Impact of E-Cigarettes on Oral Wound Healing

Thesis

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Abstract

Introduction: E-cigarettes are small, battery-powered devices that deliver a mixture of nicotine, propylene glycol, glycerol, and flavoring agents as aerosols. These devices are being used by ex-smokers, current cigarette smokers, and previous nonsmokers. Previous studies in our laboratory and others have demonstrated that e-cigarette use is associated with high pro-inflammatory response, hypokeratinization of oral epithelium, and increased cytotoxicity, suggesting that these devices can increase the risk for poor postoperative healing outcomes.

Objective: The study was aimed to assess oral wound healing in orally and systemically healthy e-cigarette users.

Methods: A parallel-arm, prospective clinical study was conducted. 8 subjects using e-cigarettes and 8 age- and gender-matched non-vapers were recruited. Punch biopsy wounds of 5 mm of diameter were created bilaterally in the palatal mucosa opposite to the 2nd premolar. Pre-wounding palatal mucosal swabs were also collected for metabolomics profiling of the microbiome at all time points. Subjects were followed up at 1, 2, 4, 7, 14 and 21 days post-operatively, and 3 mm punch biopsies of the healing wounds were collected from the right and left palate at 1- and 3- weeks respectively. Biopsies were subjected to immunohistochemistry to quantify the levels of vimentin, keratin and filaggrin.

Results: Statistically significant differences in overall healing were observed between control and test groups, between visits, as well as different trends shown by the groups over the visits. Significant differences were observed between groups and between visits in terms of bleeding and swelling, while only significant differences between visits were seen for epithelialization and redness. Pain perception and difficulty to obtain hemostasis were more observed in the test group. Pathway analysis between week 1 and 3 revealed that carbohydrate and lipid metabolism was significantly higher in e-cigarette users while synthesis of secondary metabolites was higher in the control group. Immunohistochemistry revealed significantly lower vimentin, filaggrin and keratin scores over all time points in the e-cigarette users when compared to controls.

Conclusion: E-cigarette use represents a significant risk to postoperative oral wound healing, affecting keratinization of epithelium, and altering the metabolic profile of the oral microbiome.

Dedication

Dedicated to my parents, Antonio and Gabriela, who have been extremely supportive and loving throughout my dental and periodontal training and to my siblings for always being with me in every step of the way. Their love comes a long way, even if thousands of miles separate us. I also want to dedicate this thesis to Richard, my fiancé, for his daily kindness and support.

Mereces lo que sueñas - Cerati

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Fields of Study

Major Field: Dentistry

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Chapter 1. Introduction

A *wound*, regardless of the cause of injury, is a disruption of normal tissue continuity and integrity. *Healing* is the process of restoring the integrity of the wounded tissue. The nature of the native involved tissue determines whether regeneration or repair will ensue. Regeneration results in tissue that is structurally and functionally the same as the original tissue, whereas repair results in the re-establishment of tissue integrity through the formation of a fibrous, connective-tissue scar. Wound healing is a protective function of the body that focuses on quick recovery and involves four highly integrated and overlapping phases[1,2] that are explained in further detail in the present chapter.

Wound healing is a complex and highly coordinated pathophysiological process orchestrated by a variety of known and unknown factors. Although cutaneous and oral wound healing follow the same wound healing stages, oral wounds show accelerated healing compared to cutaneous wounds.[3,4] Buccal mucosa, gingiva, and palate show similar accelerated wound healing when compared to cutaneous wounds, despite all having different keratinization and terminal differentiation profiles.[5,6] The oral cavity is a remarkable environment in which wound healing occurs in the presence of a warm oral fluid, namely saliva, that contains millions of microorganisms.[7] Despite this hostile environment, oral wounds tend to re-epithelialize more rapidly with minimal or no scar formation compared to cutaneous wounds.[2] *In* vitro and animal model studies have

attributed this to a variety of mechanisms including differential inflammatory response, distinct modulation of stem cells, proliferative and differentiation programs, and more efficient epithelial remodeling in oral wounds.[8] In a comparative analysis between human oral and cutaneous wound healing, Iglesias-Bartolome used paired and sequential biopsies during the repair process.[8] With the use of molecular profiling, it was determined that wound-activated transcriptional networks are present at the basal state in the oral mucosa, priming the epithelium for wound repair and contributing to rapid wound resolution. It was also demonstrated that oral mucosa wound-related networks control epithelial cell differentiation and regulate inflammatory responses.[8] The study identified eight transcriptional factors that define oral keratinocytes, and particularly SOX2 and PITX1, regulate networks involved in wound closure.[8] Oral wounds exhibit less inflammation than skin wounds, including a lower neutrophil, macrophage, and T-cell at the wound site.[8] The proinflammatory cytokines, interleukin -6 (IL-6) and tumor necrosis factor- α (TNF-α) are all decreased in oral wounds when compared to skin counterparts. [9] Chemokine levels are also altered in the oral mucosa inflammatory response as reduced levels of transforming growth factor-β1 (TGF-β1) are linked with the minimal oral mucosa scarring. There is also a threefold increase in transforming growth factor-β3 (TGF-β3) production compared with unwounded tissue by 24 hours post-wounding. The altered ratio of TGF-β1 and β3 has been suggested to play a role in the enhanced healing of the oral mucosa.[9]

Two different kinds of wound healing have been primarily described in the literature: primary and secondary intention healing. In the former, the lining tissues are closely approximated surgically to cover all underlying injured tissues. This type of healing

involves minimal scarring, is quicker and has a lower risk of infection than secondary intention healing. The latter occurs in areas which are not covered by normally epithelialized tissue due to intentional exposure (extraction sockets, apically repositioned flaps) or accidental (wounds with full thickness loss of substance), or due to an insufficient amount of lining tissue to be used for coverage. A third term, tertiary intention, is used to define delayed healing occurring in the previously described types of healing after an infected wound is left exposed for several days until the infection resolves and is completely closed by the overlying tissue. A fourth type of wound healing involves a partially lost overlying tissue due to abrasion or intentionally removed (free gingival graft), so that a de-epithelialized connective tissue layer is exposed and heals by re-epithelialization from the intact adjacent epithelium.[10,11]

Physiology of wound healing

The skin and mucous membranes represent the first line of defense against any insult that may disrupt homeostasis.[12] Both tissues comprise an underlying basement membrane and superficial epithelia, containing keratinocytes attached by desmosomes, melanocytes, Merkel cells, and Langerhans cells, which provide protection against body fluid loss, exposure to toxins, and microbial invasion.[12,13] Despite the general similarities between oral mucosa and skin, there are critical structural, functional, and homeostatic differences.[14] The epithelial lining of the skin develops from the ectoderm, whereas the oral epithelium derives from both the ectoderm and endoderm.[15] Cutaneous skin consists of a keratinized epidermal layer, dermis, and hypodermis. It harbors additional appendages, such as hair

follicles, nails, sweat and sebaceous glands.[16] On the other hand, the oral mucosa consists of stratified squamous epithelium followed by layers of the basal lamina, lamina propria, and submucosa.[12] The oral epithelium is generally thicker compared to the skin, as the palate and buccal mucosa consists of more cell layers and a higher proliferation rate in the basal lamina compared to skin.[17]

The epithelium of the palatal masticatory mucosa is orthokeratinized and approximately 0.36 mm thick.[18] However, epithelial thickness varies significantly between canine and posterior teeth, being thicker around the canines than the molar region.[18] Thin mucosa (1.8-2.7 mm) occurs at the first and second maxillary molars due to the presence of palatal root prominences.[18] Located immediately below the epithelial layer, the lamina propria is a dense bilayered connective tissue rich in type I and III collagen fibrils. The thickness and composition of the lamina propria differs depending on the thickness of the palatal mucosa. Thicker palatal mucosa has thinner lamina propria and higher proportions of adipose and glandular tissue. In the canine area, the lamina propria is significantly thicker and decreases progressively in posterior sites. As the distance from the gingival margin increases, the thickness of the lamina propria decreases, ranging from 1.41-1.99 mm and 0.86-1.39 mm at the marginal and apical regions, respectively.[19,20] Beneath the lamina propria, the submucosa is a connective tissue band overlying the periosteum, which contains a large concentration of glandular and adipose tissue. In areas lacking submucosa, the dense lamina propria binds directly and intimately to the periosteum, which overlies the bone. The periosteum has three zones: the innermost cambium, or osteogenic layer that

is attached to the bone; a highly vascularized fibrous layer containing fibroblasts and fibroblast progenitors; and an outermost fibrous layer composed of dense collagen fibers.[21]

Wound healing involves diverse cellular activities including chemotaxis, phagocytosis, mitogenesis, migration, and extracellular matrix (ECM) synthesis/remodeling.[22] While the rates and patterns of healing depend on diverse local, systemic, and surgical factors, the phases of *hemostasis, inflammation, proliferation,* and *remodeling* are similar for all other tissues.[2]

The first phase of *hemostasis* begins immediately after wound creation, with vascular constriction and fibrin clot formation.[1] Vasoconstriction occurs to stop further blood loss and is followed by platelet activation. Platelets regulate primary hemostasis during the aggregation phase and secondary hemostasis during the coagulation phase. Platelets are capable of producing biologically active products such as vasoactive mediators and chemotactic factors.[7] Released key mediators include transforming growth factor- β (TGF-β), platelet-derived growth factors (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF).[1,2] Vasoactive mediators such as serotonin increase microvascular permeability, leading to exudation of fluid into the extravascular space resulting in tissue edema, which is further evident clinically in the *inflammation* phase.[22] Platelets adhere to form platelet plugs that are reinforced by fibrin polymerization to create a fibrin clot that seals the wound against dehydration and infection.[12,20] The clot, comprising fibrin, fibronectin, vitronectin, von Willebrand factor, and thrombospondin, provides support as a temporary extracellular matrix that allows epithelial cells and fibroblasts to migrate into the wound site.[12,20,22]

The open wound site provides an ideal environment for pathogens to colonize, form biofilms, express virulence factors, and subsequently infect the host with related morbidity.[14] Once bleeding is controlled, the *inflammation* phase initiates with the recruitment of inflammatory cells that remove damaged tissue and bacteria from the injured site in response to chemoattractants at the site of the injury.[2,12] Damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) in the wound bed trigger toll-like receptor (TLR), receptor for advanced glycation end products (RAGE), and inflammasome signaling, leading to a cytokine and chemokine cascade released by resident cells.[14] The inflammatory response peaks at 24 to 48 hours post-injury and can last for up to a week. Polymorphonuclear leukocytes (PMNL) migration is stimulated by platelets, TGF-β, complement components such as C5a, and bacteria. Neutrophils are the first to migrate to the wound site to debride damaged ECM components and to secrete protease like matrix metalloproteinase (MMP).[12] These cells also produce substances such as proteases and reactive oxygen species (ROS), which cause some additional bystander damage.[1] A more rapid influx of neutrophils has been found in oral wounds compared to skin wounds and might be the result of increased platelet activation and consequent release of chemokines such as CXCL4. Another major neutrophil chemoattractant that is increased in early wound healing is CXCL8 (IL-8). Keratinocytes increase expression of intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule used by neutrophils to facilitate extravasation and migration into the wounded area.[14] During the early *inflammation* phase, neutrophils initiate a cascade of cytokine secretion and growth factors to recruit other immune cells, including monocytes, which aid in

initiating re-epithelialization. After the wound bed is clear of microbes, neutrophils exit the wound through extrusion, apoptosis, and phagocytosis.[12] Neutrophils are rapidly outnumbered by macrophages derived from circulating monocytes[2], serving as the dominant cell type through "proinflammatory" M1 macrophage polarization.[12] Macrophages play major roles in initiating collagen synthesis and in the formation of endothelial cells and fibroblasts[7] as a result of cytokine secretion including IL-1, IL-6, FGF, PDGF, EGF, and TGF-β. During the late inflammatory phase, macrophages lead proliferative healing through "anti-inflammatory" M2 macrophage polarization and continue to secrete regenerative cytokines such as IL-10.[12] Following the immune cell mediated removal of pathogens, there is an increase of blood vessel permeability with plasma exudation from capillaries, and decreased blood flow, leading up to the *proliferation phase*.[12,23]

Proliferation generally overlaps with the *inflammation* phase and is characterized by epithelial proliferation and migration over the provisional matrix within the wound through the process of re-epithelialization.[1] *Proliferation* also involves angiogenesis, granulation tissue formation, and collagen deposition, and takes place from day 4 to day 14 after the injury.[7] Within the wound bed, fibroblasts proliferate and produce granulation tissue, collagen, as well as fibronectin, glycosaminoglycans and proteoglycans, which are major components of the ECM.[1] Fibroblasts interact with their surrounding matrix via receptors known as integrins that regulate the level of collagen gene expression and collagenase induction. Collagen restores the integrity of the repaired tissue, whereas proteoglycans function as moisture storage.[2] Hypoxia is a result of stagnation of blood flow

in the wounded area and is an important trigger for neovascularization or angiogenesis during this phase.[7,14] Angiogenesis is the process by which new blood vessels sprout from existing vascular networks to restore tissue perfusion, establish microcirculation, and increase oxygenation to support collagen crosslinking and wound maturation.[12] Macrophages and keratinocytes in the hypoxic wound bed secrete various proangiogenic factors such as hypoxia-inducible factor 1 α (HIF-1α) and vascular endothelial growth factor (VEGF)[24] to stimulate endothelial cell proliferation and migration, resulting in a large production of leaky vessels that provide the granulation tissue with nutrients and immune cells.[14] FGF-2 has also been found to promote wound angiogenesis. Szpaderska suggested that oral mucosal injuries may exhibit less robust angiogenesis as it may keep the fibrotic response in check by reducing available nutrient support, reducing scar formation.[5] Given this reduced angiogenic response in oral mucosal wounds, VEGF production within the wound is low during the entire healing process.[9] Once normoxia is attained, the production of VEGF diminishes, signaling an end to the pro-angiogenic period.[5]

