data not shown). The relative expression of individual genes tested in the RT^2 Profiler PCR Array was determined using individual well CT values reported by the StepOnePlus and the $\Delta\Delta$CT method after normalization. Genes were reported as altered using the method of Huggins et al. 2008 [25] which uses both fold change of ≥ 1.3 and $p < 0.2$ to establish biologically relevant changes in gene expression. Altered genes were mapped to cancer

and inflammatory pathways using REACTOME an open-source, open access, manually curated and peer-reviewed pathway database [26, 27].

2.2.9 Statistical analysis

All reported results represent the mean ± SEM of data obtained in three independent experiments. Statistical analysis was performed with the one-way ANOVA and Tukey's HSD post hoc tests. $P \leq 0.05$ was considered statistically significant.

2.3 Results

2.3.1 HGEP differentiation determination

Keratin 10 and Keratin 14 gene and protein expression were determined at 3 time points during the differentiation process. Keratin 14 was identified in HGEP just prior to the initiation of the differentiation process (Figure 2.1A, 2.1C and 2.1D). Cultures were tested for expression of Keratin 10 and Keratin 14 at 24, 48 and 96 hours after the addition of differentiation media. Keratin 14 gene and protein expression varied minimally during the differentiation process (Figure 2.1A, 2.1C and 2.1D). In sharp contrast, Keratin 10 gene and protein expression increased significantly during HGEP differentiation and continued to increase through the 96 hour time point, with a significant increase in expression at 24 hours ($p \leq 0.014$, gene), 48 hours ($p \leq 0.0023$, gene) and 96 hours ($p \leq 0.0009$, gene, $p \leq 0.0001$, protien) compared to undifferentiated HGEP (Figure 2.1B, 2.1C and 2.1D).

2.3.2 COX2 gene expression during HGEP differentiation

COX2 gene expression was measured in undifferentiated HGEP and at 3 time points, 24, 48 and 96 hours, during the differentiation process. COX2 gene expression was detected in HGEP just prior to the initiation of the differentiation process (Figure 2.2). As one would predict, the expression of COX2, which is associated with mesenchymal traits, decreased upon

differentiation of the epithelial cells (Figure 2.2). After 24 hours in differentiation media, COX2 gene expression had decreased by approximately 9 fold, and maintained the reduced expression through the 96 hour time point with differentiation media.

2.3.3 Cell viability of exposed differentiated HGEP

To determine the effect on cell viability of artificial saliva, and artificial saliva extracts of tobacco, punk ash or iqmik, with or without dilution in differentiation media, differentiated HGEP were exposed for 24 hours to these various treatments. Extracts of air-cured tobacco ($p \leq 0.0001$) and artificial saliva ($p \leq 0.0001$) diluted 1:1 in differentiation media were significantly less cytotoxic than pure extract (Figure 2.3). HGEP treated with artificial saliva extracts of iqmik ($p \leq 0.0001$), air-cured tobacco ($p \leq 0.0001$) and saliva alone ($p \leq 0.0001$) diluted 1:1 in differentiation media exhibited significantly less cytotoxicity than the control dead samples (Figure 2.3).

2.3.4 COX2 gene expression in exposed differentiated HGEP

Differentiated HGEP were exposed for 4 hours to artificial saliva or saliva extracts of iqmik or tobacco, and COX2 gene expression was measured by qPCR. Short-term exposure to iqmik resulted in an up-regulation of COX2 gene expression (Figure 2.4). Iqmik induced 1.9 fold greater COX2 gene expression than tobacco exposure, although not statistically significant, and a significantly greater up-regulation of COX2 gene expression than the media control ($p \leq 0.0001$).

2.3.5 NF-κB signaling target gene expression

NF-κB signaling target gene expression was measured with RT^2 Profiler PCR Arrays, containing PCR primer pairs for a total of 84 genes. Iqmik treatment altered gene expression of 41 of the NF-κB signaling target genes compared to the saliva treatment alone using the parameters defined in Huggins et al. (2008) (Table 2.1). Genes altered by iqmik treatment are involved in

evading and inducing apoptosis, angiogenesis, blocking differentiation, proliferation, coagulation cascade and inflammation. Tobacco treatment altered 31 of the NF-κB signaling target genes compared to the saliva treatment alone (Table 2.2). Many of the iqmik altered genes mapped to cancer and inflammatory pathways in the REACTOME, a peer-reviewed database (Table 2.3). The cancer-associated pathways include sustained angiogenesis, coagulation cascade, proliferation, blocking differentiation and pro-apoptosis/anti-apoptosis. The inflammation-associated pathways include TNF family, IL-1 family, interferons, cell recruitment, hematopoietins, coagulation cascade and IL-6 signaling.

2.4 Discussion

The oral epithelium basal layer includes cells with a self-renewing capacity. These cells are responsible for renewing the epithelium lining and regenerate rapidly when damaged. These cells differentiate as they move through the layers of epithelium and become keratinocytes in the gingival area. HNSCC likely arises from an accumulation of mutations within the renewing cell population of the basal layer or from differentiated cells that have regained the capacity to self-renew [28].

COX2 is not expressed under normal physiologic conditions, but is widely overexpressed in pathophysiologic conditions, such as cancer [29, 30]. COX2 activation is an early event during carcinogenesis. COX2 overexpression is evident in oral mucosal lesions with an increasing level of COX2 expressed from hyperplasia to dysplasia with the highest levels reported in HNSCC [31]. The overexpression of COX2 alters cell adhesion, alters the response to signals from growth regulators and inhibits apoptosis [32]. Products of COX2 enzyme activity, the prostaglandins, activate nuclear hormone receptors that are responsible for intracellular levels of calcium and increase cellular proliferations rates [29]. Prostaglandins also promote angiogenesis

and inhibit immune surveillance [29, 33]. COX2 deficiency has been demonstrated to protect against the formation of epithelial tumors [23, 34]. The reduced tumor formation is in response to premature terminal differentiation in keratinocytes. Keratins 1 and 10, indicators of commitment to differentiate, are both expressed in basal keratinocytes in COX-2 deficient mice [34].

NF-κB signaling is suggested to play an important role in the early stages of head and neck cancer. Protein levels of NF-κB gradually increase from premalignant lesions to invasive cancer [28]. NF-κB activation can stimulate STAT3, another key transcription factor in HNSCC, through IL-6 signaling. HNSCC has many aberrantly activated, interrelated signaling pathways [35].

Studies have found that long-term SLT exposure can cause disturbances in the differentiation process of the oral epithelium. Three-dimensional epithelial cultures exposed to long-term treatments of Swedish snuff showed weaker expression of keratin 10 than the control samples that expressed keratin 10 in the upper two thirds of the epithelium [36, 37]. In our study we demonstrated differentiation of the HGEP prior to treatment by the increasing expression of keratin 10. A future study should investigate long-term iqmik treatments effect on keratinization markers such as keratin 10 and involucrin [36].

High levels of IL-1α and β have been observed at the SLT placement site of habitual users, indicating an inflammatory response and aberrant epithelial proliferation [38]. Studies that utilized human volunteers demonstrated development of SLT lesions in over half the volunteers by day 7, as well as, a significant increase in IL-1α and prostaglandin E_2, a downstream product of COX2 enzymatic activity [37-39]. Animal studies have demonstrated an up-regulation of COX2 at the site of SLT application [21]. In our experiments, short-term exposure to iqmik

resulted in what appears to be an up-regulation of COX2 expression greater than tobacco exposure alone, but is not statistically significant.

Prominent features of oral cancer include alterations to host immunity, inflammation, angiogenesis and metabolism and are associated with increased tumor growth, metastasis and decreased survival rates. Tumor development requires proinflammatory, proangiogenesis and immunoregulatory signaling. NF-κB is an early response gene that is elevated by tobacco use and chronic inflammatory conditions. The overexpression of NF-κB correlates with the overexpression of proinflammatory, proangiogenic and anti-apoptotic signaling which are prevalent in HNSCC [40].

