THE ROLE OF THE ORAL SPIROCHETE
TREPONEMA DENTICOLA IN PERIODONTITIS AND
ORODIGESTIVE CARCINOGENESIS

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1. LIST OF ORIGINAL PUBLICATIONS

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2. ABBREVIATIONS

AEC 3-amino-9-ethylcarbazole
CD68 Cluster of Differentiation 68 (monocytes, macrophage)
CD8 Cytotoxic T cell
CTLP Chymotrypsin-like proteinase
DAMP Danger-associated molecular pattern
DNA Deoxyribonucleic acid
EDTA Ethylenediaminetetraacetic acid
EMT Epithelial to mesenchymal transition
ERK1/2 Extracellular signal-regulated kinase ½
GEC Gingival epithelial cell
GERD Gastroesophageal reflux disease
HMGB1 High mobility group 1
HPV Human papillomavirus
hβD Human β-defensin
Ig Immunoglobulin
IL Interleukin
LPS Lipopolysaccharides
MAPK Mitogen-activated protein kinase
MMP Matrix metalloproteinase
Msp The major outer sheath protein
MTSCC Mobile tongue squamous cell carcinoma
NF-κB Nuclear factor kappa B
OSCC Oral squamous cell carcinoma
PAMP Pathogen-associated molecular pattern
PD-L Programmed death-ligand
PGE Prostaglandin E
PRR Pattern recognition receptor
RANKL Receptor activator of nuclear factor kappa-B ligand
RNI Reactive nitrogen intermediates
ROS Reactive oxygen species
RT Room temperature
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT3 Signal transducer and activator of transcription 3
Th17 T-helper 17
TIMP Tissue Inhibitors of metalloproteinase
TLR Toll-like receptor
TNF-α Tumor necrosis factor-α
TUNEL Terminal TdT-mediated dUTP-biotin nick end labeling
α-1-AC Alpha-1-antichymotrypsin
3. ABSTRACT

Periodontitis is an inflammatory disease of the tooth supporting tissue, including gingiva, bone and periodontal ligament structures. Chronic periodontitis is highly prevalent in adult population and it has been linked to various systemic diseases, such as cardiovascular diseases. Certain members of the oral microbiome, the so called periodontopathogens play a major role in its pathophysiology.

Chronic inflammatory conditions can be considered to be predisposing factors for cancer. Periodontitis have been associated with elevated oral and gastrointestinal cancer risk and mortality. However, the mechanisms are still largely unknown.

Periodontopathogen and spirochete *Treponema denticola* (*T. denticola*) possesses plethora of enzymes, such as chymotrypsin-like protease (CTLP), that facilitate its invasion into adjacent tissues and integration into microbial communities.

The first study investigated the role of *T. denticola* in chronic periodontal inflammation. The study focused on evaluating *T. denticola*–induced cell death and associated danger signals and key inflammatory cytokines. The analysis compared the occurrence of these factors in patients with either gingivitis, periodontitis or in health using immunostaining approaches. In the second study, the presence of CTLP was evaluated in various oral and gastrointestinal cancers, including oral, tonsillar, esophageal, gastric, pancreatic and colon cancer. In addition, the presence of CTLP in the non-digestive tract cancer tissues including lung, breast and thyroid were analysed. The immunomodulatory actions of CTLP were investigated under laboratory conditions. In third study, CTLP was compared to several major clinical parameters such as depth of invasion and cancer size. The tissue analyses were carried out using immunohistochemistry.

The results show that *T. denticola* induces and contributes to apoptosis via endogenous danger signals released from the late apoptotic cells. *T. denticola* CTLP was found in several cancer tissues in the orodigestive tract, but not in non-digestive tract cancer tissues. CTLP was able to activate key immunomodulatory mediators for tumor microenvironment such as pro-matrix metalloproteinases (proMMPs). The presence of CTLP in tongue
cancer correlates with a number of key clinical aspects such as the degree of tumor invasion and size. In addition, high immunopositivity of CTLP was associated with the early cancer relapse in tongue cancer patients.

This study helps to understand the mechanisms underlying *T. denticola* induced chronic inflammation in periodontal tissues. In addition, the study shows that the key virulence factor of *T. denticola* CTLP is present in multiple different orodigestive cancers. The results also elucidate potential actions CTLP possess in cancer microenvironment. These findings underline the fact that prevention and early diagnosis of periodontitis is not only vital for the oral but also for the general health.
4. INTRODUCTION

Periodontitis is one of the most common oral chronic inflammatory diseases that can cause loss of both soft and hard tissue supporting tooth, and eventually lead to tooth loss. A dysbiotic oral microbiome in conjunction with an aberrant host response leads to periodontitis (Hajishengallis, 2014). Periodontitis has been linked to various chronic systemic diseases including cancers (Fitzpatrick et al., 2010; Hajishengallis 2015; Nazir 2017). However, the exact mechanism underlying these associations has remained unclear. Causality needs to be clarified to have a better understanding of both pathogenesis and treatment.

Recent reports have linked various members of the oral microbiota with cancers in the orodigestive organs. Bacteria are thought to contribute to oral carcinogenesis through different pathways e.g. the regulation of apoptosis, proliferative signaling, cell migration and invasion, inflammatory processes, and the production of carcinogenic metabolites (Hoppe et al., 2016; Perera et al., 2016; Gholizadeh et al., 2016). The chronic inflammation induced by periodontal pathogens could activate the normal cells to undergo uncontrolled growth or it could induce mutations as well as potentiating carcinogenesis. Periodontal pathogens also can contribute in a direct manner to carcinogenic transformations (Meyer et al., 2008).