Re-epithelialization starts from the wound edges, where epithelial cells lose their hemidesmosomal connections and migrate through the provisional fibrin-fibronectin matrix through the wound until they encounter identical cells. Targeted migration and proliferation through a loose underlying network require an efficient, balanced, and enzymatically supported procedure of “cutting and pasting”.[7] Oral fibroblasts produce more hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), which are known to induce keratinocyte migration and proliferation. This may explain the increased

rate of re-epithelialization of oral wounds compared to skin in vivo.[14] Saliva is another source of growth factors and has been shown to stimulate re-epithelialization of freeze wounds in reconstituted human gingiva and skin, due to their content rich in bioactive proteins and peptides, such as histatin.[14] However, it has been shown that only large palatal wounds were affected by the absence of saliva, while smaller wounds healed normally. It is unlikely to fully account for the rapid scarless healing of the oral mucosa to saliva; therefore, intrinsic characteristics of the tissue come into play in oral wound healing.[9]

Remodeling/Maturation is the process in which the ECM architecture approaches that of the normal tissue.[1] Macrophages secrete growth factors, MMPs, and tissue inhibitors of metalloproteinases (TIMPs) to stimulate myofibroblast-mediated remodeling of the ECM.[14] The number of macrophages in oral wounds gradually decreases after peaking 14 days post-wounding.[14] This final remodeling phase of wound healing may take weeks to months and involves a contraction phase, in which the distance between wound edges is closed, reducing the wound surface, and expediting wound closure. This process occurs due to differentiation of fibroblasts and other progenitor cells into myofibroblasts due to the secretion of cytokines and growth factors (TGF-β) by macrophages.[14] The latter possess an actin-enriched cytoskeleton and provide matrix constriction. Wound contraction is followed by the remodeling process, in which matrix production stops, fibroblasts are degraded, and myofibroblasts enter apoptosis.[7,14] The tensile strength of the wound gradually restores as the collagen fibers are realigned and are increasingly cross-linked to each other.[2] The maximal tensile strength of a healed wound is reached in 6 to 12 months

following injury, but never reaches the strength of unwounded tissue.[2] Eventually, active collagen synthesis achieves equilibrium with collagenolysis.[2]

Factors that affect oral wound healing and their mechanisms

The factors that can lead to impaired wound healing can be categorized as local and systemic. Local factors are those that directly influence the characteristics of the wound itself, whereas systemic factors are the overall health or disease conditions of the individual that affect his/her ability to heal. Many of these factors may be interrelated and the systemic factors act through local effects affecting wound healing.[1]

Local factors

Infections are the most common cause of impaired wound healing. Infection occurs only when the virulence or the number of bacteria exceeds the ability of local tissue and host defenses to control them, although oral wounds are always colonized by bacteria. Bacteria provoke varying degrees of inflammation at the wounded site by releasing endotoxins, metalloproteinases, and breakdown products that inhibit the activities of regenerating cells and the scavenger macrophages.[2] This can lead to the prolonged elevation of pro-inflammatory cytokines such as IL-1 and TNF-α and elongate the inflammatory phase. If this continues, the wound may enter a chronic state and fail to heal.[1] The presence of *foreign bodies* may also prolong the inflammatory phase, delaying wound repair.[20]

Edema in the wound restricts the oxygen and nutrient supply to the wound by enlarging the diffusion distance.[7] *Oxygenation and venous sufficiency* are crucial for optimal wound healing. Hypoxia stimulates wound healing such as the release of growth factors and angiogenesis, while oxygen is needed to sustain the healing process. In wounds

where oxygenation is not restored, healing is impaired. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing.[1] *Postoperative bleeding* disturbs granulation tissue formation and slows the healing process.[25]

In cell cultures and laboratory animals, *local anesthetics* exhibit an inhibitory effect on wound healing, which is mainly reflected in the inflammatory and proliferation phase of wound healing.[7]

Systemic factors

Wound healing is a complex process involving a varying array of key-pathways regulating the response of numerous gene panels. The genetic component is a prominent, non-modifiable risk factor that may contribute to wound healing. Genetic diseases such as Down Syndrome and ataxia-telangiectasia are known to lead to non-physiologic and impaired wound healing.[26] Rare genetic diseases are known to be responsible for the occurrence of venous ulcers in about 10% of cases.[26] Disorders of the immune system are known risk factors of impaired wound healing, due to their role in the inflammatory phase. One such example includes Leukocyte adhesion deficiencies (LADs), which result in decreased migration of neutrophils to the wounded tissue and lack of phagocytosis ability in the wound site.[26] This leads to an increased susceptibility to infections and risks of severe wound complications.[26]

The influence of *age* on wound healing probably results from the general reduction of tissue metabolism that may be manifested by multiple physiologic problems as the subject ages. Wound healing is enhanced in younger subjects than in the elderly.[2]

Numerous clinical and animal studies at the cellular and molecular level have examined age-related changes and delays in wound healing.[1] The latter is associated with an altered inflammatory response, such as delayed T-cell infiltration into the wound area with alterations in chemokine production and reduced macrophage phagocytic capacity.[1] Results from these mechanisms include delayed re-epithelialization, collagen synthesis and angiogenesis.[20]

Sex hormones play a role *in aged individuals* undergoing wound healing deficits. Compared with aged females, aged males have been shown to have delayed healing of acute wounds.[1] This may be explained due to the effect of estrogen, which affects wound healing by regulating a variety of genes associated with regeneration, matrix production, protease inhibition, epidermal function, and the genes primarily associated with inflammation.[1]

The impact of *stress* on wound healing may be influenced by factors that can be classified into two broad categories: health impairing behaviors, such as poor oral hygiene; and factors that have pathophysiological effects, such as altered cytokine profiles and increased levels of glycocorticoids. These effects may result in impaired cell-mediated immunity at the wound site and delayed healing.[20,27] Marucha and colleagues conducted a study in which medical students had punch biopsy wound inflicted on the hard palatal mucosa.[28] Wounds in the test group were made during "stressful" examination periods, whereas control groups had the wound created during vacation. Wounds placed on the hard palate in the test group healed 40% more slowly than those during the vacation period.[28]

Individuals with *diabetes* exhibit a documented impairment in the healing of acute wounds, which can be attributed to the development of diabetic microangiopathy.[1] Local ischemia, secondary to poor oxygen delivery at the tissue level, and small vessel occlusion play a role in the pathogenesis and delayed healing of diabetes.[1,2] Poor healing has also been related to mechanisms such as fibroblast and epithelial cell dysfunction, high levels of metalloproteinases, decreased host immune resistance, neuropathy and damage from reactive oxygen species and advanced glycation end-products.[1,20]

Medications such as systemic glucocorticoids are frequently used as anti-inflammatory agents and are well known to inhibit wound repair through anti-inflammatory effects and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis. This may lead to a healing process with incomplete granulation tissue and reduced wound contraction.[1] Matrix metalloproteinases and vascular endothelial growth factor are suppressed with glucocorticoid use.[29]

Alcohol consumption reduces host resistance, and ethanol intoxication at the time of injury is a risk factor for increased susceptibility to wound infection.[1] With alcohol intake, accompanying mechanisms include increased insulin resistance, higher blood sugar levels, and high risk of protein malnutrition.[20] Acute ethanol exposure can lead to impaired wound healing by altering the early inflammatory response, inhibiting wound closure, angiogenesis, and collagen production, and altering the protease balance at the wound site.[1]

Smoking has a detrimental impact on wound healing. Nicotine reduces blood flow through vasoconstriction, stimulates release of proteases that accelerate tissue destruction, suppresses the immune response, and leads to an increased risk of infection.[29] Carbon

monoxide in cigarette smoke causes tissue hypoxia and aggressively binds to hemoglobin, resulting in a decreased fraction of oxygenated hemoglobin in the bloodstream. In the *inflammation* phase, smoking causes impaired white blood cell migration, resulting in lower numbers of monocytes and macrophages in the wound site, and reduces neutrophil bactericidal activity.[20] Lymphocyte function, cytotoxicity of natural killer cells, and production of IL-1 are depressed, and macrophage sensing of Gram-negative bacteria is inhibited.[1] All these mechanisms may lead to a poor immune response, increased risk of infection, and higher chances of flap necrosis, wound dehiscence, and infection.[20]

Obesity may lead to wound complications, including skin wound infection, dehiscence, hematoma and seroma formation, pressure ulcers, and venous ulcers.[1] A higher rate of surgical site infection occurs in patients with obesity.[1] Many of these complications may be a result of a relative hypoperfusion and ischemia occurring in the subcutaneous adipose tissue.[1] In surgical wounds, the increased tension on the wound edges also contributes to wound dehiscence. Wound tension increases tissue pressure, reducing microperfusion and oxygen availability to the wound.[1] Increased production of adipokines, proinflammatory cytokines, and chemokines have a negative impact on the immune and inflammatory response, leading to delayed healing.[1,20]

Malnutrition is a common problem in the elderly population and may lead to delayed wound healing.[29] Nutritional deficiencies that produce hypoproteinemia hinder wound healing and impair the immune defense by limiting the availability of the amino acids that are critical for collagen synthesis and other proteins. Methionine is a key amino acid in wound healing and plays an essential role in the inflammatory, proliferative, and

remodeling phases of wound healing.[2] Vitamin C (L-ascorbic acid), vitamin A (retinol), and vitamin E (tocopherol) show potent antioxidant and anti-inflammatory effects. Vitamin C deficiency leads to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, increased capillary fragility, impaired immune response, and increased susceptibility to wound infection.[1] Macrophages increase with vitamin A intake; therefore, a lack of macrophages leads to reduced collagen synthesis and inhibits wound healing.[7] Vitamin A properties include antioxidant activity, increased fibroblast proliferation, modulation of cellular differentiation and proliferation, increased collagen and hyaluronate synthesis, and decreased MMP-mediated extracellular matrix degradation.[1]

HIV/Chemotherapy/Immunosuppression/Radiotherapy: The risk for complications significantly increases when the number of CD-4 lymphocytes is less than 400 per microliter.[7] Chemotherapy targeting oral cancer can result in oral mucositis during the active treatment phase and *in vitro* studies indicate altered wound healing during chemotherapy.[7] Patients who undergo radiation therapy for oral cancer of more than 50 Gy have a greater risk of complications with wound healing, such as osteoradionecrosis, particularly after tooth extractions.[7]

Hereditary and bleeding tendencies give rise to altered wound healing in the presence of pathological bleeding.[7]

Apart from intrinsic local and systemic factors, external factors such as humidity, saliva, mechanical tension, microbial burden, and ecology have been shown to contribute to wound healing and influence healing outcome.[1]

The oral microbiome

It has been well documented that the human body is extensively harbored by trillions of microbes, including bacteria, fungi, and viruses[30] and these are estimated to outnumber eukaryotic cells by 10-fold.[31]

The human microbiome is a collection of microorganisms living in association with the body, inhabiting all exposed surfaces including the respiratory tract, skin, genital organs, gastrointestinal system, and the oral cavity.[4] The term microbiome describes the whole community as a "superorganism" in the context of their habitats. As described by Kumar, the microbiome is the collection of microbiota and their genes in a particular niche or habitat.[32] In the context of the oral cavity, the oral microbiome comprises bacteria, fungi, viruses, archaea, and protozoa.[30] Of these microorganisms, the most widely studied is bacteria.[33]

The normal microflora of the oral cavity is diverse and abundant, initiating with the colonization of Gram-positive bacteria and later shifts to Gram-negative anaerobes, particularly in subgingival plaque. The oral microbiome is the second most diverse microbiota after the colon [34] and comprises more than 700 species of bacteria, with no significant difference based on gender, race, age, or geographic location of the host.[30] About 400-500 oral taxa have been detected in the subgingival crevice alone. The remaining taxa are distributed on the different oral habitats including the tongue, tooth surface, buccal mucosa, tonsils, soft and hard palate, and labial vestibule. The salivary microbiome is essentially composed by a mixture of microbes sloughed off from all sites.[35]

The healthy oral human microbiome is predominantly composed of members of the phyla *Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes*, and *Fusobacter* and *Spirochaetes* are present in lower numbers. Diverse factors modify the composition of the oral community, including physical and chemical interactions, environmental pressures, antimicrobial peptides, host factors in saliva and gingival crevice fluid (lysozyme, secreted antibodies, and other immune mediators), host genetics, maternal transmission, dietary habits, oral hygiene practice, and systemic factors.[30,34] The ecological niches provided by the soft and hard tissues within the mouth also determine the diversity and abundance of the oral microbiome. Supragingival and subgingival plaque harbor 14 common genera: *Streptococcus, Corynebacterium, Capnocytophaga, Haemophilus, Aggregatibacter, Fusobacterium, Prevotella, Leptotrichia, Veillonella, Neisseria, Rothia, Actinomyces, Lautropia,* and *Porphyromonas*.[30]

The human microbiome has been studied over a span of several decades. Advancements in technology have led to an increased understanding of the human microbiome. The targeted gene approach and the metagenomics approach are two commonly used methods to study the microbiome, and both utilize next generation sequencing as their main platform of analysis. Targeted gene analysis involves the use of one or a couple hypervariable regions and is best used for highly specific sequencing, while shotgun metagenomics is a widespread, untargeted approach that incorporates a variety of genetic information and is best used in the absence of reference genome. Another method for microbiome characterization is metabolomics, which is a comprehensive analysis that utilizes mass spectrometry and is best used to gain information on the role of small

metabolites in cell function. Additional methods include metaproteomics and metatranscriptomics. The former uses mass spectrometry to provide information on macromolecules and is best used when inquiring about protein interaction with the system, while the latter is an approach that uses microbes within their natural environment to provide additional information on the overall function of the community.[33]

The microbial community in humans plays a vital role in health, tissue homeostasis, and disease prevention, leading to a state of "eubiosis"[4] or as defined elsewhere in the literature, "a functional balance within the ecosystem as well as one between the ecosystem and the host; and is characterized by diversity (richness of species as well as functions in the system), resistance (the ability to withstand perturbation), and resilience (the capacity to return to the pre-perturbed state following removal of the pressor".[36] The oral microbial ecosystem is constantly exposed to exogenous foreign substances, which may set the stage for founding microbes and their ability to persist and make for distinct relationships between host and microbe.[34] An eubiotic oral microbial community can be described as a house of cards that is highly regulated and precisely assembled, possessing structural complexity, and demonstrating cooperativity between the resident members and the oral immune-inflammatory apparatus.[36] A change in one or more of these organizing principles can result in expansion of selected resident species, loss of keystone species, or an increase in diversity due to random changes in species abundance. If any of these events occur, combined or isolated, this house of cards is determined to collapse into a state of "dysbiosis".[36] Dysbiosis has been reported in e-cigarette users compared with never-

smokers and tobacco cigarette smokers[37] and this topic will be explained in detail in a further subtopic.