Iqmik exposure altered the gene expression of 41 of the tested NF-κB signaling target genes (Table 2.1) compared to the saliva treatment alone as defined by the parameters of Huggins et al. [25], which has been found to identify more biologically meaningful sets of altered genes than p-value alone or more strict fold-change and p-value cut-offs [41]. Genes altered by iqmik exposure are involved in evading and inducing apoptosis, angiogenesis, blocking differentiation, proliferation, coagulation cascade and inflammation. Tobacco exposure altered 31 of the test genes (Table 2.2) compared to the saliva treatment alone. Iqmik treatments had a unique NF-κB signaling target gene expression profile, 9 genes altered by both iqmik exposure and tobacco exposure were upregulated by iqmik, but down regulated by tobacco exposure, and 24 of the altered genes were more significantly altered by iqmik than by tobacco, based on the Huggins et al. [25] significance parameters.

2.5 Conclusion

Although Iqmik is perceived as a safer alternative to other tobacco products [42], it induces more cancer associated genes than tobacco alone including many of the key NF-κB-dependent

inflammatory and proangiogenic cytokines, such as, IL-1, IL-6, IL-8, GM-CSF and members of the TNF family. Tobacco use is a significant risk factor for oral disease, specifically oral mucosal lesions, periodontal disease and oral cancers, such as HNSCC, which is especially prevalent in Alaska. The findings reported herein make iqmik a unique health hazard compared to other tobacco products.

2.6 Acknowledgements

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2.7 Role of the funding source

Funding sources played no role in the work presented herein.

2.8 Conflict of interest

None declared

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Table 2.1 Iqmik altered NF-κB signaling target genes.

Gene	Name	Fold Regulation	*p*-value
EGR2	Early growth response 2	67.0	0.17
CSF2	Colony stimulating factor 2	46.6	0.08
NCOA3	Nuclear receptor coactivator 3	13.5	0.13
CCL2	Chemokine (C-C motif) ligand 2	12.6	0.22
SNAP25	Synaptosomal-associated protein, 25kDa	12.1	0.06
MYC	V-myc myelocytomatosis viral oncogene homolog	11.6	0.17
IL1R2	Interleukin 1 receptor, type II	10.7	0.07
IL8	Interleukin 8	10.5	0.19
IL1A	Interleukin 1, alpha	8.2	0.15
NR4A2	Nuclear receptor subfamily 4, group A, member 2	8.0	0.15
SELE	Selectin E	7.2	0.11
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	7.0	0.19
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	6.7	0.02
CSF3	Colony stimulating factor 3	6.0	0.21
IL1B	Interleukin 1, beta	6.0	0.08
IL6	Interleukin 6 (interferon, beta 2)	5.3	0.02
CD83	CD83 molecule	4.7	0.02
CCL22	Chemokine (C-C motif) ligand 22	4.6	0.18
REL	V-rel reticuloendotheliosis viral oncogene homolog	4.4	0.08
IFNB1	Interferon, beta 1, fibroblast	4.2	0.15
IL1RN	Interleukin 1 receptor antagonist	4.2	0.22
BIRC2	Baculoviral IAP repeat containing 2	3.4	0.01
F3	Coagulation factor III (thromboplastin, tissue factor)	2.9	0.13
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.8	0.11
XIAP	X-linked inhibitor of apoptosis	2.8	0.07
EGFR	Epidermal growth factor receptor	2.7	0.03
CXCL2	Chemokine (C-X-C motif) ligand 2	2.7	0.04
IL15	Interleukin 15	2.6	0.03
NQO1	NAD(P)H dehydrogenase, quinone 1	2.6	0.03
AGT	Angiotensinogen	2.3	0.14
CCND1	Cyclin D1	2.1	0.23
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	2.1	0.16
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.1	0.09
ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	1.7	0.17
CSF1	Colony stimulating factor 1	1.7	0.19
IRF1	Interferon regulatory factor 1	1.7	0.15
STAT3	Signal transducer and activator of transcription 3	1.6	0.07
PLAU	Plasminogen activator, urokinase	1.6	0.16
PDGFB	Platelet-derived growth factor beta polypeptide	-2.0	0.15
ADM	Adrenomedullin	-2.6	0.02
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	-4.9	0.22

Table 2.2 Tobacco altered NF-κB signaling target genes.

Gene	Name	Fold Regulation	*p*-value
CSF2	Colony stimulating factor 2	8.2	0.13
IL1B	Interleukin 1, beta	6.7	0.00
IL1A	Interleukin 1, alpha	6.5	0.01
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	5.4	0.05
CSF3	Colony stimulating factor 3	5.0	0.01
NR4A2	Nuclear receptor subfamily 4, group A, member 2	4.7	0.06
NCOA3	Nuclear receptor coactivator 3	4.0	0.00
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity	3.5	0.17
IL8	Interleukin 8	3.5	0.08
IL6	Interleukin 6 (interferon, beta 2)	3.1	0.21
BCL2A1	BCL2-related protein A1	3.0	0.19
REL	V-rel reticuloendotheliosis viral oncogene homolog	2.9	0.03
EGFR	Epidermal growth factor receptor	2.7	0.01
SNAP25	Synaptosomal-associated protein, 25kDa	2.6	0.20
PLAU	Plasminogen activator, urokinase	2.4	0.06
CCL22	Chemokine (C-C motif) ligand 22	2.3	0.08
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.3	0.03
FASLG	Fas ligand (TNF superfamily, member 6)	2.2	0.07
MYC	V-myc myelocytomatosis viral oncogene homolog	2.0	0.23
BIRC2	Baculoviral IAP repeat containing 2	1.9	0.01
XIAP	X-linked inhibitor of apoptosis	1.7	0.16
NQO1	NAD(P)H dehydrogenase, quinone 1	1.6	0.09
IL15	Interleukin 15	1.5	0.23
RELA	V-rel reticuloendotheliosis viral oncogene homolog A	1.5	0.11
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	1.4	0.24
IFNB1	Interferon, beta 1, fibroblast	-2.4	0.19
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-2.7	0.14
ADM	Adrenomedullin	-2.8	0.00
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	-4.4	0.17

Table 2.3 Iqmik altered genes in cancer and inflammatory pathways.

Iqmik Altered Inflammatory Genes		**Iqmik Altered Genes in Cancers Pathways**		
Coagulation Cascade	**Hematopoietins**	**Coagulation Cascade**	**Proliferation**	**Pro-apoptotic**
F3	IL6	F3	IκBα	TRAIL/TNFSF10
F8	CSF1, 2, 3	F8	NFκB	IL1
PLAU	IL15	PLAU	c-Myc	TNFR1
			Cyclin D1	LTA
Interferons	**IL6 Signaling**	**Sustained Angiogenesis**	p21	AGT
IFNB1	AGT		NCOA3	
	IκBα		EGFR	**Anti-apoptotic**
TNF Family	NFκB	PDGFB	PDGFB	IAPs
LTA	IL6	IκBα	AGT	BCL-xL
TNFRSF1B	STAT3	NFκB		IL15
TRAIL/TNFsf10		COX2	**Blocking Differentiation**	IκBα
	Cell Recruitment	STAT3		NFκB
IL-1 Family	IL8	IL8	IL6	
IL1A & IL1B	SELE	c-Myc	C-Myc	
IL1R2	CXCL2	EGFR	CSF1, 2, 3	
IL1RN/RA	CCL2	NCOA3		
	CCL22			