*T. denticola* is a periodontal pathogen that has been associated with a severe and aggressive form of periodontitis (Meyer-Bäumer et al., 2014). It is a highly invasive, motile microorganism, and it is an obligate anaerobe. It possesses several virulence factors with immunomodulatory and tissue destructive potential. Chymotrypsin-like proteinase (CTLP) is one of the key virulence factors possessed by *T. denticola*. *T. denticola* CTLP can degrade several host proteins and hydrolyze bioactive peptides, thus enhancing the integration of *T. denticola* into a dental plaque biofilm colony and contributing to its penetration into the epithelium. (Grenier et al., 1990; Mäkinen et al., 1995; Cogoni et al., 2012). *T. denticola* can also modulate immunity and inflammation, and trigger apoptosis in various cell types (Uitto et al., 1995; Ding et al., 1996). Due to these characteristics of *T. denticola*, we hypothesized that this pathogen may be
important in contributing to chronic inflammatory periodontitis and in orodigestive carcinoma progression, especially in mobile tongue squamous cell carcinoma.

Several studies have addressed the role of other oral pathogens in cancer i.e. *Fusobacterium nucleatum* (*F. nucleatum*) (Kostic et al., 2013), *Porphyromonas gingivalis* (*P. gingivalis*) (Gallimidi et al., 2015), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) (Hoppe et al., 2016), and *Tannerella forsythia* (*T. forsythia*) (Kruger et al., 2013). However, there are so far very few studies investigating the association between *T. denticola* and malignancies (Narikiyo et al., 2004; Shin et al., 2017). In our study, we examined whether *T. denticola* plays a role in orodigestive cancers.
5. REVIEW OF THE LITERATURE

5.1 Oral microbiome in health and disease

5.1.1 The healthy oral microbiome

The human mouth is a harbor for diverse microorganisms including bacteria, fungi, viruses, protozoa, and archaea. It has been shown to be one of the most complex microbial flora in the body, after the colon (Huttenhower et al., 2012). It contains more than 700 species and approximately 50% of those have not been cultivated (Paster et al., 2006). Different sites in the oral structures have displayed distinct microbial communities (Mager et al., 2003, Aas et al., 2005). Under homeostasis conditions, the oral microbial communities often maintain a relatively stable composition and exist in a commensal and mutualistic relationship with the host (Do et al., 2013). The host provides the environment while the oral microbes protect the host from invasion by pathogenic microorganisms by preventing their adherence and growth. There are few available adherence sites for pathogens since most of all mouth surfaces are colonized by normal flora. (Wade 2013; Zenobia et al., 2003).

5.1.2 Dysbiotic oral microbiome

The presence of the oral microbiota in the mouth contributes to both oral and general health. However, if the balance of the composition between the species changes, microbial homeostasis will be disrupted (a phenomenon called dysbiosis) leading to oral diseases which can also impact on general health as a consequence (Wade 2013).

The oral cavity is a main entrance to the human body for food. Oral microbes have a high probability to spread onto the adjacent epithelial of the respiratory and orodigestive tracts. Some oral microbes have been shown to cause oral infectious diseases, such as caries (Tanner et al., 2011), periodontitis (Socransky and Haffajee 1991), endodontic infections (Munso et al., 2002), and tonsillitis (Swidsinski et al. 2007). There is accumulating evidence linking oral bacteria with several systemic diseases, including cardiovascular disease (Beck et al., 2005), preterm birth (Han et al., 2004),
diabetes (Kampoo et al., 2014), pneumonia (Kikutani et al., 2014), and cancer (Börnigen et al., 2017; Heikkilä et al., 2018).

The oral microbiome has long been known to be a source of infections at other distant body sites (Han and Wang 2013). Oral bacteria can gain access to the blood circulation during tooth brushing or in dental procedures and they have been reported to be a cause of infectious endocarditis (Ross et al., 2018), as well as brain (Mueller et al., 2009) and liver abscesses (Yoneda et al, 2011; Ganju et al., 2017). In addition, it has been thought that the composition of the oral microbiome may well be related to oral and systemic diseases where it can act as either a causative agent or as a consequence of some systemic changes in the body (Wade 2013). Recently, oral microbiomic profiles paired with salivary biomarkers have been claimed to be a useful diagnostic biomarker for other diseases (AlMoharib et al., 2014; AlRowis et al., 2014; Kerr and Tribble, 2015).

The oral microbiome exists as multispecies microbial communities which grow in structurally and metabolically organized communities of interacting species termed dental plaque biofilms on mucosa and dental surface (Kolenbrander et al., 2002). An excessive accumulation of dental plaque biofilm around the gingiva will alter the normal microbial composition of the biofilm (Abusleme et al., 2013). This condition will activate an inflammatory host response leading to oral diseases, such as periodontitis (Marsh 2006; Darveau 2010; Herrero et al., 2018). A. actinomycetemcomitans, P. gingivalis, T. forsythia and T. denticola have been identified as the oral bacteria most likely to play an etiologic role in the development of periodontal disease (Pihlstrom et al., 2005; Kinane et al., 2017; Kakuta et al., 2017). T. denticola has been convincingly associated with the severity and the aggressive form of periodontal diseases including periodontitis and acute necrotizing ulcerative gingivitis (Loesche et al., 1982; Moore et al., 1991, Socransky and Haffajee 1991; Fenno and McBride, 1998; Byrne et al., 2009; Pandit et al., 2016).

5.2 Oral spirochetes

Oral spirochetes classified as treponemes are present in the oral cavity along with over 600 other bacterial species and exist as a part of a polymicrobial dental plaque biofilm
attached to the dental surface in the gingival crevice (Dewhirst et al., 2010). If there are very low numbers of treponemes present, they can be considered as a normal part of the flora, but they also can cause opportunistic infections if their numbers increase (Moter et al., 2006; You et al., 2013). Spirochetes have been implicated in several diseases which share some important characteristics, such as having both a chronic and an episodic nature, and the destructive impact of the bacterial infection triggered by the host’s immune response (Fenno and McBride, 1998; Dashper et al., 2011; Visser and Ellen, 2011).