E-cigarettes and oral wound healing

Electronic Nicotine Delivery Systems (ENDS)

Vapes, vaping, vaporizers, vape pens, electronic cigarettes, e-cigarettes, and e-cigs are synonyms to describe ENDS, which are non-combustible tobacco products[38] that produce an aerosol for users to inhale by heating the e-liquid in its cartridge.[39] This product was patented by a Chinese researcher in 2003 [40] and later became commercially available in North America and Europe in 2006.[41] Tobacco use has decreased nationally over the last decades; however, electronic cigarette use is dramatically increasing in the US, especially among adolescents, teens, and young adults where it has risen sharply since 2011.[42] These devices are being used by ex-smokers, current cigarette smokers, and previous nonsmokers.[43]

There are more than 460 brands of e-cigarettes and more than 7,000 flavors. As of 2017, evidence suggested that 2.8% of adults used e-cigarettes and that 10.5% of middle school students and 27.5% of high school students reported e-cigarette use in the last month.[43] As of 2020, the number of users seemed to increase in the United States, since over 15% of adults have used an e-cigarette, with the majority being between the ages of 18 and 44 years of age.[41]

Predominantly among teens and young adults, the use of e-cigarette has become socially acceptable on account of fashion sense, desirable flavors, cost-effectiveness, user-friendly functions, and the thrill of secretive use in prohibited indoor and outdoor

locations.[39,44] Flavored tobacco products predominantly attract young users (aged 12-24 years) and 81% of youth that currently use e-cigs cited appealing flavors as a primary reason for first using a tobacco product.[42] Although e-cigarettes have been deemed by some as a safer alternative to conventional cigarettes, statistical data insinuate an exponential rise in e-cigarette usage among college students, and its co-use with alcohol can contribute to negative consequences in the current younger generation.[37] Adolescent e-cigarette users were 4 times more likely to initiate cigarette smoking, and the odds of quitting smoking were lower; in many instances, quitting was delayed for those using e-cigarettes.[45]

There are four generations of e-cigarettes, all of them are battery-operated devices and cartridge-based products containing fluid with varying levels of nicotine and flavoring, as well as several toxicants such as heavy metals.[38] E-cigarettes exist in several formats, such as cig-a-likes (resemble traditional cigarettes, are low-capacity), tank systems (resemble a pen, include larger batteries, and have refillable fluid reservoirs), consumer-modifiable devices (or mods), and small devices that look like external storage devices for computers.[43,46] In these products, a solution of "e-liquid" held in a cartridge is heated to a temperature of 100-300 °C and over 350 °C depending on the e-cigarette category, e-liquid composition, and power output to create an aerosol that is inhaled by the user. This liquid is generally composed with/without nicotine, water, propylene glycol (PG), vegetable glycerin (VG) or glycerol and flavoring components.[38] E-cigarette use devices are not exclusively used to vape a liquid but may also be used to vape tetrahydrocannabinol (THC), dried cannabis, tobacco products, or psychoactive substances.[47]

Unlike traditional cigarettes with tobacco filling, approximately 24 mg of nicotine per pack or 1.2 mg/cigarette, e-liquid contains nicotine that varies between 6 and 48 mg/mL and is not meant to be smoked completely in one sitting. Each nicotine cartridge in an e-cigarette can provide an average of 200 puffs, which may be equivalent to 1-3 packs of cigarettes.[37] Nicotine in aqueous solution can be found in three forms: diprotonated, monoprotonated, and unprotonated. The newer e-cigarette generation called "pod-mods" (such as JUUL) use the protonated formulation derived from nicotine salts. In the e-liquid of the "pod-mods", nicotine can be found in concentrations 2-10 times higher than in traditional e-cigarettes, and in a high concentration of 65.2 mg/L.[38] Nicotine is extremely addictive and has a multitude of harmful effects. Nicotine has significant biologic activity and adversely affects several physiological systems including the cardiovascular, respiratory, immunological, and reproductive systems, and can also compromise lung and kidney function.[48]

Present in commercial refill liquid and e-liquid, PG is odorless and tasteless, whereas VG has a sweet taste, and together are used as a solvent in the e-liquid.[38] These compounds are used as humectants to prevent the e-liquid from drying out and are classified by the Food and Drug Administration (FDA) as "Generally Recognized as Safe".[48] During e-liquid heating, aerosol is formed, and the main components PG and VG can be disintegrated into hazardous toxic substances.[38] PG can induce respiratory irritation and increase the probability of asthma development. Both PG and VG from e-cigarettes might reach concentrations sufficiently high to potentially cause airway irritation.[48]

After PG and VG are thermally decomposed, toxicants are produced such as carbonyls, formaldehyde, acetaldehyde, acrolein, crotonaldehyde, epoxides, and glycidol. At a fixed e-cigarette power output, PG-based e -liquids form more toxic carbonyls than VG-based e-liquids. Nonetheless, the main part of carbonyl compounds is due to the thermal decomposition of flavoring compounds or flavor-catalyzed decomposition of PG. Formation of ROS in e-cigarettes was also reported to depend on the kind of flavoring chemicals present in e-liquids.[42]

The range of e-liquid flavors available to consumers is extensive and attracts both current smokers and new e-cigarette users. However, some of these flavoring agents have demonstrated cytotoxicity. Bahl et al. demonstrated cytotoxic effects on pulmonary fibroblasts with flavors such as menthol or caramel.[49] Those with cinnamon Ceylon flavor were the most cytotoxic in all cell lines.[48,49] Another study by Allen et al. found a significant amount of other flavoring-related compounds that are associated with respiratory complications, such as diacetyl, 2, 3-pentanedione or acetoin.[50] In addition, PG-VG-derived aerosols contain pulmonary irritants and constituents that can induce endothelial dysfunction thereby increasing concern that e-cigarette users, independent of nicotine or flavorings, likely increase cardiopulmonary risk. Formaldehyde, as an abundant thermal degradation product of PG-VG, is an irritant and induces endothelial dysfunction, suggesting that formaldehyde may contribute to the deleterious effects of inhaled aerosols.[51]

E-cigarette use has renormalized cigarette-like behaviors, such as inhaling and exhaling smoke.[45] There is rising evidence that points out e-cigarettes to have a variety of

risks, diseases, and disruptions of normal physiology, including asthma exacerbations, chronic bronchitis, e-cigarette-associated lung injury, and cardiovascular disease.[52] Like conventional cigarettes, the first point of contact, and therefore the first system to be affected by these devices, is the oral cavity.[45] However, studies on potential oral cavity injuries are still sparse, with some controversy about their safety.

Effects of e-cigarettes in the oral cavity

The assessment of the clinical effects of e-cigarettes on oral health has become a challenging task since a longer period of exposure is required. Chronic diseases such as periodontitis, caries, and cancer take several years to manifest as clinical signs and symptoms.[45] Decades might be necessary until extended epidemiological studies could accurately estimate the impact of electronic cigarettes. Moreover, numerous e-cigarette users are former conventional tobacco smokers or dual users, complicating the distinction between the effects of traditional and e-cigarettes.[53] However, what we know about conventional tobacco products can be harnessed to further understand the mechanisms by which e-cigarettes could impact oral health.[45]

The interaction of nicotine and other chemicals in the aerosol produced by e-cigarettes and the human body occurs first in the oral cavity, where they are expected to be most active and have potent effects on the oral microbiome and oral epithelial cells. Nicotine is one of the main components responsible for the toxicity of e-cigarettes. Approximately 45% of the nicotine released from e-cigarettes is deposited in the oral cavity, and its concentrations in saliva are 10.5 times higher than those in plasma.[53] E-

liquids with nicotine have demonstrated a higher degree of cytotoxicity in comparison to nicotine-free samples.[53]

PG is broken down to acetic and lactic acids that are toxic to enamel and soft tissues. PG can lead to xerostomia and promote carious lesions.[54] Vegetable glycerin with other flavoring agents can increase microbial adhesion to enamel, promote biofilm formation, and decrease enamel hardness.[54] The viscosity of the e-liquid enhances the colonization of *Streptococcus mutans* and leads to rampant tooth decay.[54]

A cross sectional study by Huilgol et al., observed a link between poor oral health, increased odds of permanent tooth loss, and daily use of e-cigarettes.[55] Oral symptoms reported in e-cigarette users included decreased salivation, dryness, and irritation of the mouth and throat. Other symptoms include burning, bad taste, bad breath, pain, oral mucosal lesions, black tongue, and burns.[56] Other adverse outcomes were increased inflammatory response and cellular senescence[57], compromised oral wound healing, and masked signs of periodontal disease, such as bleeding on probing.[43] In various studies included in a systematic review by Ralho et al., bleeding on probing was increased in non-smokers when compared with conventional cigarette smokers and e-cigarette users, and no differences were recorded between the latter two groups.[58] This may be due to the presence of nicotine, which has a vasoconstrictive effect on the gingival blood vessels, reducing hemorrhage and cellular healing and inhibiting the early signs and symptoms of gingivitis. Vasoconstriction also leads to a slight decrease in the gingival crevicular fluid flow, which may impair the immune response to bacterial growth.[58]

A study by Al-aali et al., found that e-cigarette users showed an increase in plaque index, probing depths, bone loss, volume of gingival crevicular fluid, and localized inflammatory markers.[59] However, other studies demonstrated that e-cigarette user plaque indexes were lower than conventional smokers, but higher than non-smokers.[41]

An *in vitro* study by Willershausen et al., demonstrated that some additives from e-cigarettes could cause considerable damage to cell proliferation.[60] While some flavorings such as lime or hazelnut did not seem to exert a harmful effect on cell viability and proliferation, the menthol e-liquid preparation had a significant negative impact on periodontal ligament fibroblasts.[60]

Studies by Al-aali, Alqahtani, and ArRejaie support the hypothesis that peri-implant clinical and radiographic parameters are worse and that proinflammatory cytokine levels are higher among e-cigarette smokers and waterpipe smokers than among never-smokers.[59,61,62]

In a pilot study by Reuther, the effect of e-cigarettes (of which half contained nicotine and half did not) on blood flow in the buccal mucosa in 10 volunteers immediately after vaping.[63] Their findings widely varied and a small, but significant capillary perfusion rise was observed as a result of nicotine vaping, but this fell to the same levels as baseline within 30 minutes. However, these results must be interpreted with caution.[63]

The temperature of aerosols may also play a role in e-cigarette carcinogenic potential on oral tissues. High temperatures can result in aldehyde release, causing oxidative stress and have been associated with pre-cancerous nicotine stomatitis.[56]

Intraoral explosion injuries from e-cigarettes have been reported and represent an uncommon complication of e-cigarette use.[64] Explosion blast injuries from overheating of the internal lithium-ion battery during the vaping process have resulted in several oral injuries that include tooth fracture, tooth avulsion, dento-alveolar fracture, hematoma formation, traumatic ulceration, intraoral burns, and subsequent necrosis, palatal perforation with extension into the nasal cavity, and extensive soft tissue deficits that may warrant considerable cosmetic and functional corrective surgery.[46,64]

What do we know about the effects of e-cigarettes on wound healing?

A study by Alanazi et al., in which biopsies of healthy gingival connective tissue were collected and exposed to e-liquid vapor and cigarette smoke, demonstrated that both types of vapor condensate negatively modulate gingival fibroblast migration and proliferation as well as wound healing.[65] However, the damage to gingival fibroblasts was greater with conventional cigarette smoke condensate than with nicotine-rich e-vapor condensate. It was also demonstrated that chemicals other than nicotine present in the e-liquid present a certain level of toxicity to the cells.[65]

A study by Shaikh et al., evaluated the effects of e-cigarettes on the proliferation of normal and cancerous monolayer and 3D models of human oral mucosa and oral wound healing after short-term (3 days) and medium-term (7 days) exposure.[66] Their findings show that medium-term exposure to high concentrations of e-liquid (10%) prolonged the wound healing of normal human oral fibroblasts and oral keratinocytes oral mucosa cells.[66]

Effects of e-cigarettes in the oral microbiome

It has been demonstrated that bacteria are relevant in the process of wound healing, by influencing healing responses through effects on epithelial cell migration, proliferation and apoptosis, endothelial motility, and by contributing to complicated postoperative outcomes through high bacterial loads.[67]

Both beneficial and detrimental effects have been attributed to host-microbial interactions during wound healing.[14] Studies using germ-free mice have shown that priming the skin with commensal microbes prior to wounding improves wound closure via Th17-mediated cytokine release. In line with the hypothesis that oral tissue is primed toward a more inflammatory state by the continuous microbial exposure, ex vivo gingiva biopsies secrete higher amounts of proinflammatory cytokines (IL-6, IL-8, IL-1B, IL-10, TNF-α) as compared to skin biopsies.[14] In contrast, sterile cultured reconstructed human skin and gingiva without microbes or immune cells secrete similar levels of the aforementioned cytokines. Exposure of sterile cultured reconstructed human gingiva to a commensal biofilm again stimulates IL-6 and IL-8 release.[14] The oral microbiome is readily exposed to a moist and protein-rich environment during homeostasis and possibly remains much more stable upon wounding. The resident host immune cells may be better primed toward the oral wound microbiome, resulting in a more efficient and rapid immune response in oral as compared to in skin wounds.[14]

A longitudinal study by Xu et al., investigated the effect of e-cigarette use on the bacterial community structure in the saliva of 101 periodontitis patients.[68] The data demonstrated that e-cigarette use altered the oral microbiome in periodontitis patients,

enriching members of the *Filifactor, Treponema,* and *Fusobacterium* taxa and that e-cigarette and cigarette smokers shared similarities in their oral bacterial composition.[68] This research also demonstrated a positive correlation between certain genera such as *Dialister, Selenomonas,* and *Leptotrichia* and levels of proinflammatory cytokines, including IFN-γ, IL-1β, and TNF-α in the e-cigarette group, which contribute to oral microbiome dysbiosis.[39] From a population with mild periodontitis, Thomas showed similar results in that the e-cigarette microbiome shared numerous characteristics with the microbiome of conventional smokers and some with non-smokers, yet it maintained a unique subgingival microbial community enriched in *Fusobacterium* and *Bacteroides*.[68]