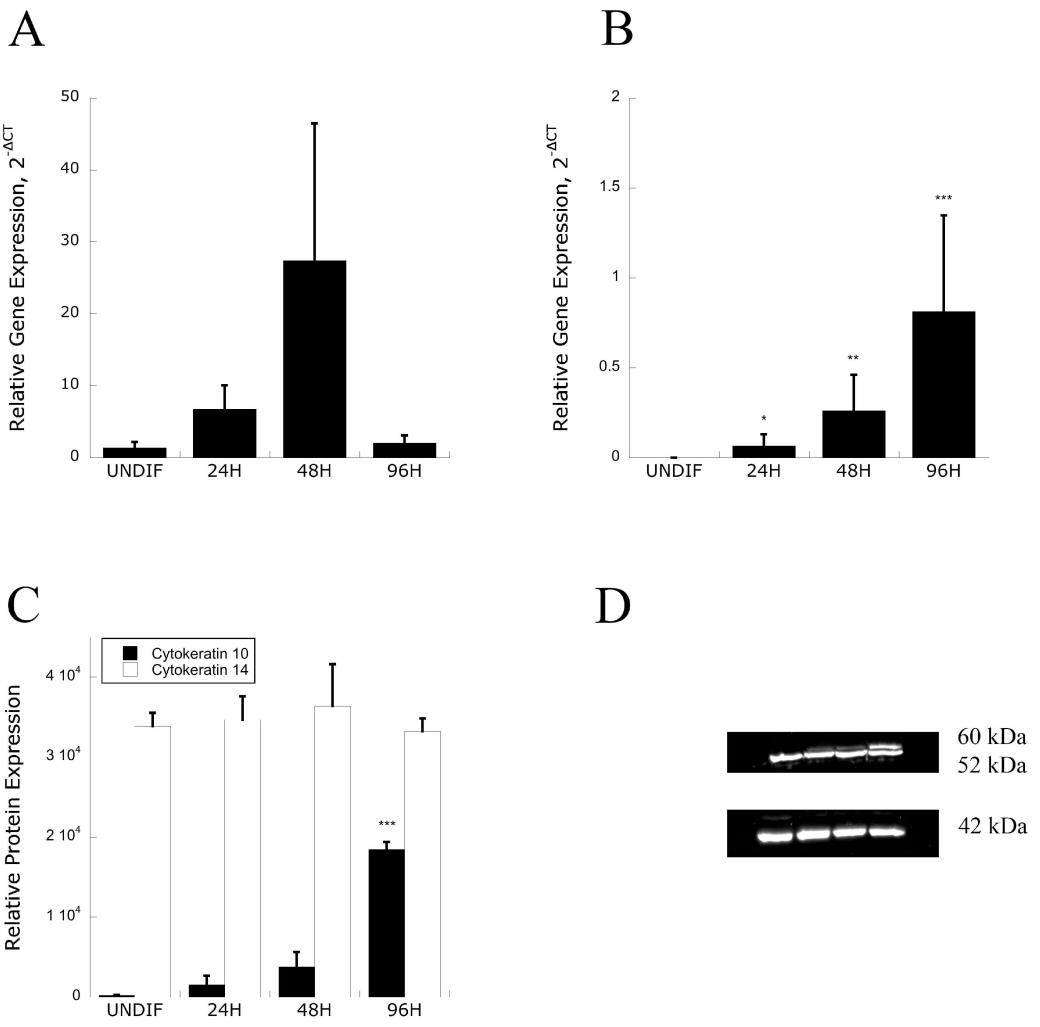

Figure 2.1 Keratin 10 and 14 gene and protein expression in HGEP differentiation. Keratin 14 (A) and keratin 10 gene (B) and protein (C) expression was determined by qPCR and western blot of keratin 14 (52 kDa), keratin 10 (60 kDa) and β-actin (42 kDa) (D), as described in Materials and Methods during HGEP differentiation, just prior to starting, and then at 24, 48, and 96hrs after the addition of differentiation media. Gene data are representative of triplicate samples and are expressed as $2^{-\Delta CT}$. Data are representative of mean ± SEM. Significance represents comparison to undifferentiated (UNDIF) control for all data, *$p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

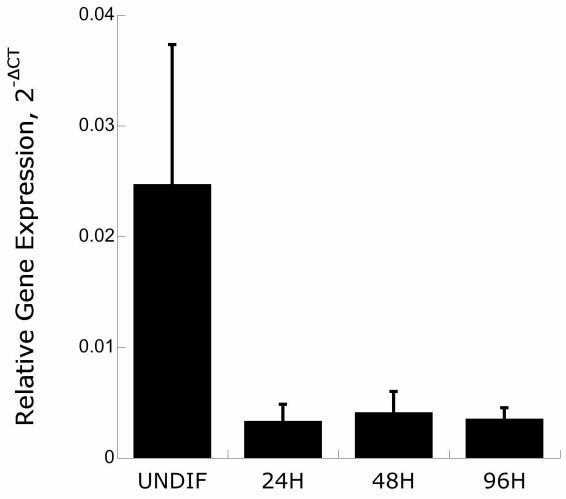

Figure 2.2 COX2 gene expression during HGEP differentiation. COX2 gene expression was determined by qPCR during HGEP differentiation just prior to starting, and then at 24, 48, and 96hrs after the addition of differentiation media as detailed in Material and methods. Data are representative of triplicate samples and are expressed as $2^{-\Delta CT}$. Data are representative of mean ± SEM. Data are not significant, but represent a trend in gene expression.

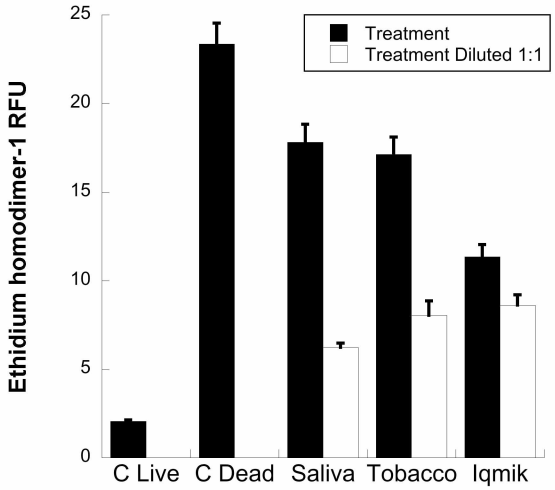

Figure 2.3 Cell viability following saliva extract exposure. Cell viability was measured with the Live/Dead Cell Assay after differentiated HGEP were exposed for 24hrs to artificial saliva, artificial saliva extracts of iqmik, air-cured tobacco or punk ash or extracts diluted 1:1 in differentiation media as detailed in Material and methods. C represents control. Data are representative of mean ± SEM of triplicate samples.