Only four species of oral spirochetes have been widely cultivated; these consist of *Treponema pectinovorum*, *Treponema socranskii*, *Treponema vincentii*, and the most extensively characterized, *T. denticola*. There is evidence that there are at least a dozen oral spirochetes, some of which may play a role in the pathogenesis of periodontal infections, but these species are difficult to characterize due to their inability to grow (Wardle 1997). However, current advances in genomic sequencing may clarify the pathogenicity of oral spirochetes (Seshadri et al., 2004). Spirochetes represent as a dominant microbe detected in the subgingival plaque from patients with acute necrotizing ulcerative gingivitis and generalized aggressive periodontitis, while they are much less common, i.e. less than 1%, in healthy sites (Chan and McLaughlin, 2000; Abusleme et al., 2013; Han et al., 2017).

*T. denticola* is the most readily cultivated species from clinical samples and it has thus been the best characterized as compared to other spirochetes found in gingival lesion (Fenno and McBride, 1998). The complete genome sequence of *T. denticola* together with the recently developed techniques of genetic manipulation will provide an opportunity for further analysis of the genes involved in the pathogenicity linked with this microorganism (Visser and Ellen, 2011).

### 5.2.1 *Treponema denticola*

*T. denticola* is an obligate anaerobic, gram-negative spirochete bacterium which is motile, slender with a helical shape. *T. denticola* almost always exists in association with other oral microbes, including known periodontal pathogens (Söder et al., 1993; Faveri
et al., 2009). \textit{T. denticola} coaggregate with a number of these microbes with the strongest coaggregation being found between \textit{P. gingivalis} and \textit{T. denticola} in subgingival plaque samples (Simonson et al., 1992; Yao et al., 1996). In fact, it has been claimed that the existence of \textit{T. denticola} at a site of periodontitis might be presumed by the presence of \textit{P. gingivalis} (Simonson et al., 1992). Furthermore, immunohistochemical techniques have revealed that \textit{P. gingivalis} is predominant beneath \textit{T. denticola} in human subgingival plaque, but their coexistence has been found in deeper subgingival plaque (Kigure et al., 1995). These various observations have been confirmed in polymicrobial dental plaque biofilm investigations, revealing that \textit{P. gingivalis} acts as the initial colonizer and then subsequently colonization occurs by \textit{T. denticola}; their synergistic relationship is mediated by the presence of gingipains, CTLP, fimbriae, and Msp (Yamada et al., 2005). Their cooperative synergism in colonization extends to pathogenesis, i.e. their combined proteolytic activities are more effective in interfering with blood clotting (promoting bleeding) than their individual activities (Cogoni et al., 2012).

\textit{T. denticola} is believed to be one of the most important periodontal pathogens (Mineoka et al., 2008; Byrne et al., 2009) and it can also be a pathogen in root canal infections (Montagner et al., 2010).

5.2.1.1 Role in periodontal diseases/periodontitis

\textit{T. denticola} exists as the predominant spirochete in subgingival plaque (Kolenbrander et al., 2002). Since it is anaerobic, \textit{T. denticola} occupies the depth of the plaque adjacent to and directly in contact with the subgingival area (Zijinje et al., 2010). This microorganism colonizes the gingival crevice and multiplies using components from the gingival crevicular fluid as its energy source. The association of \textit{T. denticola} with \textit{P. gingivalis} and \textit{T. forsythia} forms the red complex species, which act in synergy to improve each other’s growth and enhance their virulence. Each of the species releases distinct proteolytic and other enzymes; their presence exhibits a very strong association with the severity (as measured by probing depth and the extent of bleeding) of several periodontal diseases including chronic periodontitis, acute necrotizing ulcerative
gingivitis, and also other oral diseases such as endodontic infections, and acute dental abscesses (Socransky et al., 1998).

*T. denticola* is frequently isolated from the most severe site of periodontal inflammation and its increasing level parallels the destruction of periodontal tissue (Kumawat et al., 2016). This may due to its ability to suppress the proliferation of fibroblasts as well as enhancing collagen phagocytosis by gingival fibroblasts and activating both the classic and the alternative human complement pathways (Pandit et al., 2016).

### 5.2.1.2 Virulence factors

*T. denticola* expresses several virulence factors, such as the major surface protein (Msp) (Weinberg and Holt, 1991), a cell-associated lipooligosaccharide (Grenier 2013), chymotrypsin-like protease (CTLP) also known as dentilisin (Uitto et al., 1988), peptidoglycan (Grenier and Uitto, 1993), cystalysin (Krupka et al., 2000), several peptidases (Mäkinen et al., 1995; Fenno et al., 2001), and phosphatase (Ishihara and Kuramitsu, 1995). These factors facilitate the integration of *T. denticola* within oral dental plaque biofilms as well as promoting its adherence and subsequent invasion into the host’s cells (Inagaki et al., 2016). Previous analyses have characterized some of these factors, but not all of the molecules involved in *T. denticola* pathogenicity are known because of difficulties in the isolation of these molecules from *T. denticola* (Ishihara 2010).

The adherence of human pathogens to host tissue is the critical step in tissue invasion. *T. denticola* has been shown to adhere to different sites in the oral cavity surface including teeth, host soft tissue (different type of cells and extracellular matrix), and microbes in the dental plaque biofilm although *T. denticola* does not have any specific adherence structure such as fimbriae (Fenno and McBride, 1998). This adherence capacity is facilitated by various adherence factors possessed by *T. denticola* such as Msp, lipooligosaccharide, and CTLP (Bamford et al., 2007; Edwards et al., 2005; Ishihara 2010; Grenier 2013).
*T. denticola* penetrates the epithelial cell by disrupting cellular tight junctions. This penetration is facilitated by CTLP and the outer membrane vesicle virulence factors possessed by *T. denticola*. (Chi et al., 2003). In addition, motility and chemotaxis are also involved in the tissue penetration of *T. denticola* (Lux et al., 2001).

*T. denticola* exerts cytopathic activities toward both epithelial and fibroblast cells which are representatives of periodontal tissue. The cytopathic activities were able to cause membrane damage, vacuolization, cell detachment, inhibition of proliferation, loss of tight intercellular contact and cytoskeletal rearrangements, as well as loss of volume control (Uitto et al., 1995). These activities are facilitated by the *T. denticola*’s Msp, CTLP, and peptidoglycan (Fenno et al., 1998). *T. denticola* also induces the lysis of erythrocytes by secreting hemolytic factors (Fenno and McBride 1998).