Ganesan et al. investigated the effects of e-cigarettes on the subgingival microbiome of periodontally healthy subjects and found that e-cigarettes exert a powerful, detrimental effect on the subgingival ecosystem, altering the immunotolerance of the host.[40]

The metagenome of e-cigarette users demonstrated greater virulence signatures than controls and there were significant correlations between proinflammatory cytokines and genes encoding for stress response, environmental response regulation, and transport of heavy metals.[40] In this group, quorum sensing was also found to be induced, as well as expression of genes encoding for pellicle proteins.[40]

In a study by Pushalkar et al., *in vivo* effects of e-cigarette aerosol and its influence on human salivary microbiome and immune health were reported.[37] A higher abundance of *Porphyromonas* and *Veillonella* (specifically with species *V. atypica* and *V. rogosae*) was observed among vapers.[37]

The microbial profiles of 10 nonsmokers, 10 cigarette smokers, and 10 e-cigarette users with mild to severe periodontal disease were analyzed via 16s amplicon sequencing in a study by Kumar et al.[45] The oral microbiome composition differed significantly between smokers (e-cigarette and cigarette) and never smokers. Smokers showed lower relative abundance of *Bacteroidetes* and higher abundance of *Actinobacteria* in the saliva as compared with never smokers.[45] *Firmicutes* were enriched in the saliva of current smokers and *Proteobacteria* was significantly higher in e-cigarette smokers. Opportunistic pathogens *Rothia* and *Haemophilus* were significantly enriched in e-cigarette smokers.[45]

A study by Park et al., observed a significant increase in α-diversity in the saliva and subgingival sites for e-cigarette users compared to non-users, suggesting that exposure to e-cigarette vapor increases microbial diversity.[69] Additionally, a greater significant increase in α-diversity in the subgingival sites compared to saliva samples was noted, suggesting that the subgingival niche is richer in microbial diversity compared to saliva.[69]

Oral candidiasis was significantly more prevalent in e-cigarette users compared to non-vapers and the prevalence of candidiasis did not differ from that of conventional cigarette smokers.[70] Alanazi et al. demonstrated that exposure to e-cigarette vapor with or without nicotine promoted the growth and hyphal content of *C. albicans*.[65] Bardellini found that e-cigarette users had a significantly higher frequency of hyperplastic candidiasis compared to former conventional cigarette smokers.[71]

Working Hypothesis

The proposed study will test the hypothesis that e-cigarettes have a significant impact on palatal wound healing in orally and systemically healthy subjects.

Specific aims:

Aim 1: To measure the rate of palatal wound healing after injury in e-cigarette users (clinical impact).

Aim 2: To assess the relationship between the metabolomic profile and clinical parameters in e-cigarette users (metabolomics).

Aim 3: To quantify the inflammatory changes associated with palatal wound healing in e-cigarette users (host impact).

Chapter 2. Materials and Methods

A prospective, case-control clinical study was conducted to determine the aforementioned aims. The study recruited periodontally and systemically healthy adult volunteers, including 16 never-smoker subjects; 8 e-cigarette users (tests) and 8 non-e-cigarette users (controls). The risks to human subjects were minimized by having strict inclusion and exclusion criteria.

Recruited participants were adults (18-50 years). Age limits were placed due to evidence of delayed wound healing in older adults and of the greater likelihood of older adults taking systemic medications and/or having systemic disease.[72] Recruited subjects were either e-cigarette users or never smokers. A never smoker is defined as a person who never smoked or has smoked less than 100 cigarettes in his/her lifetime, and who has not had a cigarette in over 10 years. Smoking/e-cigarette status was assessed by a screening questionnaire. Subjects enrolled in the test group agreed to use a specific e-cigarette device and e-liquids (Voopoo brand, Doric style vape; SynchNic nicotine shot [2 bottles of 15 ml each and 1 bottle of 90 ml of e-liquid with tobacco flavoring]) to ensure a consistent agent. All recruited subjects were willing to provide informed consent.

Exclusion criteria included the following: history of adverse reactions to the use of local anesthetics (Lidocaine), have had antibiotic therapy within the previous 3 months, require antibiotic prophylaxis prior to professional oral cleaning; history of periodontal

surgery; are taking immunosuppressant medications, bisphosphonates, or steroid medications, greater classification than American Society of Anesthesiologists (ASA) II; planning to conceive or currently pregnant or lactating; have had active oral lesions/open sores in the past month or currently; have cavitated carious lesions; a Periodontal Screening and Recording (PSR) score of 3 in two or more sextants or a PSR score of 4 in at least one sextant; are taking medications impacting immune responses or medications investigated for the prevention of tobacco-related cancers; and being on an anticoagulant regimen (warfarin, apixaban, dabigatran, and/or rivaroxaban).

Since this is a pilot investigation, there was no robust method to estimate sample size. This estimation was based on a previous wound healing study conducted in smokers. In that study, a final sample size of 15 in each group allowed for effects of 1% to be detected with a confidence of 80%.[67] Allowing for an attrition rate of 25%, 20 subjects were recruited in each group. However, for the purposes of the present thesis, 8 e-cigarette users and 8 non-smokers were randomly selected.

Interested participants were given a screening questionnaire, which aim was to primarily assess systemic health, lifestyle, demographics, as well as e-cigarette and tobacco exposure. Those who satisfied the inclusion criteria, signed informed consent. Eligible subjects were enrolled in the study after completing a dental and periodontal screening. E-cigarette users were provided with a device and e-liquids, and they were instructed to use the device 2 times per day for over 1 hour each, in the morning and evening, with 20 puffs per one-hour session. After completion of study, e-cigarette users were instructed to return remaining e-liquids and devices.

Participants attended seven study visits after the screening/consent visit (visit 1): first surgical visit (visit 2) involving bilateral wounding in the premolar area; second surgical visit (visit 6) involving a unilateral wounding on the right side; third surgical visit (visit 8) involving a unilateral wounding on the left side; and six follow-up visits. The latter visits took place 24 hours (visit 3), 48 hours (visit 4), 4 days (visit 5), 7 days (visit 6), 14 days (visit 7), and 21 days (visit 8) after the first surgical visit (visit 2).

The study protocol, informed consent, study forms, and questionnaires were approved by the Institutional Review Board of The Ohio State University (Protocol #2020H0074). All clinical procedures were performed at the Graduate Periodontology Clinic at The Ohio State University College of Dentistry.

Smoking status was assessed by a questionnaire and confirmed by exhaled carbon monoxide measurements.[67] Vital signs were obtained at the screening appointment, at the beginning of every visit and prior to palatal wounding. The biological samples were collected at each visit and included unstimulated saliva, supragingival and subgingival plaque, gingival crevicular fluid (GCF), and palatal biofilm. GCF and supragingival/subgingival plaque samples were collected from Ramfjord teeth (3, 9, 12, 19, 25, and 28) or the immediate mesial tooth if any of the latter are missing. A scaler was used to collect supragingival and subgingival plaque samples. GCF was collected using two sterile paper strips per tooth, which were inserted in the gingival crevice for at least 1 mm or until resistance was felt and were left in for 30 seconds. A previously calibrated electronic volume quantification unit (Periotron 8000 ®) was used to determine the collected volume in each strip. The samples were placed in sterile vials and stored at -20°C

until processing. Supra/subgingival plaque samples and GCF were not included for the purposes of the present study.

Palatal biofilm was collected with one pass of a sterile swab over the palatal tissue and bilaterally. Palatal and supragingival/subgingival samples were stored in *RNAlater*. All samples were stored frozen at -20°C until processing.

To minimize differences related to the surgical technique, all surgical procedures (wound creation and biopsy harvest) were performed by the same investigator (NRL). Wound creation and biopsy harvest were conducted after achievement of profound local anesthesia. First, both right and left sides of the palate were dried with a sterile gauze followed by topical anesthetic (20% benzocaine) application with a cotton applicator for two minutes, over the area corresponding to the greater palatine foramen. Once located, sufficient pressure was applied to blanch the tissue for 30 seconds. Greater palatine block anesthesia was then performed using ¼ to ⅓ of a 1.7 ml cartridge of Lidocaine Hydrochloride 2% with 1: 100,000 epinephrine. A 5 mm diameter partial thickness (1.5 mm depth without a standardized method) palatal wound was made 3-5 mm from the free gingival margin in the bicuspid region of both right and left palate with a sterile disposable biopsy punch (Integra Miltex). Wound biopsies were immediately sectioned in two halves with one half stored for gene expression analysis (*RNAlater)* and the other was stored in 10% neutral buffered formalin for histology and immunohistochemistry. Hemostasis was ensured and recorded after wound creation. Hemostasis was determined as difficult after observing continuous bleeding after 5 minutes of pressure with a moist gauze. The same anesthesia technique, wound creation, processing, and storage was conducted for visits 6

and 8, for the right and left side palate, respectively. For these sites, a 3 mm diameter partial thickness (1.5 mm depth) palatal wound was made 3-5 mm from the free gingival margin in the bicuspid region and including healing margins, with a sterile disposable biopsy punch (Integra Miltex).

Wound size was captured by photography at completion of wounding procedures and at all post-operative visits. Photographs were taken with a macro 100 mm F/2.8 lens (Canon, Japan), keeping a consistent focusing distance. Photographs were edited (Adobe Photoshop, USA) to obtain the corresponding right and left sides of the palate since the image is a mirror view.

Written and verbal postoperative instructions were provided, and these included standard oral hygiene measures with no prescribed mouthwash use, together with analgesics as needed (acetaminophen first, ibuprofen as rescue analgesic).

Wound site assessment

The Pippi modification of the Landry index[10] was used to evaluate clinical healing. The following evaluation parameters were scored for each wound by applying a dichotomic score (0/1) with a possible total score of 7: presence/absence of redness; presence/absence of granulation tissue; presence/absence of suppuration; presence/absence of swelling; degree of tissue epithelialization (partial/complete); presence/absence of bleeding; presence/absence of pain on palpation. Patients were asked about their pain experience from each site during the healing period as a dichotomous variable (yes/no).

Complete epithelialization was scored clinically after visual inspection. Peroxide testing (Figures 1 and 2) was employed to determine the extent of epithelization (wound closure) on visit 3 (24 hours after first bilateral wounding), visit 4 (48 hours after first bilateral wounding), visit 5 (4 days after bilateral wounding), visit 6 (7 days after bilateral wounding), visit 7 (14 days after bilateral wounding and 7 days after right side wounding), and visit 8 (21 days after bilateral wounding and 14 days after right side wounding). After drying the area to be evaluated with a gentle stream of air, 3% H_2O_2 was applied on the wound with a cotton swab. The appearance of bubbles suggested that the surgical site was not completely epithelialized. Evidence of epithelialization was documented photographically in all wounds at every visit, starting from the 24-day postoperative visit.

An intraoral optical scanner (iTero) was used to obtain maxillary arch digital models as STL files in the subsequent six postoperative visits, starting at the 24-hour appointment (third visit) after first wounding to further assess volumetric changes of the palatal wounds. These files are not included for the purposes of the present study.

Metabolomics

Palatal swab samples were removed from the -20°C freezer, thawed at room temperature, and vortexed to ensure a homogenous solution. Next, a matrix solution of 90% methanol, 0.01% tissue necrosis factor α (TNF), and 20mg/mL 2,5 dihydroxybenzoic acid (DHB) was prepared. Clean PCR tubes were labeled with their corresponding sample numbers and a 1:1 ratio of sample: matrix was added to each. After mixing the contents of each tube by aspirating the solution up and down, 2mL were spot-plated on a 96-spot Matrix-Assisted

Laser Desorption Ionization (MALDI) plate which was then placed under vacuum seal in the desiccator.

Once the spotted samples were adequately dry, the spot plate was loaded into the MALDI for mass spectrometry analysis using the following settings: positive ion polarity, scan range of 20m/z – 2500m/z, and a laser application setting of "MS Dried Droplet" at an intensity of 100%.

Data Processing and Metabolite Annotations:

The raw data was processed with the SCiLS lab 2021a (Bruker USA). To assign the regions a mass range for each subject saliva sample was 20-2000 m/z. To achieve the segmentation in SCiLS, the "peak list" option was selected, and the windows moved till maximum intervals was obtained. The peak list was saved, and the data normalized by root mean square transformation. Regions were created from the desired segments, and this was repeated till all regions were created.

The region file was then exported to Metaboscape 2021b (Bruker, USA) for annotation and further downstream processing. The subsampling parameters of Metaboscape region of interest (ROI) were as follows: width-5, height-5, maximum number of speckles/ROI-500 and intensity threshold-500. Following this the regions were checked, and m/z points were annotated. The annotation was done using the following libraries: HMDB library 2.0_Kyoto Encyclopedia of Genes and Genomes (KEGG), Lipids Human Brain metabolites library, Lipids Mouse Kidney metabolites library, Small Molecules metabolites library, N-Glycan human library, Cell culture nutrient library, Fatty

acids library, HMDB plasma metabolites library, Lipid maps library, Natural products metabolites library, and the CCS compendium library. Additional annotation was done with help of range of mass spectral libraries available with Metaboscape, such as Bruker NIST 2020 MSMS Spectral Library hr-2, MSDIAL- Tandem Mass Spectral Atlas libraries for the positive ions. The m/z: 2.0-5.0 ppm, msigma 25–500, and CCS 2.0-5.0% were the parameters used for annotation. Same values were also used to annotate the metabolites against the Lipid class.

Initially, bucket labels for each experiment were exported from Metaboscape 2021b and dimensionality reduction performed using nonmetric dimensional scaling (NMDS) of Bray-Curtis Dissimilarity distance with the Vegan package in R (https://www.r-project.org/). ANOSIM was used to test the statistical significance of clustering. Student's t-test with False Discovery Rate (FDR) adjusted p-values (Benjamini-Hochberg procedure for multiple comparisons) was used to identify metabolites that differed significantly between groups. 50 metabolites that demonstrated the most significant differences as well as the largest peak intensities were identified, and peak intensities log-transformed and normalized by mean subtraction. The transformed peak intensities of these 50 metabolites were plotted against correlation-based clustering of columns in a heat-map. The 50 metabolites were further annotated with help of The Natural Product Atlas (https://www.npatlas.org/discover/overview) and Metabolomics Workbench (https://www.metabolomicsworkbench.org/about/index.php).