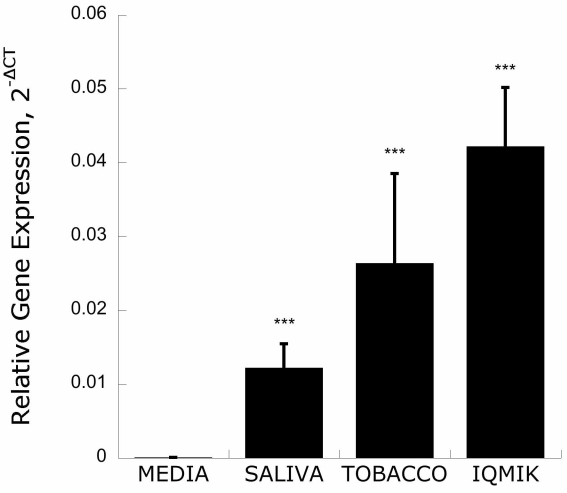

Figure 2.4 COX2 gene expression in differentiated HGEP exposed to diluted iqmik and tobacco saliva extracts. COX2 gene expression was determined by qPCR after differentiated HGEP were exposed for 4hrs to the indicated saliva extracts as detailed in Material and methods. Data are representative of triplicate samples and are expressed as $2^{-\Delta CT}$. Data are representative of mean ± SEM. Significance represents comparison to media control, *$p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Chapter 3. Biomarkers for iqmik smokeless tobacco use: future research directions[3]

Abstract

Alaska Natives have an oral cancer incidence rate of 16.0 cases per 100,000 per year. The incidence of oral cancer is estimated to be rising annually by 2.6% in Alaska, and for Alaska Natives the incidence is rising by 6.5% annually. Nearly half of patients diagnosed with oral cancers have already developed metastases prior to their diagnosis. This stresses the need for early detection of pre-malignant oral lesions to improve patients' survival. Several diagnostic methods are currently in development for earlier diagnosis of oral cancers, including saliva biomarkers. The advantage of saliva biomarker analysis is the easy and noninvasive collection procedure. This article proposes a case-control study to determine biomarkers for oral cancer in tobacco users in Alaska and to develop an accurate method for earlier detection of oral cancer by testing the level of inflammatory cytokines in saliva samples. This proposed pilot study will provide a foundation for future tobacco and oral cancer biomarker research in Alaska focused on addressing health concerns related to tobacco use in Alaska Native peoples.

[3] Dwyer, G.K. and Knall, C. Biomarkers for iqmik smokeless tobacco use: future research directions. Prepared for submission in the Hypotheses/Commentaries section in the journal Cancer Epidemiology Biomarkers & Prevention.

3.1 Introduction

Head and neck cancers are the sixth most common cancer in the world and are especially prevalent in Alaska (1-3). Oral squamous cell carcinomas (OSCC), which arise from the epithelium lining of the oral cavity, make up more than 90% of all oral cancers (4). Alaska Natives have an oral cancer, age-adjusted, incidence rate of 16.0 cases per 100,000 per year compared to 10.8 cases per 100,000 per year for non-Natives with an average of 71 cases per year in Alaska. The incidence of oral cancer is estimated to be rising annually by 2.6% in Alaska and by 6.5% among Alaska Native peoples. Among Alaska Native people the mortality rate from oral cancer is 6.1 cases per 100,000 per year which is more than twice that at of Non-Native Alaskans (5).

Tobacco and alcohol are identified as important etiological agents in the development of OSCC. (6-9). Smokeless tobacco (SLT) users have a 4 - 6 times higher risk for developing oral cancer than non-users and risk of oral cancer increases with years of use. The likelihood of developing oral cancer is related to the amount of tobacco products used in a dose-response manner (10). SLT is used by 6% of all adult Alaskans compared to the national average of only 5% of all adults. The percentage of Alaska Natives who use SLT is 4 times that of non-Native Alaskans. The use of SLT is concentrated in Southwest Alaska where 32% of all adult Alaska Natives use SLT. Out of all adult Alaska Natives who use SLT, 35% use only iqmik, a form of SLT unique to Alaska (11).

The development of oral cancer consists of several stages of oral premalignant lesions with varying degrees of dysplasia. These oral lesions precede the majority of oral cancers. A minority of oral cancers, approximately 30%, appear de novo from histologically normal oral epithelium (12, 13). Oral cancer is an aggressive cancer that commonly metastasizes to other organs, and

nearly half of all patients diagnosed with oral cancers present with metastatic disease. HNSCC is a significant problem because of its poor 5-year survival rate, about 62%, and functional defects associated with treatments (4, 14). Early detection of oral lesions has a significant effect on survival rates, but a more effective method to detect oral cancers at an earlier stage is needed (15).

The most common diagnostic tool for detecting oral cancers is by visual inspection. However, there is little evidence that this form of screening reduces the incidence of or mortality from oral cancers (10, 12). Therefore, a new method of screening, based on evidence, is needed to reduce the incidence and mortality associated with SLT use.

Only a small percentage of oral mucosal lesions, of which leukoplakia is the most common epithelial precancerous lesion, will transform into cancer with a malignant transformation rate of 12.1%, world-wide (16). Identification of true preneoplastic changes in the oral cavity would benefit from molecular and biochemical detection methods. A practical application of these markers, implemented in the clinic, has the potential to provide a more accurate detection of oral lesions which will undergo malignant transformation (12).

Several diagnostic methods are currently in development for earlier diagnosis of oral cancers, including saliva biomarkers. A saliva based assay to test for pre-malignancy would more easily and accurately identify patients that will develop oral cancer. Saliva contains constituents that reflect the disease state of the body and particularly for the oral cavity (12, 17, 18). The advantage of saliva biomarker analysis is the easy and noninvasive collection procedure, compared to a blood draw or biopsy (4). Biomarker data should be collected that is biologically relevant for the population of patients being screened.

NF-κB dependent cytokines, interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor alpha (TNF α) are elevated in oral squamous cell carcinoma (OSCC) tissue samples and in saliva samples from patients with OSCC and oral pre-malignant lesions (OPML) compared to health controls (19-22). Cyclooxygenase (COX) 2, another NF-κB mediated signaling target, and its product prostaglandin E2 (PGE2) are highly active in tobacco induced carcinogenesis of OSCC (23).

Our laboratory has demonstrated that artificial saliva extracts of the Alaska specific SLT iqmik elevated NF-κB dependent proinflammatory cytokine expression (Figure 3.1) to a greater extent than saliva extracts of tobacco alone (Dwyer and Knall, unpublished observations). We therefore propose a study design to test the hypothesis that there is a difference in proinflammatory cytokines expressed in whole unstimulated saliva among users of tobacco types in Alaska and those with oral cancer.

3.2 Strategic research design

To identify biomarkers for oral cancer in tobacco users in Alaska, and to develop an accurate method for earlier detection of oral cancer in users of the Alaska specific SLT iqmik, a case-control study will be performed to test the level of inflammatory cytokines in saliva samples collected from participants with diagnosed oral cancer who self-report using iqmik, commercial SLT, cigarettes or no tobacco use. A control group of participants that are oral cancer-free will also be recruited into each group of tobacco use.

The case-control study is a mainstay in molecular epidemiology studies because of its ability to target rare diseases, and its strength in focused questionnaire information and specimen collection (24). The benefit of a case-control study is that it can be used to compare the disease, oral cancer, to healthy controls. The levels of inflammatory biomarkers can be quantitatively

analysed between the cases and the healthy controls. Biomarker data between exogenous exposures, cigarettes, SLT or iqmik, will be valuable in determine the inflammatory profile between cases and healthy controls of each exposure group. Some of the disadvantages of a case-control study are that biomarker data is only collected one time and because of the incidence rate cannot be determined from a case-control study unless all cases from the target population are recruited. Case-control studies are retrospective studies and are susceptible to recall bias. The participants may not accurately remember the length of tobacco use. Selection bias is another common problem with this study design and can be a particular problem with so few cases and controls recruited into each group. The goal of the control group is to be representative of the population from which the cases are recruited. (25-27).

A longitudinal study, such as a prospective cohort study would allow for continual biomarker collection through the full range of oral cancer development from tobacco use. The cohort study design compensates for many of the biases that can arise from a case-control study and allows for nested case-control designs, which is more ideal for biomarker studies, allowing for immediate analysis after collection (28). The disadvantage of a cohort study is that it is more costly and the latency from tobacco exposure/use to disease development is lengthy (27).

3.3 Sample size calculation

A previous study performed by Rhodus et al., 2005 demonstrated significantly increased levels of cytokines IL-1, IL-6, IL-8 and TNF α in saliva samples collected from HNSCC participants compared to HNSCC-free participants. A sample size of 9 participants in each group will provide 95% power to detect a significant difference between specific biomarker group means assuming a two-tailed $\alpha = 0.05$ test, if the cytokine data has a similar distribution to the Rhodus et al., (20) study.