The immunomodulation activity of *T. denticola*, facilitated by its major antigenic factors including Msp and CTLP, is involved in its pathogenicity, i.e. it was capable of inducing a strong antibody response in young adult patients with localized juvenile periodontitis (aggressive periodontitis), inhibiting the chemotaxic activities of neutrophils, and suppressing the properties of human peripheral mononuclear cells and fibroblasts. *T. denticola* virulence factor also induces the production of various cytokines, including interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α), and metalloproteinase 9 (MMP-9) (Miyamoto et al., 2006; Ishihara 2010; Gaibani et al., 2010).

### 5.2.1.3 Chymotrypsin-like proteinase (CTLP)

CTLP is a major virulence factor of *T. denticola*: it is a true proteinase with similar characteristics to chymotrypsin in terms of its specificity and inhibition properties (Mäkinen et al., 1995). CTLP is found on the cell surface and has a mass of about 95 kDa (Uitto et al., 1988).

CTLP mediates the adherence and aggregation of *T. denticola* to other potential periodontal pathogens. These steps play an essential role of CTLP in the interactions of *T. denticola* with other oral microbes, leading to mutual interactions in the development of microbial communities and host tissue pathogenesis (Cogoni et al., 2012). CTLP
facilitates the spirochetes to penetrate into epithelial cells (Fenno et al., 1998; Ellen et al., 2000; Chi et al., 2003); this enzyme can degrade multiple host proteins and hydrolyze bioactive peptides (Uitto et al., 1988; Grenier et al., 1990 Mäkinen et al., 1995). CTLP also induces the activation of certain MMPs (MMP-1, MMP-2, MMP-8), thus providing another mechanism through which T. denticola can disrupt tissues and invade deeper tissue layers (Sorsa et al., 1992; Miao et al., 2011). CTLP also has been reported to modulate immune responses by degrading several inflammatory mediators including IL-1β, TNF-α, and IL-6 (Miyamoto et al., 2006) and has a broad range of cytopathic activities (Uitto et al., 1995; Fenno et al., 1998; Ellen et al., 2000). This may be the reason why T. denticola infection results in a longer duration of inflammation than many other periodontopathogens.

5.3 Periodontium

The periodontium is the tissue surrounding and supporting the tooth; it comprises gingiva, the alveolar bone that surrounds the tooth root, the periodontal ligament connecting the root of the tooth with the alveolar bone socket, and the root cementum that strengthens the attachment of periodontal ligament on the tooth root surface (Figure 1). These tissues interact dynamically and influence each other in order to perform their function. The gingiva is an adaptation of oral mucosa covering the alveolar bone. It acts as a barrier between the subgingival space and the pathogenic microbial flora of the oral cavity, and it initiates the immune response against microbial intrusion. (Nanci and Bosshardt, 2006). Gingiva consists of epithelium and the underlying connective tissue. The gingival epithelium is divided into three compartments i.e. keratinized oral epithelium which faces the oral cavity, the nonkeratinized sulcular epithelium which lines the gingival sulcus facing the tooth, and the nonkeratinized junctional epithelium which lies at the base of gingival sulcus. The junctional epithelium has an essential role in sealing off the periodontal tissues from the oral environment. (Nanci and Bosshardt, 2006).

The gingival connective tissue consists of highly organized collagen fibers (60-65 %); the other molecules are mainly glycoproteins and proteoglycans (Hassell 1993). The cellular
component is composed of fibroblast cells, inflammatory cells, blood and lymph vessels and nerves. The lamina propria (the upper connective tissue beneath the epithelium) supporting the junctional epithelium has a different structure from that supporting the oral and sulcular epithelium. The lamina propria beneath the junctional epithelium has an extensive vascularity. Inflammatory cells such as polymorphonuclear leukocytes continually extravasate from this dense vascular network and migrate through the junctional epithelium into the sulcus and the oral fluid. (Nanci and Bosshardt, 2006). Both the oral and the sulcular epithelium are supported by a similar structure of the lamina propria.

![Figure 1. Structure of periodontium illustrated with a dental plaque biofilm (modified from Servier Medical Art).](image)

### 5.4 Periodontitis

Periodontitis is the most common chronic oral infectious disease of the supporting tissues of teeth, causing tooth loss with a prevalence of 10-15% in adult populations all
around the world (Petersen and Ogawa, 2012). Dental plaque biofilm microbes are the primary etiological factors initiating inflammation and the subsequent tissue destruction is caused by an interplay between bacterial activity and the host’s immune response (Darveau, 2010).

5.4.1 Immunopathogenesis

The change from periodontal health to disease has been related to a shift from a symbiotic microbial community (mostly Gram-positive bacteria) to a dysbiotic microbial community (mostly Gram-negative bacteria) (Hajishengallis 2015). The dysbiotic oral microbiota is equipped with several virulence factors to help them counteract the inflammatory response mounted against them in oral environment. This dysbiotic microbiota are present in the dental plaque biofilm in the subgingival crevice. In this subgingival area, the host immune response attempts to maintain host-microbe homeostasis by controlling the bacterial overgrowth. The bacteria colonization in this site leads to gingival inflammation called gingivitis that can be reversed back to the healthy condition by removing the dental plaque biofilm-associated bacteria. Untreated gingivitis may lead to periodontitis, which is a more severe inflammation and associated with tissue destruction. (Hajishengallis and Lamont, 2012; Hajishengallis 2014).