The m/z values of all metabolites were ranked based on the FDR-adjusted p-value, then exported to MetaboAnalyst 5.0 (Wishart Research Group, University of Alberta,

Canada) to obtain functional pathway information using default filtering parameters. Pathways were assigned using Homo sapiens (Human) KEGG library. A minimum of 3 metabolite entries was needed for a pathway to be considered.

Histological analysis

Formalin-fixed paraffin-embedded specimens were subjected to 4 μm thick sections and were stained with hematoxylin and eosin for histologic confirmation of clinical diagnosis and other evaluations. These sections were used for grading overall wound healing (Figures 3, 4, and 5).

Immunohistochemistry protocol:

Eight-micron-thick sections were mounted on poly-L-lysine (PLL) slides and stained with monoclonal antibodies to vimentin (Vimentin (Rabbit) CST#5741, Invitrogen USA), keratin (Pan-Keratin (Mouse) CST#4545), and filaggrin (Filaggrin (Rabbit) Invitrogen #PA5-83129), using a standard avidin-biotin-peroxidase complex method. Enzymatic predigestion with proteolytic enzymes (Proteinase K) was done for greater staining intensity and uniformity on formalin-fixed tissue sections. Diaminobenzidine (DAB, Zymed) was then used as the substrate for localizing the antibody binding. The preparations were counterstained with the Harris hematoxylin, mounted with a neutral mounting medium and examined under light microscope for immunoperoxidase reactivity. Positive staining of tonsil tissue was considered as positive control, while negative staining of epithelial tissue was considered as negative control for staining. The sections were

studied in detail by three observers independently and the tissue sections were graded as follows.

The sections were viewed initially in low power and the connective tissue stroma was divided into two zones—the superficial zone and deep zone. Each zone was considered separately and scored on a scale of 0–3 with 0 indicating no staining, 1 indicating mild staining, 2 indicating moderate staining, and 3 indicating intense staining. The tissue sections were later viewed under high power and the cellular localization of the staining was studied. The staining for fibroblasts and endothelium was considered separately and scored on a scale of 0–3 with 0 indicating no staining, 1 indicating mild staining, 2 indicating moderate staining, and 3 indicating intense staining. The overall intensity of staining was noted in all the cases and scored on a scale of 0–4 with 0 indicating no staining, 1 indicating mild staining, 2 indicating moderate staining, 3 indicating intense staining, and 4 indicating the most intense staining.

Data analysis

Clinical parameters were analyzed using JMP (JMP Statistical Discovery LLC, 100 SAS Campus Drive Cary, NC, USA) and inter-group comparisons made using Wilcoxon's Method. The minimum level of significance for all comparisons was set at $p < 0.05$. Analysis methods for metabolomics are described in that section.

Table 1. Study appointment sequence and procedures per-visit.

Study visits	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**	**Visit 6**	**Visit 7**	**Visit 8**
	Initial	Bilateral wounding	24-hour follow-up	48-hour follow-up	4-day follow-up	7-day follow-up and right-side wounding	14-day follow-up	21-day follow-up and left-side wounding
Enrollment								
Informed consent	X							
Screening questionnaire	X							
Vital Signs	X	X	X	X	X	X	X	X
Intervention								
Wound creation		X				X		X
Biological samples (Plaque, GCF, palatal biofilm, saliva)	X	X	X	X	X	X	X	X
Assessment								
PSR	X	X	X	X	X	X	X	X
Healing Score Index			X	X	X	X	X	X
Peroxide test			X	X	X	X	X	X
Photographs		X	X	X	X	X	X	X
Intraoral scan			X	X	X	X	X	X

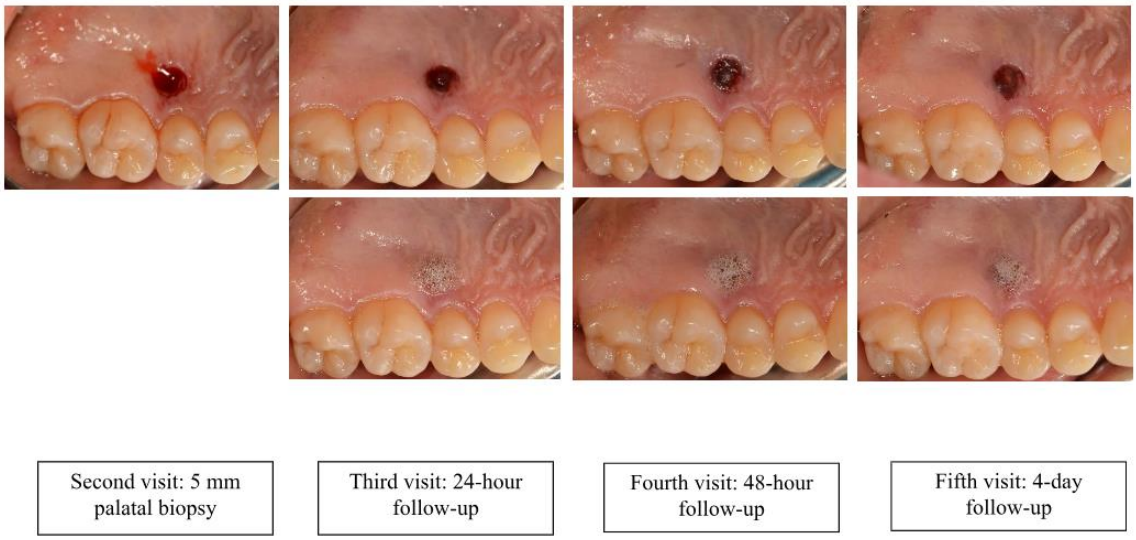


Figure 1. Representative clinical image of a test subject undergoing hydrogen peroxide test for epithelialization assessment.

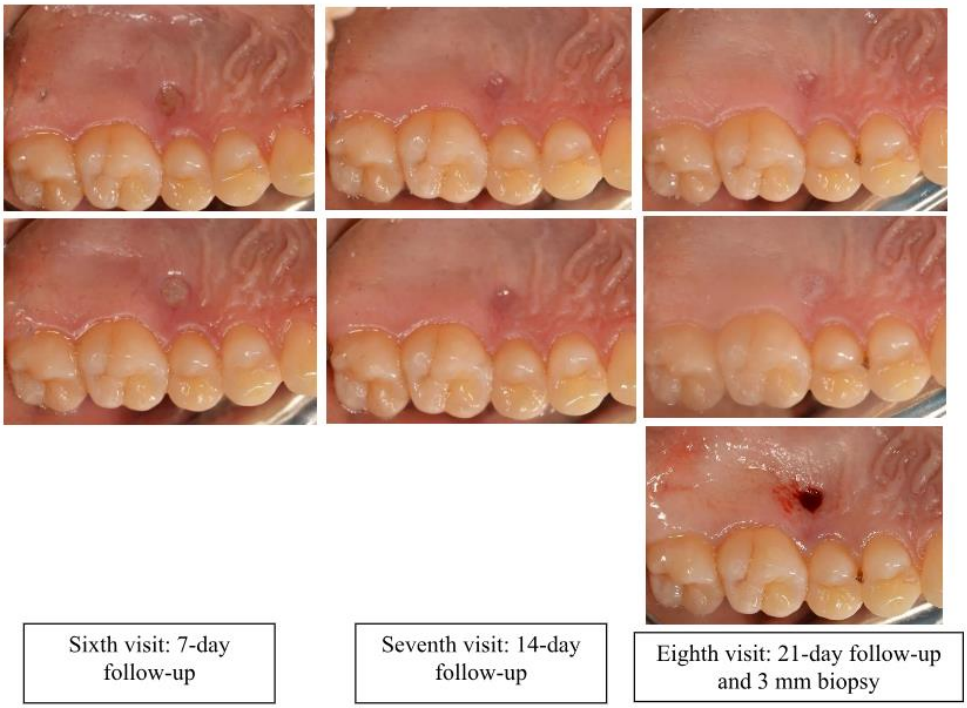


Figure 2. Continuation of clinical course of a test subject undergoing hydrogen peroxide test for epithelialization assessment.

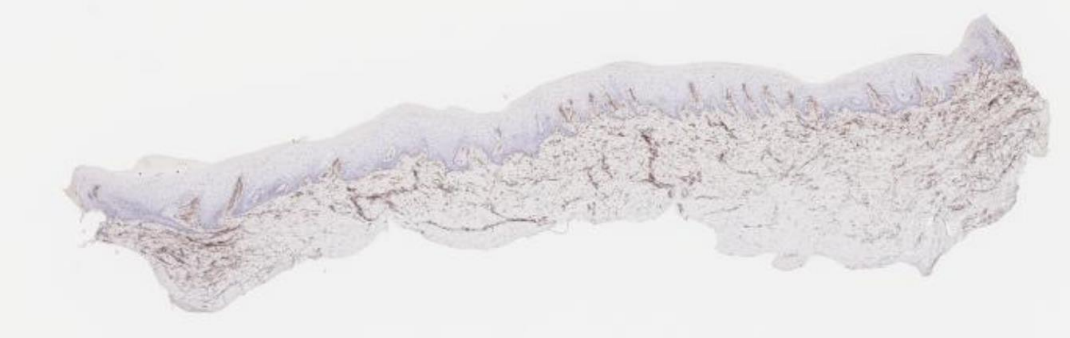


Figure 3. Representative histological slide showing vimentin staining in the lamina propria.

Figure 4. Representative histological slide showing keratin staining in the epithelium.

Figure 5. Representative histological slide showing filaggrin staining in the stratum corneum of the epithelium.

Chapter 3. Results

Study population

Sixteen individuals were recruited in the present parallel arm, prospective clinical study. Periodontally and systemically healthy adult volunteers, including 8 non-e-cigarette users (controls) and 8 e-cigarette users (test) were considered for final analysis. The average age of the control group was 28.6 ±2 years and 25.5 ±1.7 years for the test group. Sex distribution was equal between the two groups with 4 males and 4 females in each group. Ethnicity distribution of the study population included Caucasian, African Americans, and Asian individuals. The control group included 5 Caucasians and 3 Asians. The test group encompassed 6 Caucasians, 1 African American, and 1 Asian (Table 2). Regarding occupation, all 8 control subjects were students. Of the test subjects, 6 were students, 1 was a contractor, and 1 worked in digital marketing.

Of the 8 control subjects, 7 stated having no significant systemic conditions, 1 subject with a history of eosinophilic esophagitis and asthma. Of the 8 test subjects, 3 stated having no significant systemic conditions, 1 stated a history of mild Von Willebrand disease that required no anticoagulant treatment, 1 stated asthma during childhood and sinusitis, 1 had a history of seizures as a teenager, 1 stated exercise-induced asthma, and 1 had rarely occurring migraines with auras.

Regarding smoking status, all 8 control subjects were never smokers. Of the 8 test subjects, all engaged in vaping, 7 were never smokers, and 1 was a former smoker over 10 years ago.

An interesting finding was related to the number of drinks per week and vaping status. Of the 8 control subjects, 5 stated not consuming alcohol, 2 stated consuming one alcoholic drink per week, and 1 stated consuming 1-2 drinks per week. On the other hand, of the 8 e-cigarette users, 1 stated consuming 2 drinks per week, 1 stated consuming 2-3 drinks per week, 2 stated consuming 3 drinks per week, 1 stated consuming 3-4 drinks per week, 2 stated consuming 4 drinks per week, and 1 stated consuming 6 drinks per week. All the test subjects mentioned that they tend to engage more in vaping during the evening and the weekends.

Clinical findings: palatal wound assessment

Healing Score Indices

The Pippi modification of the Landry index (Healing Score Index or HSI) was used to evaluate overall clinical healing. The HSI utilized a dichotomic score (0/1), with a total score of 7, which describes the most positive clinical outcomes. Figures 6 and 7 show a representative case for a control and test subject, respectively. Table 3 demonstrates the average scores on each side per visit. Figures 8 and 9 describe the trend in clinical healing based on the total score of the HSI from visit 3 (24 hours after bilateral wounding) to visit 8 (21 days after bilateral wounding and 14 days after right side wounding). In the right palate (RP) (Figure 8), the test group tended to show a slightly average lower score in all visits compared to the control subjects, with visit 6 (7-days postoperative) showing worsened scores in the test group. However, these differences were not statistically significant. All subjects in the control group showed a score of 7 by the 21-day

postoperative appointment (visit 8), while subjects in the test group averaged a total score of 6.25 in the RP. In the left palate (LP) (Figure 9), a more consistent increase in scores was seen between both groups over time, with the test group showing slightly lower scores compared to the control group. All subjects in the control group showed a score of 7 by the 21-day postoperative appointment (visit 8), while subjects in the test group averaged a total score of 6.83 in the LP; these differences were not statistically significant. No significant statistical difference was observed in the total Healing Score Index between the two groups.

As for the individual components of the Healing Score Index, no control and test sites exhibited suppuration at any follow-up visit.

The least square regression model (Figure 10) was used to predict the behavior of dependent variables. The red line indicates the actual fit of the variables when compared to the actual fit in blue. Four variables were inducted into the model. This representation is the result of the whole model based on all 4 variables in the next figures. The data demonstrated that there were significant differences between groups in terms of the whole model ($p=0.0034$), as well as differences over each visit ($p<.0001$). Moreover, the interaction between the visit and the groups was also statistically significant ($p<.0001$). This means that each group was different from the other at each time point, within each group, the healing was significantly different at each visit, and in each group, the trend in healing over (i.e., each visit) was different.

The least square regression model for redness as a single variable (Figure 11) showed statistically significant differences over each visit ($p<.0001$). However, both groups demonstrated non-significant differences in redness at each visit ($p=0.0967$).

Moreover, the interaction between the visit and group is also not statistically significant. This means that, in each group, the trend in redness over time (i.e., each visit) was not different ($p=0.7313$).

The least square regression model for bleeding as a single variable (Figure 12) demonstrated that there were statistically significant differences over each visit ($p=0.0058$) and both groups demonstrated significant differences in bleeding at each visit ($p<.0001$). However, the interaction between the visit and group was not statistically significant ($p=0.0544$), which means that in each group, the trend in bleeding over time (i.e., each visit) was not different.