3.4 Participants

The majority of Alaska Native peoples, from throughout Alaska, with oral cancer cases are diagnosed and treated in Anchorage, Alaska (Personal communication, Janet Kelly). This study will work with the appropriate tribal authorities to obtain tribal approval of the proposed research involving Alaska Native peoples, and to obtain approval from the governing Institutional Review Boards for research with human subjects to recruit participants in the Anchorage area.

Targeted enrolments will be focused on Alaska Natives, as they represent the vast majority of users of iqmik, and have higher rates of mortality for oral cancer. The goal is to recruit SLT (iqmik or other SLT) users, cigarette users and non-tobacco users with and without oral cancer. There are approximately 71 cases of oral cancers diagnosed each year in Alaska, of those between 20-25 cases are diagnosed at the Alaska Native Medical Center (ANMC) in Anchorage (Personal Communication, Matthew Olnes). Recruitment will occur at ANMC over an 18 month period for a total of 60 participants (Figure 3.2) accounting for an estimated 10% rate of attrition for a final sample size of 54 participants, 9 participants in each sub-group (oral cancer participants, or oral cancer-free participants into SLT, cigarette and non-user sub-groups). Oral cancer participants will be recruited through the Ear, Nose and Throat department at ANMC, where most oral cancer diagnoses occurs (Personal Communication, Matthew Olnes). Control participants that are age and gender matched to cases will be recruited through the Dental department at ANMC.

To be eligible to take part in the study, participants must meet the following inclusion criteria: (1) be Alaska Native (2) aged 18 years or older, (3) provide written informed consent. An additional inclusion criterion for oral cancer participants is a primary untreated oral cancer. The exclusion criteria for oral cancer participants include previous chemotherapy, radiotherapy and

previous oral surgery other than oral biopsy, and the inability to properly consent. The exclusion criteria for oral cancer-free control individuals included previous chemotherapy, radiotherapy, systemic conditions associated with immune dysfunction, and the presence of any oral mucosal lesions. Exclusions will include individuals that are taking any drugs that would induce hyposalivation and for the non-users group any tobacco use within the last 5 years. Studies have shown that oral cancer risk decreases by 50% after 5 years of abstinence from tobacco products (29).

All potential participants will be provided the consent forms and given sufficient time to read and to address any questions that may arise before a signature is obtained, if they wish to participate. Consent for banking of samples and their use in future studies will also be independently obtained. The participants will also be asked to consent to be contacted in the future regarding their biomarker results, if they wish to participate in future cessation message development. The participants will be given a copy of the signed consent form and the principal investigator will retain the original for documentation.

At the time of enrollment, an interview-based assessment will be conducted with the participants to assess socio-demographics, duration, amount and type of tobacco used, spouse/partner tobacco use, number of household tobacco users, household restrictions on smoking and other tobacco use, duration, amount and type of alcohol use and knowledge of Epstein-Barr Virus (EBV), Hepatitis C virus (HCV) or oral Human Papillomavirus (HPV) infection. A tobacco user is defined as a participant that has used tobacco for at least 1 year, on a weekly basis. Current tobacco users will be assessed for the type and amount of tobacco used and time of last cigarette or last SLT use. Non-tobacco users will be assessed for abstinence from tobacco for at least 5 years and previous use of tobacco. The assessments of tobacco use and

other variables collected by the investigators at the time of enrollment and at sample collection will be entered into a database. Questionnaire data and biologic specimens will be assigned coded study numbers and links to personal identifiers will be kept in a password protected location that is separate from questionnaire data and biological specimens, and will only be accessible to the principal investigator.

3.5 Saliva sample analysis

Saliva samples are a minimally invasive technique that provides RNA information and key inflammatory markers. Saliva will be collected as described by Rhodus et al., (20). Briefly, subjects will expectorate all saliva into a 50 ml centrifuge tube for 5 minutes without swallowing. The final volume and flow rate of saliva will be determined gravimetrically. Saliva samples will be aliquoted appropriately for future studies, if consented for banking, and frozen at -80^{o}C. The saliva samples will be separated with centrifugation and prepared for analysis following the method described by Lisa Cheng et al., (21). Samples will be screened for cytokine expression by enzyme-linked immunosorbent assay (ELISA) (Figure 3.3) for IL-1, IL-6, IL-8, COX2 and TNF α (Thermo Scientific, Waltham, MA), as well as screening for EBV, HCV and HPV infection (Abnova, Taipei City, Taiwan) (30-32).

3.6 Statistical analysis

Nonparametric statistical tests in commercial software SPSS 10.0 (SPSS Inc., Chicago, IL) with significance level $p < 0.05$ will be used to analyze the data because of the small sample size in each group, which increases the potential to not fulfil the normal distribution requirement of a parametric test. Mann-Whitney tests will be used to compare two groups and Kruskal-Wallis test will be used to compare more than two groups.

Multiple linear regression modelling will be used to account for confounding variables, such

as length of tobacco use, alcohol use, EBV, HCV and/or HPV infection, which are etiological agents in oral cancer development (33, 34). Multiple linear regression modelling will be used to compare tobacco type and oral cancer diagnosis to biomarker levels.

3.7 Feasibility measures

The number of oral cancer cases diagnosed each year in Alaska is limited, and the majority of iqmik users reside in Southwest Alaska. Opportunities for Alaska Natives in remote villages in Alaska to be diagnosed with oral cancer early are scarce. The 18-month window for patient recruitment may be insufficient to recruit a diverse group of participants including iqmik users. Therefore, data related to participant recruitment will be recorded, including the number of potential participants referred to, approached by the investigators and the number excluded by the specific criteria of the study. A recruitment rate will be calculated as the number enrolled by the total eligible participants. The total eligible participants will include those that satisfy the inclusion and exclusion criteria and are referred to the principal investigator by the medical staff in the departments designated for recruitment at ANMC.

3.8 Expected results

Previous studies have demonstrated a significant increase in levels of NF-κB dependent proinflammatory cytokines in the saliva of oral cancer patients compared to controls (20, 21). These same cytokines have been demonstrated to be elevated in oral preneoplastic lesions compared to controls, as well. With successful recruitment in this study, a significant difference should be evident between health controls and oral cancer patients. These proinflammatory cytokines may be used as biomarkers indicating carcinogenic transformation from oral preneoplastic lesions to oral cancer enabling earlier detection and providing a screening method more relevant to the Alaska Native population and particularly iqmik users. Another use for

saliva biomarkers, such as these proinflammatory cytokines, may also be preliminary biomarkers, identified prior to treatment, for a measure of efficacy of therapy treatments for patients with oral cancer. A future study may benefit from using the biomarker levels identified in the recruited participants with primary oral cancers, before treatment, as a comparison to evaluate the effectiveness a variety of treatment methods.

Data from the different sub-groups will provide information on the pro-inflammatory environment created by different tobacco products used, such as iqmik. Our laboratory has demonstrated that NF-κB dependent proinflammatory cytokine gene expression is significantly elevated in HGEP (Figure 3.1) following exposure to iqmik saliva extracts compared to the saliva control. Our data suggests that there is a greater gene expression of the NF-κB dependent proinflammatory cytokines IL-1, IL-6, IL-8, COX-2 and TNF-α following iqmik saliva extract exposure than tobacco extract exposure, but a study of this nature has not been performed yet on iqmik users saliva. Thus, the sample size may not be sufficient to identify a significant difference between tobacco products, but can be used to determine if a larger study in the future would be feasible to identify a significant difference (19).

3.9 Conclusions and future directions

Biomarker data as a method for measuring biological events, such as the development of cancer, is extremely useful and important. The best example of this to date is the analysis of *TP53* mutations in lung cancer of smokers and non-smokers. The pattern of mutations in non-smokers is very different from the pattern found in smokers, closely resembling the pattern in other non-tobacco-related cancers (35, 36).