In periodontitis, the host immune response is dysregulated. The dysregulation occurs either because the host immune system has been disrupted by the microbial community or because of several host-related phenomena e.g. congenital or acquired host immunodeficiencies or immunoregulatory defects (Amano et al., 2008), systemic diseases such as diabetes (Karima et al., 2005) and obesity (Virto et al., 2018), environmental factors such as smoking, diet, and stress, as well as epigenetic modifications in response to environmental changes, which alone or in combination can contribute to unfavorable disturbances in the homeostatic balance (Moutsopoulos et al., 2015). Aging is also a factor that could diminish the activity of the immune system and thus it increases the risk of periodontitis (Hajishengallis 2014). Moreover, some periodontal bacteria may exploit the host immune response mechanism, such as by
disrupting neutrophils and complement function, as a way to enhance their survival (Darveau 2010).

The accumulation of dysbiotic microbes in the subgingival area is associated with an abundance of key molecular patterns on microbial organisms (pathogen-associated molecular patterns, or PAMPs) such as lipopolysaccharides (LPS), lipoteichoic acids, peptidoglycan, mannose, and glucans that are recognized as danger signals by the immune cells (Hernandez et al., 2011; Yucel-Lindberg and Bage, 2013). Periodontopathogens, as part of the oral dysbiotic microbiome, are associated with host cell apoptosis and necrosis that can lead to excessive proinflammatory responses (Leung et al., 2002; Kelk et al., 2011). Apoptosis and necrosis have been suggested to play important roles in the progression of chronic inflammatory processes and tissue destruction in periodontitis (Bantel et al., 2005; Abuhussein et al., 2014; Tunali et al., 2014). In even clinically healthy gingiva, some apoptosis may occur in the junctional epithelium as a way of regulating mucosal inflammation in order to maintain a homeostatic balance. However, the level of apoptosis has been found to be increased in the deepest pocket of gingiva during the progression of periodontitis. (Tonetti et al., 1998; Jarnbring, et al., 2002).

Injured or stressed host cells stimulated either by bacterial invasion or trauma can also release endogenous molecules called danger-associated molecular patterns (DAMPs) to warn the immune system of danger (Jun et al., 2017). HMGB1 is an endogenous molecule which normally facilitates cell transcription but can also act as a DAMP (Yamada and Maruyama, 2007). HMGB1 is normally localized in the nucleus, but if there is a change in its nuclear localization, such as its translocation into the cytoplasm and/or release into extracellular space, it can trigger inflammation (Scaffidi et al., 2002). The release of HMGB1 has been detected mainly after infection or cell necrosis/apoptosis (Qin et al., 2006; Tang et al., 2012).

PAMPs and DAMPs as danger signals are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) which are one of the most important PRRs. TLRs 2, 3, 4 and 9 are expressed in human gingival tissue, and their expressions are higher in
periodontal patient samples than in healthy individuals (Rojo-Botello et al., 2012). The PAMP/DAMP-PRR interaction results in the production of proinflammatory cytokines and in the activation of inflammatory reactions (Pöllänen et al., 2012).

Due to the challenge posed by the presence of bacteria in the subgingival area, neutrophils are recruited even in healthy conditions as they are the first defense cells to respond to the bacterial stimulus (Wellappuli et al., 2018). Complement proteins are also involved in the immune response against bacterial infections; these proteins are secreted by cells of the immune system (Olsen et al., 2017). The complement system plays crucial roles both innate and adaptive immunity and thus makes a significant contribution to inflammatory and immune responses. Certain complement proteins can also act to recruit neutrophils. A massive accumulation of neutrophils has been found in periodontitis (Pöllänen et al., 2012). Neutrophils strive to prevent bacterial invasion into the underlying tissue, however an excess of neutrophil cells can cause periodontal tissue destruction since these immune cells release degradative enzymes such as MMPs, elastase, cathepsins and cytotoxic substances such as reactive oxygen species, leading to inflammation-mediated bone loss by inducing osteoclastic bone resorption through the expression of membrane-bound receptor activator of nuclear factor kappa-B ligand (RANKL) (Cortés-Vieyra et al., 2016).

MMPs, a group of proteolytic enzymes, and their inhibitors (tissue Inhibitors of metalloproteinases or TIMPs) are involved in the homeostasis of the extracellular matrix in healthy tissue. MMPs-TIMPs imbalance can result in tissue destruction in inflammatory diseases, e.g. in periodontitis, the expression and secretion of MMPs by the host’s immune cell are increased whereas the levels of TIMPs are reduced (Yucel-Lindberg and Bage, 2013).

Some periodontal bacteria can escape from neutrophil-mediated killing while promoting inflammation, and even cause the death of neutrophil cells, thereby contributing to the dysbiosis (Olsen and Hajishengallis, 2016). If neutrophils, as the first line of defense against microbe invasion, fail to perform their defense functions, more of the chronic inflammatory cells such as lymphocytes and macrophages will be recruited, leading to
prolonged inflammation (Cortés-Vieyra et al., 2016). The increase of chronic inflammatory infiltrates is followed by the release of several proinflammatory cytokines including IL-1, IL-6, IL-8, TNF-alpha and also prostaglandins (Noh et al., 2013). IL-8 is a potent chemoattractant cytokine and activator of neutrophils at sites of inflammation. It is released from endothelial cells, gingival fibroblasts, neutrophils, monocytes, and phagocytes (Finoti et al., 2017). IL-1β is a proinflammatory cytokine which plays a central role in the regulation of the inflammatory response (Dinarello 2011). These expressions exacerbate the inflammation resulting in even more extensive tissue destruction (Pöllänen et al., 2012).

5.4.2 Diagnosis and treatment

Chronic periodontitis is usually asymptomatic until the teeth loosen. Clinically, it is characterized by gingival swelling, bleeding on probing, gingival recession, deep gingival pockets (deeper than 3 mm), and ultimately, tooth loss. The risk of tooth loss increases significantly if the patients have 7 mm or deeper pockets around their teeth. (Khan et al., 2015).

Oral fluid-based biomarkers, gingival crevicular fluid and saliva, have been widely explored due to the noninvasive and simplicity in sample collection. Some of these biomarkers are commercially available for detecting the presence of periodontitis. Saliva-based diagnostic methods for periodontal diagnosis are promising because periodontal pathogens, host antibacterial proteins, and inflammatory marker are readily detectable in saliva. (Taba et al., 2005).