In terms of epithelialization as a single variable, the least square regression model (Figure 13), demonstrated that there were non-significant differences between groups ($p=0.0834$). However, both groups demonstrated significant differences in epithelialization at each visit ($p=0.0016$). The interaction between the visit and group was not statistically significant, which means that in each group, the trend in epithelialization (i.e., each visit) was not different ($p=0.0935$).

The least square regression model for swelling as a single variable (Figure 14) showed a statistically significant difference over each visit ($p<.0001$) and both groups demonstrated significant differences in swelling at each visit ($p=0.0083$). However, the interaction between the visit and the group was not statistically significant, which means that in each group, the trend in swelling over time was not different ($p=0.0895$). The parameter of granulation was not included in the model as there were no differences.

Regarding immediate postoperative hemostasis after harvesting of the 5 mm bilateral biopsies (visit 2), 5 out of the 8 test participants showed difficult hemostasis on both RP and LP. At the 7-day follow-up (visit 6) and right-side wounding (3 mm biopsy), hemostasis was not easily achieved in 2 subjects. At the 21-day follow-up (visit 8) and left-side wounding (3 mm biopsy), hemostasis was not easily achieved in one of the latter subjects. Three of the test subjects did not have any hemostasis issues at any of the harvesting time points. Statistical analysis was not performed for these findings.

Pain perception

Pain perception was reported as present (score of 0) or absent (score of 1). From the control group, only 1 subject reported pain on palpation on the RP and LP on visit 3 (24-hour postoperative). On the other hand, pain perception in the test group was more marked. At 24 hours postoperative, 2 subjects reported pain on both RP and LP, while 2 subjects reported pain on the RP and LP, respectively. At 48 hours postoperative, the 2 subjects that previously perceived pain bilaterally, continued with pain on palpation, while 1 subject continued to experience pain on palpation only on the LP. At 4 days postoperative, 2 subjects continued to perceive pain only on the LP and 1 subject on the RP, while 2 subjects started perceiving pain on the LP. At 7 days postoperative, 3 of the subjects that initially perceived pain from visit 3 (24-hour postoperative), continued to experience pain on palpation only on the LP, while 1 subject continued to perceive pain on the RP and LP. Only 1 subject that initially perceived pain at the 24-hour postoperative visit on the RP, started to experience pain on palpation at the 7-day postoperative visit (visit

6). No pain perception was reported on visits 7 and 8 at any site in any control and test participant, despite the creation of a 3 mm wound on the right-side palate at the end of visit 6 (7-day postoperative visit after first bilateral wounding). Statistical analysis was not performed for these findings.

Metabolome analysis

The heatmap analysis (Figure 15 in Appendix A) between the E-cigarette and control group at week 1 demonstrated significant differences in the intensities of the following metabolites: 698.3983578, 908.2412269, 1439.65821, 1094.528189, 1196.375960, all of which were identified as Glycerolipids. The heatmap analysis between E-cigarette and control group at week 3 demonstrated significant differences in the intensities of the following metabolites: 1188.770843 (Glycerolipids), 763.3592801 (Glycosphingolipids), 768.416696 (Glycosyldiradylgycerols). The metabolic pathway analysis plot was generated using MetaboAnalyst 5.0. The pathway analysis (Figures 16A and 16B) was done in positive ion mode between the groups. This analysis depicts different metabolic pathway alterations in common and specific metabolites from these groups were tested. The plots are plotted against the y-axis which is -log10 of the P-value, such that the pathways that are most significantly changed are characterized by a high -10log P-value.

The blue lines are pathways that are over-represented in e-cigarette users and the red lines indicate pathways that are over-represented in the control subjects. The pathway analysis between week 1 and 3 revealed that the microbiome of e-cigarette users metabolized

carbohydrates and lipids more significantly than controls, while protein metabolism was over-represented in the control group.

Histological assessment and comparison

Immunohistochemistry revealed significantly lower vimentin, filaggrin and keratin scores over all time points in the e-cigarette users when compared to controls (Table 4). The baseline score for each protein was 4.0 ± 0. At week 1, e-cigarette users and control subjects showed a score of 0.875 ± 0.15 and 1.6 ± 0.1125 for vimentin ($p=0.01$), 1.5125 ± 0.0875 and 3.025 ± 0.237 ($p=0.03$) for filaggrin, and 1.4 ± 0.1 and 3.625 ± 0.3 ($p=0.003$) for keratin, respectively. At week 3, e-cigarette users and control subjects had a score of 1.4125 ± 0.075 and 3.5875 ±0.1625 ($p=0.008$) for vimentin, 1.6 ± 0.1625 and 3.2375 ± 0.05 for filaggrin ($p=0.003$), and 1.4 ±0.475 and 3.8 ± 0.2 for keratin ($p=0.001$), respectively.

Table 2: Clinical and demographic data of study subjects			
	Control	E-cigarettes	p-value
Age (mean ±std. dev, range)	28.6 ±2.2, 25-31	25.5 ±1.7, 23-28	0.08
Gender (number of females)	4	4	1
Race/ethnicity (White, Asian, Black)	5, 3, 0	6,1,1	0.07

Table 2. Clinical and demographic data of study subjects.

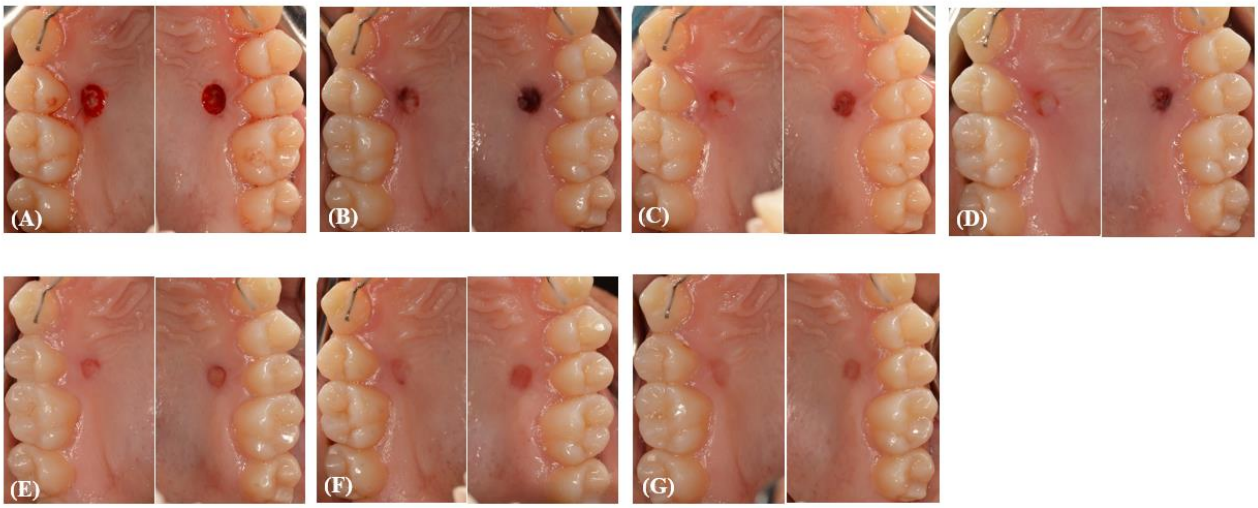


Figure 6. Clinical wound healing sequence from a representative control subject. (A) Visit 2, bilateral biopsies; (B) Visit 3, 24-hour postoperative appointment; (C) Visit 4, 48-hour postoperative appointment; (D) Visit 5, 4-day post-operative appointment; (E) Visit 6, 7-day post-operative appointment; (F) Visit 7, 14-day postoperative appointment; (G) Visit 8, 21-day post-operative appointment.

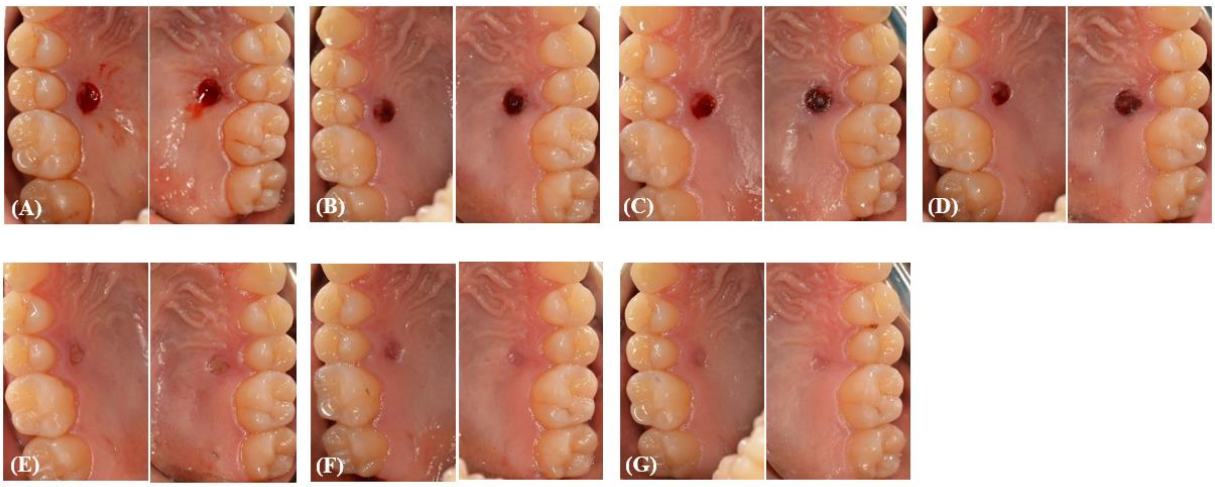


Figure 7. Clinical wound healing sequence from a representative test subject. (A) Visit 2, bilateral biopsies; (B) Visit 3, 24-hour postoperative appointment; (C) Visit 4, 48-hour postoperative appointment; (D) Visit 5, 4-day post-operative appointment; (E) Visit 6, 7-day post-operative appointment; (F) Visit 7, 14-day postoperative appointment; (G) Visit 8, 21-day post-operative appointment.

Table 3. Healing Score Index. Averaged scores per visit				
	LP		RP	
	e-cigarette	Control	e-cigarette	Control
visit 3	2.875	3.375	3.125	3.625
visit 4	3.125	3.5	3.625	4
visit 5	3.5	4.125	4.375	4.5
visit 6	4.75	5.5	4.875	6.125
visit 7	6.71428571	7	6.83333333	7
visit 8	6.83333333	7	6.125	7

Table 3. Healing Score Index. Averaged scores per visit.

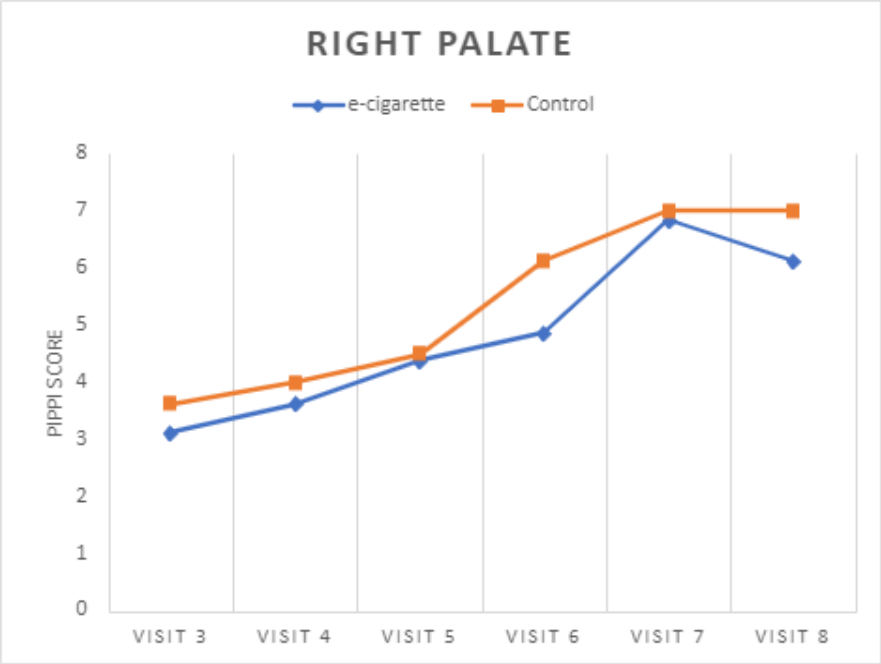


Figure 8. Right palate, Pippi (HSI) score over time.

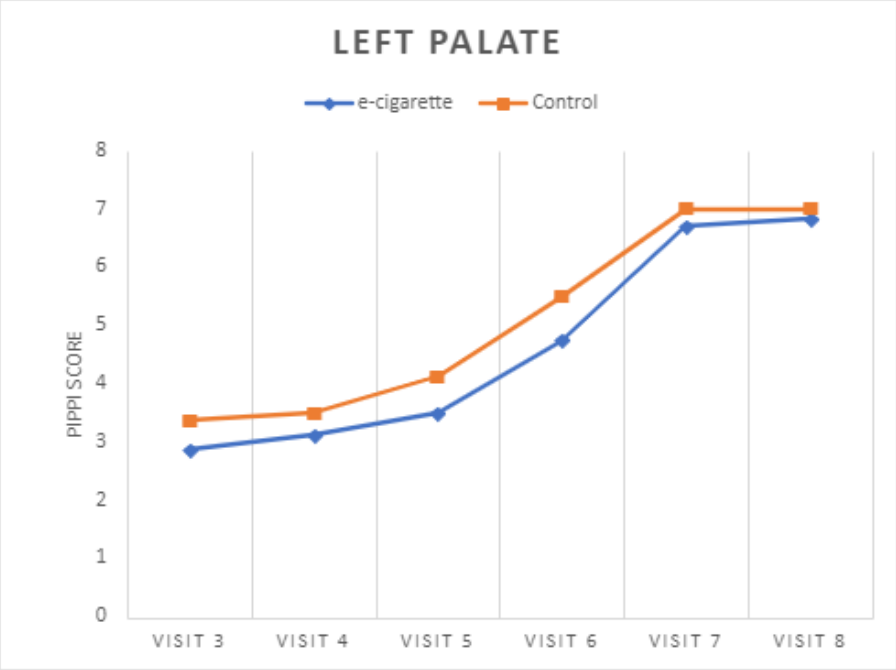
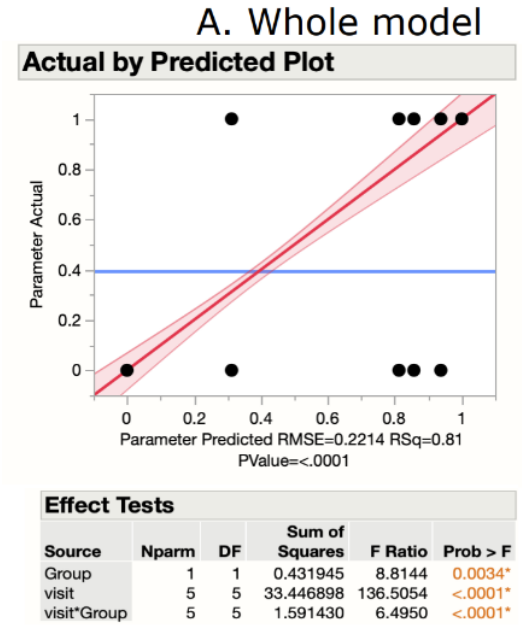


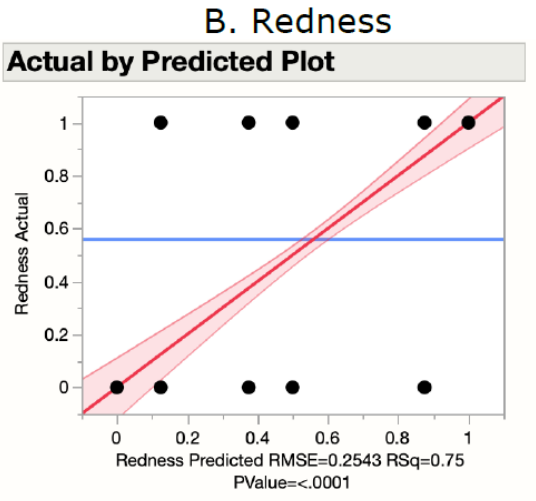
Figure 9. Left palate, Pippi (HSI) score over time.



Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Group	1	1	0.431945	8.8144	0.0034*
visit	5	5	33.446898	136.5054	<.0001*
visit*Group	5	5	1.591430	6.4950	<.0001*

Figure 10. Least square regression model of overall wound healing parameters.

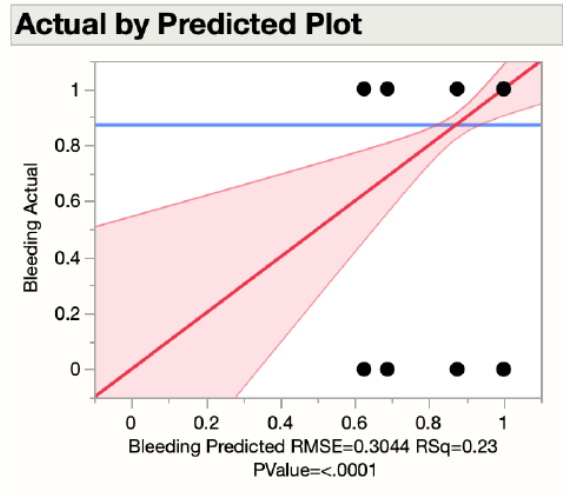


Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Group	1	1	0.180344	2.7893	0.0967
visit	5	5	34.086965	105.4423	<.0001*
visit*Group	5	5	0.180715	0.5590	0.7313

Figure 11. Least square regression model for redness as a single variable.

C. Bleeding

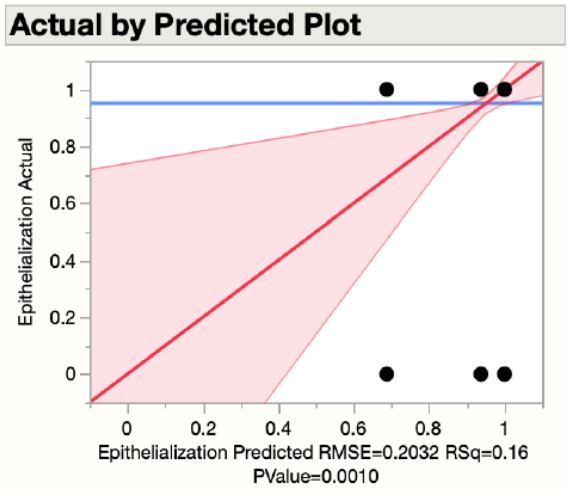


Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Group	1	1	2.0038168	21.6226	<.0001*
visit	5	5	1.5789474	3.4076	0.0058*
visit*Group	5	5	1.0287829	2.2203	0.0544

Figure 12. Least square regression model for bleeding as a single variable.

D. Epithelialization



Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Group	1	1	0.12523855	3.0319	0.0834
visit	5	5	0.84410979	4.0870	0.0016*
visit*Group	5	5	0.39633018	1.9189	0.0935

Figure 13. Least square regression model for epithelialization as a single variable.

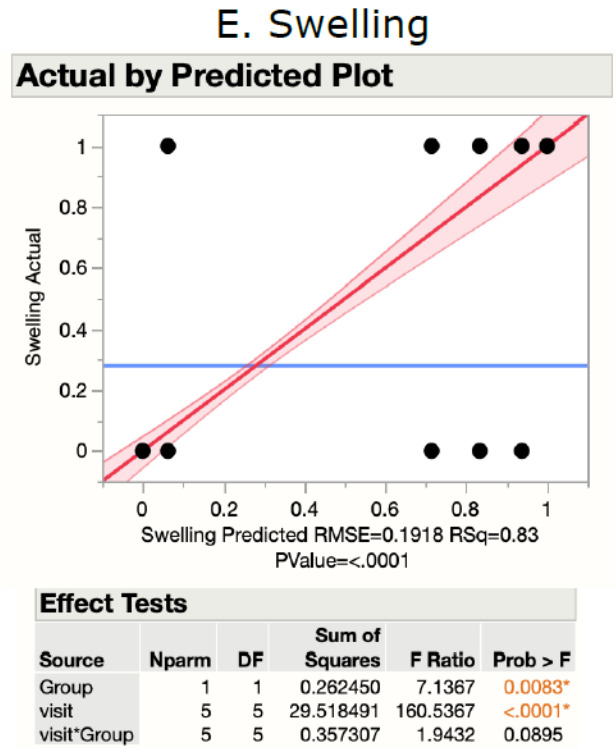


Figure 14. Least square regression model for swelling as a single variable.

	Vimentin			Filaggrin			Keratin		
	Control	E-cigarette	p-value	Control	E-cigarette	p-value	Control	E-cigarette	p-value
Baseline	4 ± 0	4 ± 0		4 ± 0	4 ± 0		4 ± 0	4 ± 0	
1-week	1.6 ± 0.1125	0.875 ± 0.15	0.01	3.025 ± 0.237	1.5125 ± 0.0875	0.03	3.625 ± 0.3	1.4 ± 0.1	0.003
3-weeks	3.5875 ± 0.1625	1.4125 ± 0.075	0.008	3.2375 ± 0.05	1.6 ± 0.1625	0.003	3.8 ± 0.2	1.4 ± 0.475	0.001

Table 4. Immunohistochemistry data of vimentin, filaggrin and keratin staining.

Chapter 4. Discussion

Findings from the present study add to the growing concern regarding the impact of e-cigarettes on normal physiology and particularly, oral wound healing. Periodontists face a significant challenge, as e-cigarettes have been shown to be associated with proinflammatory responses, increased cytotoxicity, and impaired immune defenses. These effects lead to a higher risk in patients undergoing periodontal surgery and could potentially hinder the healing response.

As a large proportion of current and former conventional cigarette smokers use e-cigarettes, it may be difficult to determine whether oral lesions/wound healing alterations are directly a result of e-cigarette use or the consequence of previous or current concomitant conventional cigarette use. Therefore, this study included e-cigarette users that never smoked conventional cigarettes. It is unknown whether the use of e-cigarettes can accentuate the effects of tobacco smoke in current conventional smokers trying to quit smoking.[45] To the best of our knowledge, this is the first study to assess and compare clinical parameters, immunohistochemistry, and metabolomics in oral wound healing in e-cigarette users and non-users.

According to the National Center for Health Sciences (NHCS), data from 2015 showed that 3.7% of the U.S. adult population were current e-cigarette smokers.[73] According to the National Health Interview Survey of the United States in 2017, approximately 2.8% of the U.S. adult population were current e-cigarette smokers.[73] In comparison with adults aged 25 years and older, young adults (18-25 years) are more likely to try e-cigarettes and report having used e-cigarettes in the past 30 days.[73] Our

demographics show that the mean age in the test group was 25.5 ±1.7 years of age, with most of these subjects having tried e-cigarettes before turning 25 years old. Also, the test group shows a higher trend of e-cigarette and alcohol dual use compared with control subjects. According to Wetzel, e-cigarette users have an increased risk for an alcohol use disorder (binge drinking or chronic use) compared with non-users.[74] Age of initiation has also been identified as a factor contributing to alcohol misuse, with a younger age of onset more likely to demonstrate lifetime alcohol use.[74]

Silva et al. demonstrated that patients who smoke display wounds that undergo delayed epithelialization; at 15 days after graft harvesting, 20% of smokers and 92% of non-smokers exhibit complete epithelialization.[75] Our clinical findings in terms of wound epithelialization an e-cigarette use seem to somewhat agree with Silva et al., since 88% of the control subjects showed complete epithelialization bilaterally at 14 days after the 5 mm biopsy creation, while 50% of the test group subjects showed complete epithelialization at the same time point. Oral wounds stay open during the entire wound healing process and are continuously exposed to saliva and the oral microbiome until complete re-epithelialization. Migration and proliferation of keratinocytes are key events in re-epithelialization.[14] E-cigarette aerosols have been noted to cause cytotoxicity in human oral keratinocytes via an oxidative stress response.[76] According to Alanazi et al., human gingival fibroblasts treated with e-vapor showed delayed migration and wound closure, particularly at higher concentrations.[65] It is interesting to note that from the test group, three out of eight subjects did not show signs of complete epithelialization at 21 days postoperative (2 subjects on the RP and 1 participant on the LP), while 5 subjects from the

same group showed complete epithelialization bilaterally at this time point. Of the control group, all subjects showed complete epithelialization at 21 days postoperative. However, based on the mixed model of epithelialization (Figure 8), statistically significant differences were only seen between visits.

An interesting area regarding adverse effects of e-cigarettes is mucosal irritation/pain. In the present study, an increased number of subjects and sites from the test group experienced postoperative pain compared to the control subjects. Evidence on the effect of nicotine and the possible effect of aldehydes (cinnamaldehyde) on pain receptors has been reported in controlled human studies.[42,77] With variability in nicotine content of e-cigarettes, the impact on oral pain perception may be higher than cigarette users. However, another study reported no significant differences in gingival pain (reported as a secondary outcome) between e-cigarette users and controls.[78]

Interestingly, our findings revealed that e-cigarette users showed more immediate postoperative bleeding than the control subjects, particularly at the time of bilateral biopsy harvesting (5 mm wound). Conversely, according to Rossmann, conventional cigarette smokers obtain hemostasis in nearly half the time as nonsmokers do, possibly because nicotine and its by-products are vasoconstrictors.[79] However, this must be further explored in exclusive e-cigarette users.

The effect of vaping on peripheral circulation exerts a significant reduction of blood flow within superficial and deep vessels, the result of which is tissue hypoxia and necrosis, particularly in surgical flaps.[52] Vaping can delay wound healing by interfering with VEGF expression, which could be the central mechanism of poor wound healing.[52] This indicates

poor wound healing in e-cigarette users can be due to impaired and weakened angiogenesis, decreased granulation tissue, and defective scar formation.[52] In the present study, differences in postoperative bleeding were statistically significant between groups and between visits. The literature is non-existent in studies performed in experimental oral wounds assessing postoperative bleeding.

Another clinical parameter that has not been explored is the persistence of redness in the wound, which showed statistically significant differences between visits in our study. Postoperative swelling was another clinical parameter explored in the present study, showing statistically significant differences between groups and between visits. Clinical studies assessing postoperative swelling after creation of experimental wounds in e-cigarette users were not found in the literature. For the present thesis, we did not explore markers of inflammation; however, according to Chatterjee et al., vaping predisposes the circulatory system to an inflammation load that may lead to an increase in oxidative stress.[80] Sayed et al. stated that e-cigarettes alter the inflammatory state of the systemic circulation, predisposing its users to increased susceptibility to infection.[81] E-vapor was shown to promote the release of inflammatory markers and result in DNA damage.[81] However, the burden of nicotine on wound healing is variable and may be influenced by its dose and time of delivery.[52]

The microbiome plays a key role in maintaining health in the oral cavity. However, disruptions to the homeostasis that exists between the beneficial bacteria and the host immune system can result in disease. In our study, the heatmap analysis between the e-cigarette and control group at week 1 demonstrated significant differences in the intensities

of metabolites that were identified as Glycerolipids. The heatmap analysis between e-cigarette and control group at week 3 demonstrated significant differences in the intensities of Glycerolipids, Glycosphingolipids, and Glycosyldiradylgycerols. The pathway analysis (KEGG map) between week 1 and 3 revealed that carbohydrate and lipid metabolism was highly significantly followed in e-cigarette users while synthesis of secondary metabolites was higher in the control group. A study by Park et al. found that, in subgingival sites exposed to e-liquids, there was a downregulation in the biosynthesis of amino acids, carbohydrates, and aerobic processes.[69] This study also found that subgingival sites in e-cigarette users exhibited an increase in metabolic pathways for obtaining energy observed in anoxic niches (for example, fermentation) and increased anaerobic metabolism in e-cigarette users.[69] Our findings are in line with what has been discovered previously by our group. Ganesan et al. revealed that the changes that appeared in the microbiome and the differences in bacterial biofilm production and architecture are more likely to be caused by the glycerol and propylene glycol present in e-liquids and not by nicotine.[40] As a result, these sugar alcohols can become a source of nutrients for bacteria.[40,53] This means that the microbiome of e-cigarette users has a higher capacity for carbohydrate metabolism. Here we are showing that, indeed, this capacity translates to action, and that these bacteria do metabolize carbohydrates. We believe that the vehicle in e-cigarettes (PG, VG) is metabolized by bacteria to large carbohydrate molecules, which then alters gene expression and covers bacteria in a capsular layer. This change in microbial phenotype causes an increased inflammatory response in the host. The next step for us is to quantify inflammation through cytokine analysis.