A case-control study allows for data collection at only one point so the data only examines one outcome and the temporal sequence of exposure can be difficult to determine (27). A nested

case-control study, while being more expensive and more involved study to execute, would allow for a more accurate temporal data collection and the study participants would be recruited into a larger cohort that would be beneficial for future study, such as measuring the effectiveness of treatments with biomarker data (28).

The results of this preliminary biomarker research will quantify the inflammatory environment present in cigarette, SLT and iqmik users oral cavity as well as the biological relevance of the NF-κB dependent cytokines as markers of cancer development in tobacco users, particularly iqmik users, which have never been studied before. The data will be accessible to design larger studies in the future to verify population and tobacco product relevant diagnostic markers in patients with OPML, as precursors to oral cancer. The information from this proposed research will be available to regional health authorities and researchers to develop cost effective assays for early detection of OPLM and oral cancer.

The biomarker data may also be used to develop a tobacco cessation intervention for the participants of the study, that consented to being contacted. Iqmik is perceived as safer alternative than other tobacco products and Alaska Native women have been reported to switch from other tobacco products to iqmik during pregnancy (37). Very little research to date has explored the health effects of iqmik use, so there is very little data available to develop a biologically informed, evidence-based cessation program for iqmik users. Additionally, banked samples can be utilized in the future to investigate the effect of tobacco products on oral cancer progression and investigate additional oral cancer biomarkers. This proposed pilot study will provide a foundation for future tobacco and oral cancer biomarker research in Alaska focused on addressing health concerns related to tobacco use in Alaska Native peoples.

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3.11 Disclosure of potential conflict of interest

No potential conflicts of interest to disclose.

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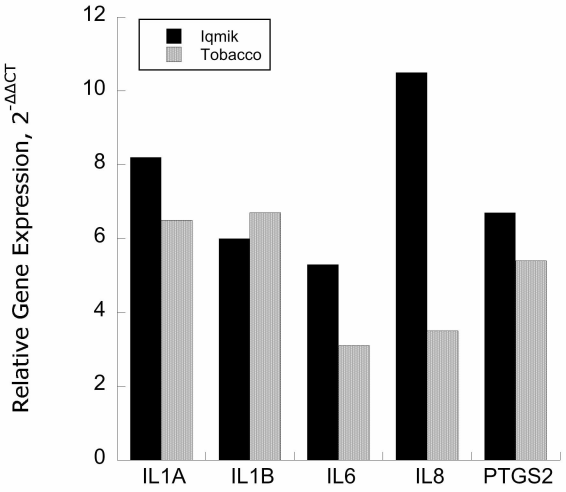


Figure 3.1 Iqmik and tobacco altered NF-κB proinflammatory gene targets. NF-κB signaling target gene expression, IL-1A, IL-1B, IL-6, IL-8 and PTGS2 (COX2) was measured by qPCR with RT^2 Profiler PCR Array following a 4hr exposure of differentiated HGEP to diluted iqmik and tobacco saliva extracts, and data analyzed using RT^2 PCR Array Data Analysis freeware. Genes were reported as altered using both fold change of ≥ 1.3 and $p < 0.2$. Data are representative of triplicate experiments and expressed as fold regulation (positive fold regulation as $2^{-\Delta\Delta CT}$ and negative fold regulation as $-1/2^{-\Delta\Delta CT}$).

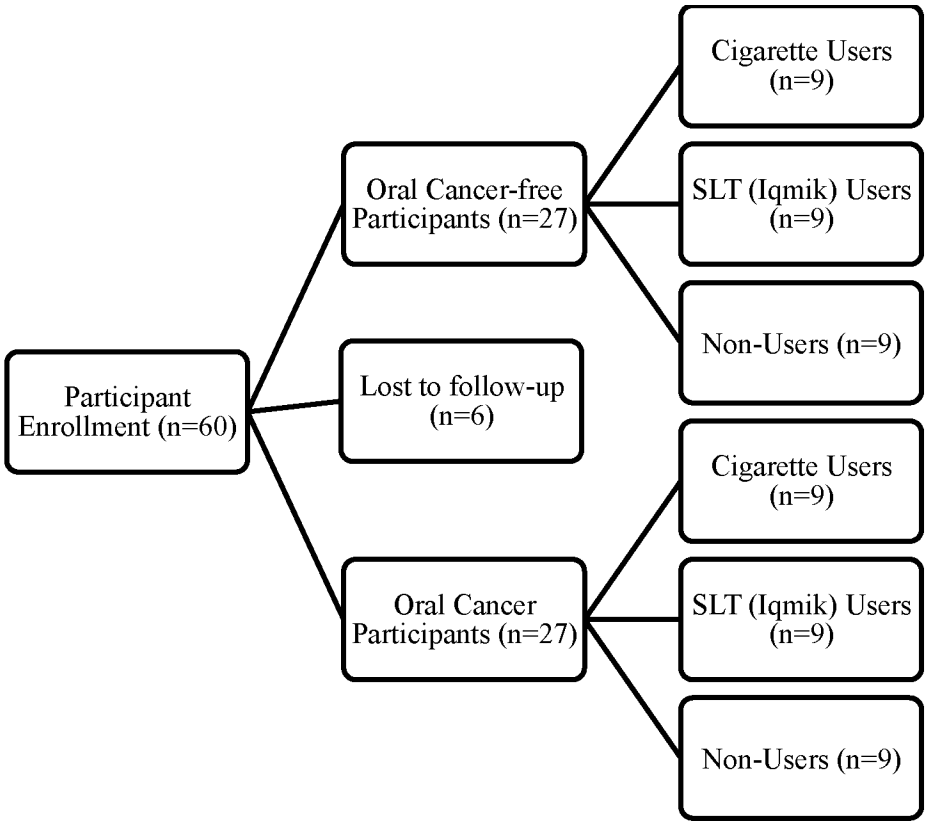
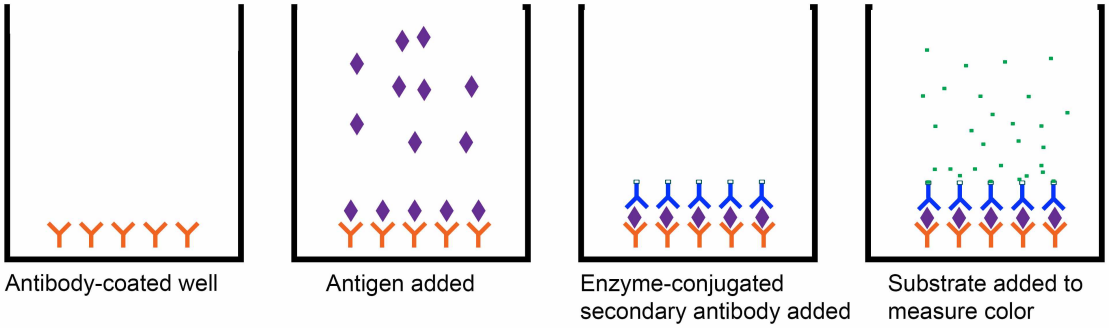


Figure 3.2 Recruitment strategy. The anticipated recruitment for all oral cancer participants and oral cancer-free participants as described in 'Participants'.