The main aim of periodontal treatment is to restore the homeostatic state in order to restore the mutually beneficial relationship between periodontal tissue and oral microbial community (Khan et al., 2015). The initial nonsurgical treatment is typically scaling and debridement to remove plaque and calculus containing dysbiotic microbes. Adjunctive antimicrobials such as oral mouthwash (chlorhexidine), systemic antibiotic treatment as well as extended-release antibiotics are sometimes used in addition to debridement. (Herrera et al., 2002). If these primary treatments are not effective in controlling the disease, periodontal surgery may be required to stop the progressive
tissue destruction and to regenerate the tissue. Periodontal surgical treatment may involve flap surgery, soft tissue, and bone grafts, or guided tissue regeneration. (Heitz-Mayfield et al., 2002).

5.4.3 Periodontitis and cancer

Furthermore, novel epidemiological and in vitro studies have linked periodontal diseases and various oral pathogens with oropharyngeal and digestive tract carcinogenesis, oral carcinoma in particular (Ahn et al., 2012; Galvão-Moreira et al., 2016). Periodontitis has been reported to increase the risk of oral leukoplakia which is thought to predispose to oral carcinoma (Maisel et al., 2012). Moreover, periodontitis was also claimed to increase the orodigestive carcinoma mortality in accordance with the severity of periodontitis (Tezal et al., 2005).

5.5 Orodigestive carcinomas

The human orodigestive tract starts from lip, oral/mouth, and continuing to the rectum and its surface is covered by some of the most rapidly renewing epithelium in the body (Vermeulen and Snippert, 2014). The human orodigestive system also contains accessory organs including the tonsils, pancreas, liver, gallbladder and biliary ducts. Orodigestive carcinomas are among some of the most frequent malignancies due to their significant cellular mass and their rapid turnover and they are responsible for around half of all cancer-related deaths (Siegel et al., 2017).

5.5.1 Oral cancer

Oral cancer, of which over 90% oral squamous cell carcinoma, involves the lips and the mouth (oral cavity) including the anterior two-thirds (the mobile part) of the tongue as defined by the International Classification of Disease 10th revision (ICD-10) C00-C09 (WHO 2016). Along with oropharyngeal cancer, it is the eight most common cancers in the world; it has been estimated globally that there were 300,400 new cases and 145,400 deaths related to this cancer in 2012, most of which occur in the developing countries (Silverman 2001; Torre et al., 2015). Thus, oral and oropharyngeal cancer becomes an important global public health problem.
Oral carcinoma is caused by a multitude of factors which vary from one population to another population. Several risk factors may act individually or interact with each other in the pathogenesis of the disease. Smoking and alcohol use cause over 80% of OSCCs (Wang et al., 2015). Dietary intake, trauma or local irritation, and oral infections are also considered as a risk factor of oral carcinoma (Pavia et al., 2006; Kusama et al., 2016; Singhvi et al., 2017). HPV has been found to be associated with oral carcinoma and is considered as a risk factor for this disease since HPV DNA has been detected in approximately 20% of oral carcinomas and 60–80% of oropharyngeal carcinomas (Rautava and Syrjänen, 2012). There is accumulated evidence also revealing an association between periodontitis and the increased risk of oral carcinoma with the putative long-term chronic inflammation postulated as the possible underlying mechanism (Meyer et al., 2008; Hayashi et al., 2010).

Surgical excision has become the main treatment for the majority of cases of oral carcinomas, especially for early-stage oral carcinoma. For more advanced carcinomas, radiotherapy and chemotherapy are employed in conjunction with surgery (Mäkitie et al., 2007). Although there have been advances in carcinoma treatment, the survival rate for patients with advanced disease is still low. Oral cavity carcinoma is associated with a high incidence of recurrence, i.e. the recurrence rate is around 32.7%, as well as with the presence of second primary carcinoma (Gonzá lez-García et al., 2008; Wang et al., 2013). This explains the majority of treatment failures (da Silva et al., 2012) and the low survival rate. The 5-year survival rate for patients with a recurrence is lower (around 30%) if compared to those without recurrence (around 80%) (Vazquez-Mahia et al., 2012; Wang et al., 2013). In addition, the low survival and poor cure rate might due to the fact that the carcinomas are mostly detected during the late stage of the disease i.e. it has been as reported that approximately up to 50% of patients already have neck metastasis at the time of diagnosis (Mäkitie et al., 2007).

Mobile tongue squamous cell carcinoma (MTSCC) is a subsite of the oral carcinomas and refers to the anterior two thirds of the tongue as defined by ICD-10 C02 (WHO 2016). If carcinoma arises in the posterior third or in the base of the tongue, it is then classified as an oropharyngeal carcinoma. MTSCC is the most common type, accounting for 25-
40% of oral carcinomas in most countries (Moore et al., 2008; Bello et al., 2010); it has been reported to have one of the poorest prognosis of all the squamous cell carcinomas affecting the oral cavity (Rusthoven et al., 2008). In Finland, the incidence of MTSCC has increased from 31 new cancer cases in 1953 to 152 in 2015 (Finnish Cancer Registry).

HPV infection does not seem to play a significant role in MTSCC (Mirghani et al., 2015; Sgaramella et al., 2015). In addition to the other identified risk factors for oral carcinomas described above, poor oral hygiene has been associated with the extensive presence of oral bacteria and claimed to be both an independent risk for and to be associated with MTSSC and other OSCCs (Subapriya et al., 2007; Zhao et al., 2017). Therefore, more additional research should be conducted to specify which oral bacteria are associated with MTSCC

5.5.2 Tonsil cancer

Most of the tonsil cancers are squamous cell carcinomas. Tonsillar squamous cell carcinoma is the most common type (more than 75%) of oropharyngeal cancer with smoking and alcohol consumption as its risk factors (Kuo et al., 2013). The survival rate of the tonsillar squamous cell carcinoma is around 69% (Jones et al., 2003). Many patients with tonsillar malignancy present with advanced disease because early lesions are generally asymptomatic when small. The lack of symptoms is responsible for 67-77% patients presenting with carcinomas larger than 2 cm and often with regional node metastasis (Mallik et al., 2013).