Immunohistochemical analyses of oral epithelia are scarce. Filaggrin is a protein localized in the stratum corneum of the skin. As in the epidermis, filaggrin is formed from profilaggrin in keratohyalin granules in the stratum granulosum of orthokeratinized oral mucosa, particularly in the hard palate and portions of the gingiva.[82] In an immunohistochemical analysis by de Benedetto et al., filaggrin is expressed in the healthy epithelium of skin and oral mucosa, thereby playing a role in barrier function at these sites.[83] Vimentin is a type III intermediate filament protein of mesenchymal cells. Evidence implicates its role in regulation of various cellular functions including cell attachment, migration, cell signaling, and tumor metastases.[84] Keratins are the predominant cytoskeletal component of stratified keratinizing epithelium.[85] To give an example of epithelium alterations after e-liquid exposure, a study by Lungova et al. found that cell adherens junctions and K13 expression in the luminal cell layers of the vocal fold epithelium were affected after exposure to 5% e-liquid.[86] In the present study, scores for filaggrin, vimentin, and keratin were significantly lower in e-cigarette users compared with control subjects at 1 and 3 weeks of wound healing. This information sheds light on the impact of e-cigarettes on hypokeratinization of palatal wounds.

The present study is not without limitations. One limitation is the sample size, which does not allow us to draw sound conclusions; however, this is a pilot investigation that allowed us to evaluate the feasibility of a larger scale study. A second limitation was the lack of standardization of the depth of the biopsies. For all biopsies, a 1.5 mm depth was attempted; however, this may be better controlled by using a colored tape to mark the 1.5 mm depth, and therefore, obtain heterogenous biopsy thicknesses. Another limitation

was the lack of control of light or heavy e-cigarette use. This can be further improved with the implementation of questionnaires aimed to document the amount of vaping use, time that the subject has been vaping, the type of device and e-liquids used, as well as other concomitant habits such as drinking, cigarette smoking, or use of recreational drugs. E-cigarette use in test subjects could not be monitored with the use of a special phone application as expected due to technical difficulties. However, the same e-liquids and devices were provided to the participants and were instructed to use these products until the end of the study. Quantification methods of e-liquid usage can be better implemented in future studies as well. Finally, cytokine analysis was not measured for the purposes of the present thesis. However, this analysis will further strengthen the results obtained from immunohistochemistry and metabolomic analyses. Strengths of the present study include the gender matched-group design, age-restricted participation, the exclusion of cigarette smokers, and the inclusion of immunohistochemistry assessment.

Chapter 5. Conclusions

Within the limitations of the present study, e-cigarettes may pose a significant risk to postoperative oral wound healing, affecting keratinization of epithelium, and altering the metabolic profile of the oral microbiome. Within this context, e-cigarettes are not safer than conventional cigarette smoking.

Further studies are required to identify specific metabolites and pathways that may be used as markers in personalized risk assessment strategies. This study highlights the urgent need for additional research regarding the impact of e-cigarettes in oral wound healing at a larger scale, as well as the need to increase awareness of complications related to delayed wound healing, to make clinicians and patients more observant toward e-cigarette initiation or cessation.

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Appendix A.

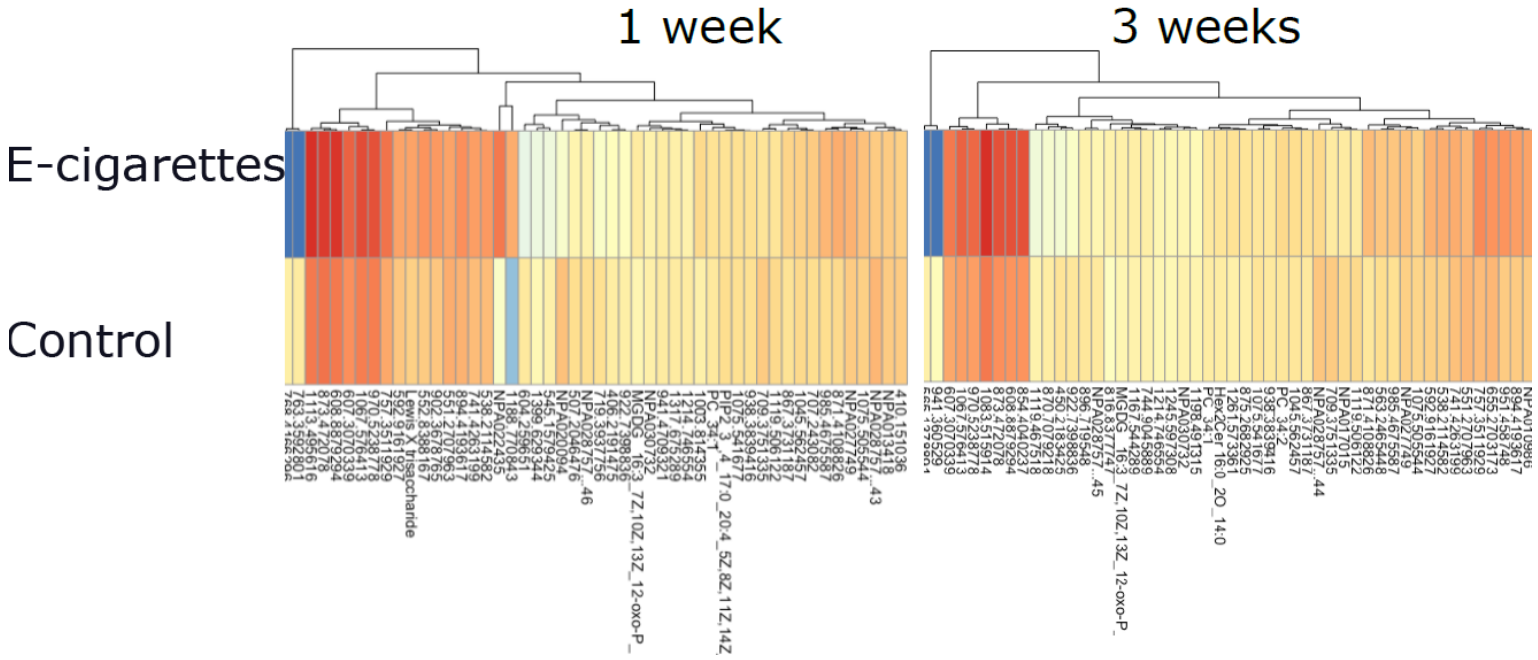

Figure 15. Heatmap analysis between e-cigarette users and control subjects.

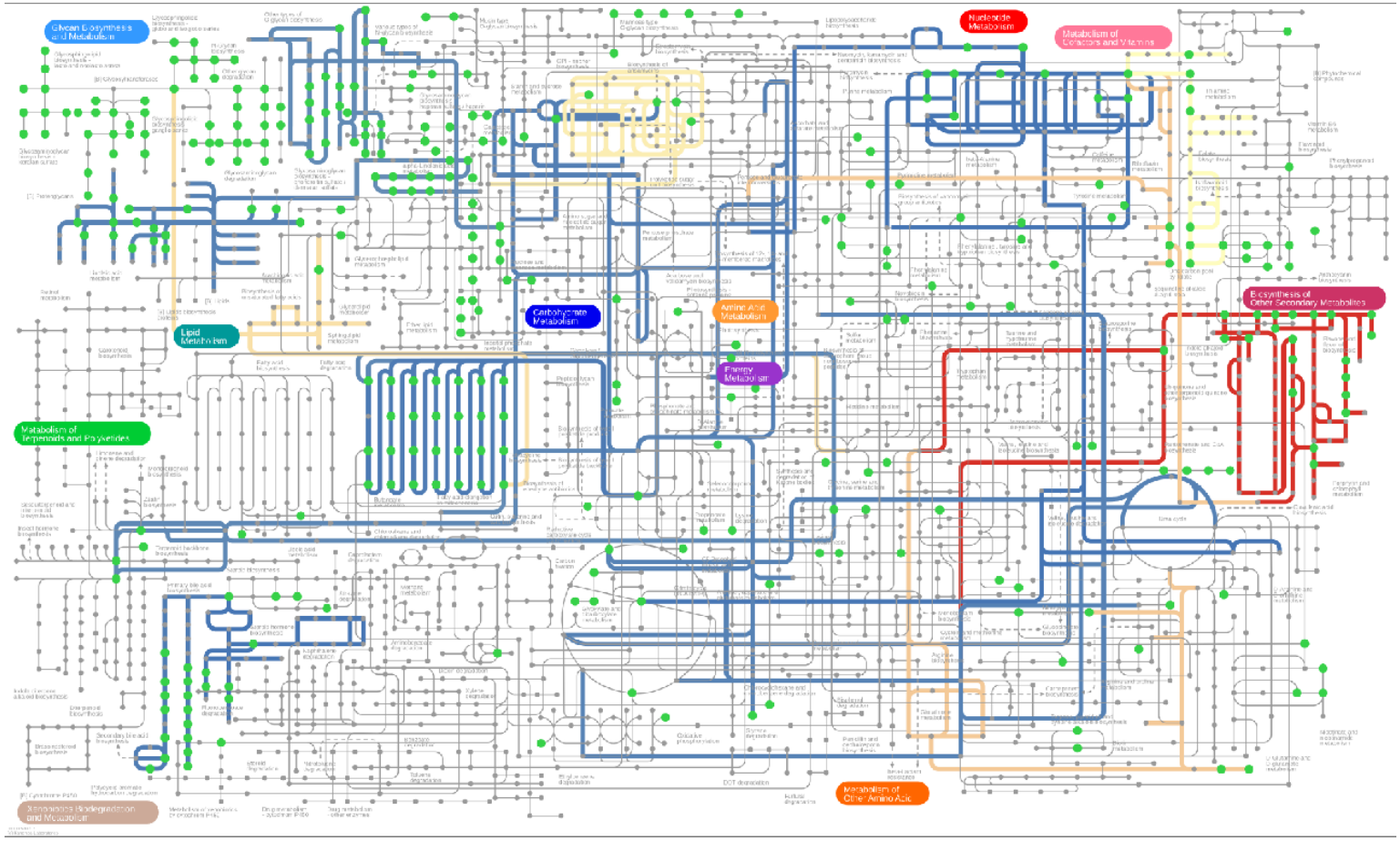

Figure 16A. Metabolic pathway map at week 1 between e-cigarette users and control subjects.

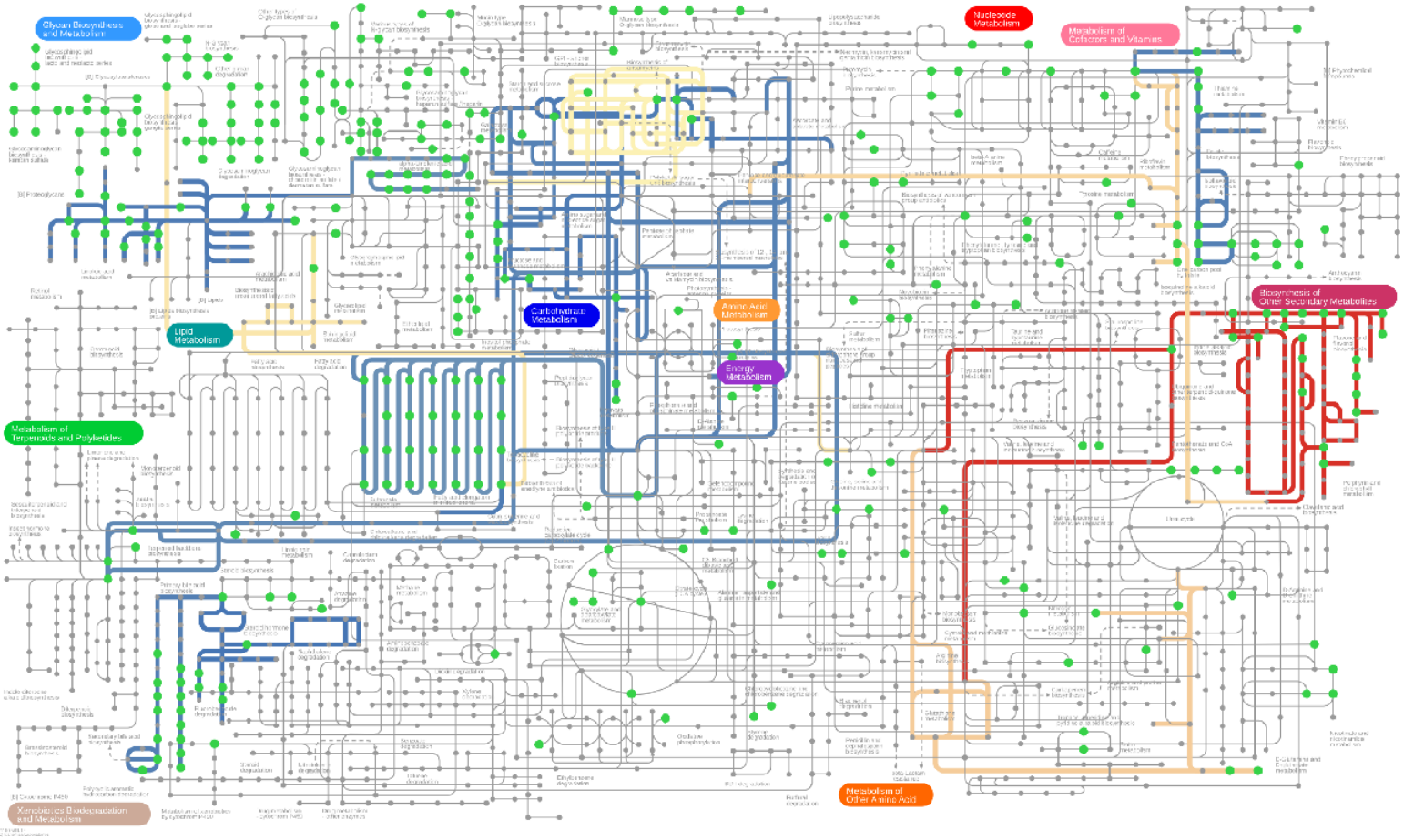

Figure 16B. Metabolic pathway map at week 3 between e-cigarette users and control subjects.