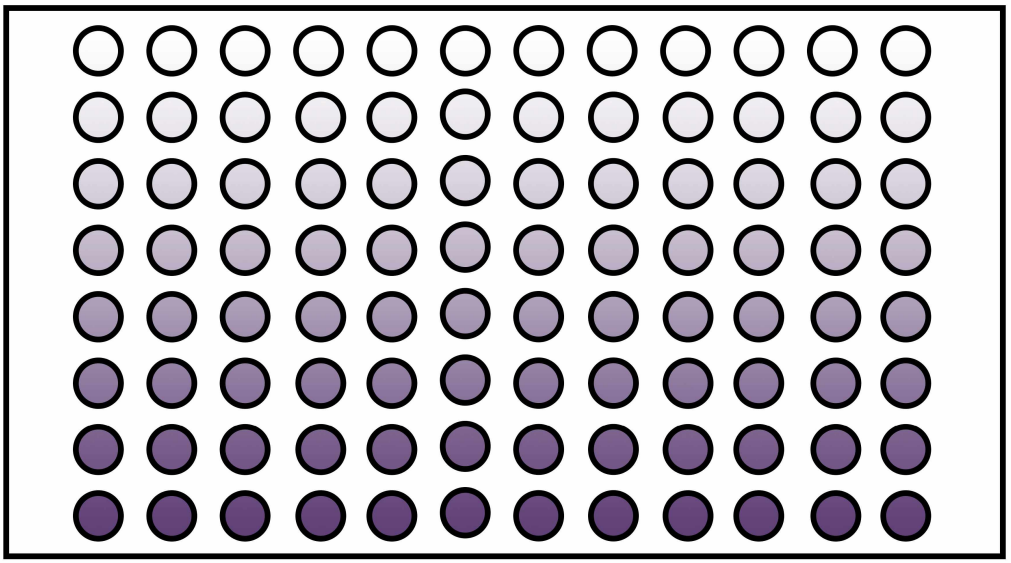


Figure 3.3 A general principle of ELISA. The saliva supernatants will be transferred to antibody-coated 96-well plates. The captured cytokine will be detected with a specific primary antibody and secondary HRP conjugate antibody. A chromogenic substrate will be added to each well and the resulting color will be read as absorbance.

Conclusion

The findings reported herein identify iqmik as a unique health hazard compared to other tobacco products. The addition of punk ash influences the biochemical make-up of iqmik, making it distinct from other smokeless tobacco (SLT) products. The punk ash and tobacco mixture has a pH of 10.02 when extracted in artificial human saliva, a similar pH to betel quid when mixed with slaked lime. The pH of the mixture influences the amount of bioavailable nicotine in the mixture and accounts for the higher levels of cotinine, the major metabolite of nicotine, found in users (1). The punk ash also introduces pro-inflammatory toxic metals into the mixture that are not found in the air-cured tobacco alone, including Cd, Co and Ni, which we have demonstrated accumulate in differentiated human gingival epithelial cells (HGEP). These metals contribute to the significant endogenous reactive oxygen species (ROS) production observed in HGEP following treatment with iqmik artificial saliva extracts. Metal toxicity is a stimulus for oxidative stress leading to inflammation through the activation of a key redox-regulated transcription factor nuclear factor-κB (NF-κB), which plays a major role in pro-inflammatory and stress responses. NF-κB and its signaling targets, such as COX-2, IL-6 and IL-8, are expressed at high levels in many epithelial cancers including head and neck squamous cell carcinoma (HNSCC) (2).

Squamous cell carcinoma accounts for 90% of all oral cancers (3). In the initial phases of oral cancer, one thought is that mutations accumulate in the basal layer of the epithelium (3). The oral epithelial basal layer includes cells with a self-renewing capacity. These cells are responsible for renewing the epithelial lining and regenerate rapidly when damaged. Under physiologic conditions these cells differentiate as they move through the layers of epithelium and become keratinocytes in the gingival area. An alternate theory for the development of HNSCC is that an

accumulation of mutations within differentiated cells leads to their regaining the capacity to self-renew (4).

Studies have found that long-term SLT exposure can cause disturbances in the differentiation process of the oral epithelium. Three-dimensional epithelial cultures exposed to long-term treatments of Swedish snuff showed weaker expression of keratin 10 than the control samples that expressed keratin 10 in the upper two thirds of the epithelium (5,6). In our study we demonstrated differentiation of the HGEP prior to treatment by the increasing expression of keratin 10. A future study should investigate the effect of long-term iqmik treatments on keratinization markers, such as keratin 10 and involucrin (5).

The main events leading to cancer are chronic inflammation leading to genetic alteration, and malignant transformation; ROS generation is associated with all these conditions (7). Oral squamous cell carcinoma (OSCC) patients have increased levels of ROS and reactive nitrogen species (RNS) in their saliva and the salivary antioxidant content is significantly reduced compared to healthy controls. Salivary DNA and proteins are more oxidized in OSCC patients than healthy controls, and the combination of ROS, RNS and depleted antioxidants promotes OSCC (8-10).

Exposure of HGEP to air-cured tobacco or iqmik artificial saliva extracts results in significantly more ROS generation than artificial saliva alone. The air cured tobacco extracts are similar to hydrogen peroxide, but iqmik extracts produce significantly more ROS than hydrogen peroxide. Exposure to punk ash alone does not significantly increase ROS. Levels of ROS seen in cells exposed to iqmik artificial saliva extracts are greater than the sum of ROS produced upon exposure to artificial saliva extracts of punk ash alone and air-cured tobacco alone, suggesting a synergistic effect of the punk ash-tobacco mixture. In regard to the relative contribution of each

metal, Cd, Co, and Ni, in ROS formation in differentiated HGEP, Co exposure produces similar levels of ROS to that of iqmik and 10 time that seen with saliva alone. The concentration of Co that differentiated HGEP are exposed to in artificial saliva extracts of iqmik is likely contributing to the high levels of ROS produced in response to iqmik exposure.

As previously identified by Pappas et al., (11) punk ash has significantly more Cd, Co and Ni than the air-cured tobacco leaf, accounting for the elevated levels of carcinogenic metals in iqmik. The extraction efficiency of Cd, Co and Ni is pH dependent. In an alkaline solution, Cd, Co and Ni form insoluble hydroxide salts (12). This trend is evident when comparing the concentrations of metals in artificial saliva extracts of iqmik, air-cured tobacco and punk ash. Studies of other alkaline ash extracts have shown similar results. Cd is minimally soluble between pH ranges 10.5 and 13 in fly ash (13). The pH of the punk ash extracts and iqmik extracts are just outside the range of minimal solubility for Cd. Based on the similarity of solubility constants for the metal hydroxides, Co and Ni are likely to have similar pH ranges for solubility. This is further confirmed by the fact that punk ash has a higher concentration of metals than air-cured tobacco alone, but the alkaline artificial saliva extracts of punk ash have lower levels of Cd and Co than artificial saliva extracts of air-cured tobacco.

The minimal risk levels published by the Agency for Toxic Substances and Disease Registry (ASTDR) are 0.0005 mg/kg/day intermediate oral exposure and 0.0001 mg/kg/day chronic oral exposure for Cd, 0.01 mg/kg/day intermediate oral exposure for Co, 0.0002 mg/m^3 intermediate inhaled exposure and 0.00009 mg/m^3 chronic inhaled exposure for Ni. Intermediate exposure is 15-364 days; chronic exposure is greater than 1 year. The minimal risk level for Ni from oral exposure has not been determined, but water sources have a median Ni range of 0.5 to 6 μg/L. The EPA requires drinking water to have less than 5 μg/L of Cd, and the average reported level

of Co in drinking water is 2 μg/L. A 30 min artificial saliva extract of iqmik contains 1/3 of the maximum recommended amount of Cd in drinking water, and exceeds the average amount of Co and Ni in drinking water by 5 and 10 fold, respectively (14-16).