HPV infection has been reported to be a major cause of tonsillar squamous cell carcinoma (Gillison et al., 2000). There is one report of an increase in the percentage of the HPV positive infections in tonsillar squamous cell carcinoma patients from 23.2% in 2005-2007 to 58.6% in 2010-2012 (Psychogios et al., 2014). The majority (80.2%) of HPV-positive patients present with early T-category (T1 T2) carcinoma but most of these (79%) have also lymph node metastases. However, HPV positive patients showed a better survival as compared to their HPV negative patients (Psychogios et al., 2014).
5.5.3 Esophageal cancer

Esophageal cancer is the eighth most common cancer in the world with approximately 456,000 new cases and 400,000 deaths recorded in 2012 (Ferlay et al., 2010). The incidence of esophageal cancer was 70/100,000 in 2008. In global terms, the majority of esophageal cancers are squamous cell carcinomas, but in some countries including Finland, United Kingdom, United States, France, and Australia, the incidence of esophageal adenocarcinoma has predominated (Parkin et al., 2005, Eslick 2009). The 5-year survival rate for esophageal cancer varies from 10% to 16% (Parkin et al., 2006).

The risk factors of esophageal squamous cell carcinomas are very similar to oral squamous cell carcinomas i.e. smoking, alcohol, betel nuts and nutritional deficiencies of vitamins and minerals (Lagergren et al., 2017). The most important risk factor for esophageal adenocarcinoma is Barrett’s esophagus as a serious complication of gastroesophageal reflux disease (GERD), with the minor risk factors include obesity, smoking and hiatal hernia (Huang and Yu, 2016).

5.5.4 Gastric cancer

Gastric cancer is still the fourth most common cancer and the third most common cause of cancer death; it has a poor survival outcome with only 22% of individuals surviving 5-years after diagnosis (Herszényi and Tulassay, 2010).

*Helicobacter pylori* (*H. pylori*) infection is the main risk factor for distal gastric carcinoma with about 90% of new cases of this carcinoma worldwide being attributed to these bacteria (Plummer et al., 2015). Other risk factors are dietary characteristics such as a high intake of salted, smoked, cured or picked food, heavily grilled meat and fish, smoking, and genetic factors, whereas GERD and obesity play important roles in the development of proximal gastric carcinoma (Herszényi and Tulassay, 2010).

5.5.5 Pancreatic cancer

Pancreatic cancer is ranked as the seventh leading cause of cancer deaths and the eleventh most common cancer worldwide. It has the lowest 5-year survival rate of any
of the orodigestive cancers, with only 3% of cases surviving 5 years from diagnosis (Siegel et al., 2018). The risk factors for pancreatic carcinoma are cigarette smoking (Iodice et al., 2008), overweight during early adulthood (Chao et al., 2018), and a family history of this disease (Hu et al., 2018). A positive association has also been found with diabetes mellitus and chronic pancreatitis, although the etiological mechanisms are unclear (Ilic and Ilic, 2016).

5.5.6 Colorectal cancer

Colorectal cancer is the most frequent cancer of the orodigestive tract and the third most common cancer occurring worldwide and the second most common cause of cancer mortality (Arnold et al., 2017). The main risk factors for colorectal cancer include diets low in vegetables and putatively those high in processed meat and fat; excess body weight, lack of exercise, possibly drinking excess alcohol, smoking, and inherited factors such familial adenomatous polyposis, hereditary non-polyposis colorectal cancer, “cancer family” syndrome, and also inflammatory bowel disease (Marley and Nan, 2016).

5.6 The oral microbiome in orodigestive carcinogenesis

In the year 2002, around 17.8% of the global cancer burden was infection attributable cancer (Parkin 2006). The association between poor oral hygiene or certain microbes in the oral cavity and various types of carcinomas, especially orodigestive carcinoma, is becoming increasingly better recognized (Meurman 2010).

A variety of oral microorganisms have been associated with the development and progression of cancers, including *P. gingivalis, F. nucleatum, Epstein -Barr virus (EBV)* and HPV as well as fungi, such as *C. albicans* (Fukayama et al., 2008; Kostic et al., 2013; Ramirez-Garcia et al., 2014; Tulay and Serakinci, 2016; Geng et al., 2017. *P. gingivalis* has been recently linked to pancreatic and *F. nucleatum* to colorectal carcinomas (Ahn et al., 2012).
5.6.1 Epidemiology

*H. pylori* was the first bacterial species that was officially recognized by the WHO as being a causative agent of cancer in humans (IARC 1994). A prominent association has been reported between *P. gingivalis* infection and pancreatic carcinoma. There are reports that the risk of pancreatic cancer and orodigestive cancer mortality is increased in patients with a high level of antibodies against *P. gingivalis* (Michaud 2013; Ahn et al., 2012). A recent review reported that significant numbers of *F. nucleatum* are present in colorectal cancer tissue and their levels are associated with metastases in the nodes (Shang and Liu, 2018).

Periodontitis and tooth loss have been associated with the risk of orodigestive carcinomas, particularly oral, esophageal, pancreatic, and gastric carcinomas (Abnet et al., 2005; Michaud 2013). Some oral microbes can reach the orodigestive tract following food ingestion or gain access to the bloodstream due to tooth-brushing, dental flossing, and dental extraction (Iwai 2009). This association also suggests that systemic (not only local) mechanisms may be contributing to oral microbe-mediated cancer (Ahn et al., 2012).

5.6.2 Mechanistic insights

Many mechanisms have been proposed to explain how microbes cause cancer; via inhibition of apoptosis, activation of cell proliferation, promotion of cellular invasion, induction of chronic inflammation, and the production of carcinogens.