The accumulation of metals in the cells shows a very different trend from the saliva extraction efficiencies. HGEP exposed to punk ash extracts have the greatest metal absorption for Cd, Co and Ni. Active transport of the metals into the cells occurs primarily in the punk ash and iqmik saliva extract treated HGEP. The pH of the punk ash and iqmik saliva extracts are both basic. To compensate for the alkalinity of the external environment, HGEP, like other cells, would try to maintain their cytosol close to physiological pH. To maintain a more neutral internal environment a net internal accumulation of H^+ must occur (17,18). Na^+/H^+ antiporters are actively involved in intracellular homeostasis, but in an alkaline environment the antiporter must be electrogenic rather than electroneutral. The internal transmembrane electrical gradient must be negative to support a larger influx of H^+ to a small efflux of Na^+ (18). The negative internal environment created within the cell may be influencing the transport of divalent transition metals across the cell membrane. Divalent metals are moved across the membrane by members of the solute carrier (SLC) group, many of which function as cotransporters. The SLC family uses the H^+ electrochemical gradient to transport a broad range of divalent metal ions, including Fe^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Pb^{2+} (19-21). It is possible that the alkaline external environment created by the exposure to punk ash or iqmik saliva extracts promotes the accumulation of Cd, Co and Ni in HGEP.

Heavy metals, such as Cd, Co and Ni, are carcinogenic through a variety of mechanisms, including oxidative damage to DNA, as well as inhibiting the repair of damaged DNA. Cd, Co and Ni all contribute to the production of free radicals in cells. Many divalent metals participate

in a Fenton-like reaction to produce free radicals in the cell (7,22). Cd also binds to sulfhydryls like glutathione thus depleting glutathione stores, contributing to its toxicity and allowing ROS to propagate in the cell (23). Metal toxicity is a stimulus for oxidative stress through the propagation of ROS leading to inflammation by activating redox-regulated transcription factors, including NF-κB, which plays a major role in inflammation, stress responses and oral cancer progression (2).

Increased tumor growth, metastasis and decreased survival rates of oral cancer are the consequence of alterations in host immune surveillance, inflammation, angiogenesis and metabolism. Tumor development requires proinflammatory, proangiogenesis and immunoregulatory signaling. NF-κB is a redox-sensitive transcription factor that is elevated by tobacco use and chronic inflammatory conditions. The overexpression of NF-κB correlates with the overexpression of proinflammatory, proangiogenic and anti-apoptotic signaling which are prevalent in HNSCC (24). Protein levels of NF-κB gradually increase from premalignant lesions to invasive cancer (4). Therefore, NF-κB signaling is suggested to play an important role in the early stages of head and neck cancer.

Iqmik exposure altered the expression of 41 of 84 NF-κB signaling target genes tested compared to the saliva treatment alone. This set of altered genes was defined using the parameters of Huggins et al. (25), which identify more biologically meaningful sets of altered genes than using p-value alone or more strict fold-change and p-value parameters (26). Genes altered by iqmik exposure are involved in evading and inducing apoptosis, angiogenesis, blocking differentiation, proliferation, coagulation and inflammation. Tobacco exposure altered 31 of the 84 genes tested compared to the saliva treatment alone. Iqmik exposure generated a unique gene expression profile. Nine genes that were altered by both iqmik exposure and tobacco

exposure showed increased expression following iqmik exposure, but decreased expression following tobacco exposure, and 24 of the altered genes were more significantly alter by iqmik than by tobacco, based on the Huggins et al., (25) significance parameters. These data support the hypothesis that iqmik differentially alters the expression profile of NF-κB regulated genes compared to tobacco leaf alone. These data also support the conclusion that iqmik is a unique health hazard which requires an exposure specific set of biomarkers for a population relevant early detection strategy.

COX2, a key signaling target of NF-κB, is not expressed under normal physiologic conditions, but is widely overexpressed in pathophysiologic conditions, such as cancer (27,28). In the experiments presented herein, short-term exposure to iqmik resulted in an up-regulation of the gene expression of *PTGS2,* the gene encoding COX2, greater than tobacco exposure alone. Animal studies have demonstrated an up-regulation of COX2 at the site of SLT application (29). COX2 activation is an early event during carcinogenesis. COX2 overexpression is evident in oral mucosal lesions with an increasing level of COX2 expressed from hyperplasia to dysplasia with the highest levels reported in HNSCC (30). The overexpression of COX2 alters cell adhesion, the response to signals from growth regulators and inhibits apoptosis (31). Prostaglandins, the products of COX2 enzyme activity, activate nuclear hormone receptors that regulate intracellular calcium levels and increase cellular proliferations rates (27). Prostaglandins also promote angiogenesis and inhibit immune surveillance (27,32). Studies in which human volunteers used SLT demonstrated a significant increase in IL-1α and prostaglandin E_2, a downstream product of COX2 enzymatic activity, as well as the development of oral lesions in over half the volunteers by day 7 (6,33,34). In contrast, COX2 deficiency in mice protects against the formation of epithelial tumors (35,36). The reduced tumor formation is in response to premature terminal

differentiation of epithelial cells into keratinocytes. Keratins 1 and 10, indicators of commitment to differentiate, are both expressed in basal cells in theses COX-2 deficient mice (35). The data on the increased expression of *PTGS2* following iqmik exposure compared to tobacco leaf alone supports the uniqueness of iqmik exposure on the pathophysiology of HGEP, and supports the idea that iqmik can create a cellular environment which increases the risk for malignant transformation.

A major public health concern is the rate of incidence and of mortality from HNSCC in Alaska Natives. The incidence of oral cancer is estimated to be rising by 6.5% annually for Alaska Natives. Alaska Natives have a mortality rate of 6.1 cases per 100,000 per year for oral cancers. The most common diagnostic tool is visual inspection to detect oral cancers, even though there is little evidence that this method reduces the incidence of or mortality from oral cancers (37,38). The data presented herein suggests that an alternate screening strategy based on a population specific set of biomarkers reflective of SLT use in Alaska could be developed to improve early detection of oral lesions which have the potential to proceed through malignant transformation.

Iqmik induces more cancer associated genes than tobacco alone including many of the key NF-κB dependent inflammatory and proangiogenic cytokines including IL-1, IL-6, IL-8, GM-CSF and members of the TNF family. The proposed case-control study to test the levels of these inflammatory cytokines in saliva samples collected from participants without or with identifiable HNSCC and who self-report the use of SLT (iqmik), cigarettes or no tobacco product use is predicted to demonstrate a significant difference between healthy controls and HNSCC patients. Previous studies have demonstrated a significant increase in levels of NF-κB dependent proinflammatory cytokines in the saliva of HNSCC patients compared to controls (39,40). These same cytokines are elevated in oral preneoplastic lesions compared to controls. These

proinflammatory cytokines may therefore be useful as biomarkers indicative of oral preneoplastic lesion to HNSCC transformation enabling earlier detection with an evidence-based screening method which may also be useful as a strategy for tracking the effectiveness of treatments for patients with HNSCC.

Although Iqmik is perceived as a safer alternative to other tobacco products (41), iqmik is a greater source of heavy metals, such as Cd, Co and Ni, than air-cured tobacco leaf. HGEP accumulate more Cd, Co and Ni from the punk ash component of iqmik than from the air-cured tobacco. These metals are identified carcinogens that have the capacity to accumulate in cells and generate and propagate the production of endogenous ROS. The results of this study help elucidate the unique properties of iqmik and stress the necessity to understand the biological response to iqmik use through future studies. The proposed case-control pilot study will provide a foundation for future tobacco and HNSCC research in Alaska to address health concerns related to tobacco use in Alaska Natives.

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Appendix

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Date: May 14, 2015

Gaelen Dwyer
3211 Providence Dr.
Anchorage, AK 99508

Dear Gaelen Dwyer:

I give you my permission to include in your master's thesis at UAF the manuscript entitled "Exposure to smokeless tobacco mixture iqmik leads to transition metals accumulation and reactive oxygen species production in human gingival epithelial cells", of which I am a co-author.

Sincerely,

Birgit Hagedorn

Birgit Hagedorn, PhD
University of Alaska Anchorage
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