5.6.2.1 Apoptosis

Several infections, particularly those in which the pathogen becomes intracellular, have been associated with the suppression of cellular apoptosis through the modulation of the expression of the B-cell lymphoma 2 (Bcl-2) family proteins that regulate cell survival and death, or inactivation of the retinoblastoma protein (Lax and Thomas, 2002). By blocking apoptosis, intracellular bacterial could survive by escaping the surveillance from the immune system which attempts to eliminate them by triggering apoptosis of infected cells. The overexpression of cyclooxygenase-2 (COX-2), an enzyme responsible
for inflammation, has also been shown to inhibit apoptosis (Choi et al., 2005). *P. gingivalis* is strongly anti-apoptotic e.g. it was demonstrated to block chemically induced apoptosis in primary cultures of gingival epithelial cells (GECs) mediated by various mechanisms (Mao et al., 2007). Caspase-3 is an enzyme which regulates inflammation and apoptotic signaling networks; it is widely used as an indicator of apoptosis in image-based assays. The activation of caspase-3 represents a critical step in the pathways leading to the biochemical and morphological changes that underlie apoptosis. (Tawa et al., 2004).

### 5.6.2.2 Proliferative signaling

Chronic infections induce cell proliferation; inflammation-induced cell proliferation is associated with DNA damage that leads to the formation of mutations (Kiraly et al., 2015). Mutations in crucial genes increase the incidence of cell transformation and the rate of cancer development. In addition to its anti-apoptotic properties, *P. gingivalis* accelerates the progression of the cell cycle in gingival epithelial cells. *F. nucleatum* also promotes cell proliferation in human epithelial cells (Whitmore and Lamont, 2014).

### 5.6.2.3 Cancer cell migration and invasion

*P. gingivalis* and *F. nucleatum* facilitate oral squamous cell carcinoma cellular invasion. It has been reported that a *P. gingivalis* infection can induce OSCC cells to produce pro-MMP-9 and furthermore bacterial gingipains (a protease secreted by *P. gingivalis*) activate this enzyme (Inaba et al., 2014). The presence of MMP-9 can promote carcinoma cell migration and invasion since this enzyme degrades the basement membrane and the extracellular matrix, thus allowing carcinoma cells to reach the blood and lymphatic circulations (Gialeli et al., 2011). Prolonged repeated exposure of *P. gingivalis* can increase the aggressiveness of OSCC by triggering epithelial to mesenchymal transition (EMT), acquisition of cancer stemness, as well as enhancing the production of MMP-1 and MMP-10 (Ha et al., 2015). In a similar manner, *F. nucleatum* exposure increases the production of MMP-2, -9, and MMP-13 from epithelial cell and those MMPs have been claimed to play a role in cancer cell migration and invasion (Gursoy et al., 2008).
5.6.2.4 Inflammation pathway

Chronic inflammation has long been proposed as a factor that contributes to cancer development (Elinav et al., 2013). Chronic inflammation, either generated by infectious or non-infectious factors, plays an essential role in many phases of carcinogenesis e.g. induction, progression, invasion and metastasis (Trinchieri 2012). Reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), and cytokines produced during inflammation processes are thought to contribute to the initiation of cancer by inducing mutations, genomic instability and epigenetic alterations (Perera et al., 2016). Inflammatory cytokines activate a key transcription factor i.e. nuclear factor kappa B (NF-kB) (as a master commander acting as cancer promoter) and thus control the transcription of DNA, cell survival, as well as evoking several cellular alterations associated with more aggressive phenotypes in the premalignant cells (Allavena et al., 2008). This, in turn, supports pro-malignant progressions including uncontrolled proliferation, resistance to apoptotic signals, angiogenesis, invasion, and metastasis (Perera et al., 2016).

Chronic inflammation has been considered as one of the potential pathways by which bacteria contribute to oral carcinogenesis (Hooper et al., 2009). This represents a rational explanation for the strong association between periodontitis and the higher risk of oral carcinoma. A pro-inflammatory involvement has been demonstrated for some oral bacterial species. F. nucleatum has been associated with an increase in the levels of inflammatory cytokines that generate cancer cell proliferation and the creation of a proinflammatory microenvironment which supports cancer progression (Shang and Liu, 2018). In gingival epithelial cells as well OSCC cell lines, P. gingivalis upregulates the expression of programmed death-ligand 1 (PD-L1) and PD-L2 receptors which confer protection on the cancer cells from immune attack, while in human oral mucosa, P. gingivalis infection increases the productions of IL-1, IL-6, IL-8 and TNF-α, all of which are known to contribute to chronic inflammation (Perera et al., 2016). PD-L1 expression drives the development of regulatory T cells (Treg), which suppresses the effector T cells contributing to immune evasion by oral cancer cells (Whitmore and Lamont, 2014).
5.6.2.5 Carcinogenic metabolites

Ethanol is not a carcinogen but its metabolite, acetaldehyde, is known to be carcinogenic (IARC 2012; Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment 2016). Acetaldehyde is associated with alcohol consumption (present in alcoholic beverages and food); it can cause sister chromatid exchanges, point mutations, DNA adducts and hyperproliferation of the epithelium (Nieminen and Salaspuro 2018). Certain oral bacteria and yeast such as Candida spp. (which are major human fungal pathogens) have the enzyme alcohol dehydrogenase (ADH). Under aerobic or microaerophilic conditions, this enzyme catalyses the production of acetaldehyde (Homann et al., 2000). Thus, these oral microbiomes play a major role in accumulation of salivary acetaldehyde (Nieminen and Salaspuro, 2018). The use of local antimicrobial mouthwash, chlorhexidine, reduced the mutagenic levels of salivary acetaldehyde (Homann et al., 1997). In addition to alcohol, tobacco is a major source of salivary acetaldehyde. Smoking can potentiate the production of acetaldehyde from alcohol by oral bacteria; these represent alcohol-tobacco interactions which are important in carcinogenesis (Ahn et al., 2012). Furthermore, oral bacteria may also play a role in amplifying the activation of the carcinogenic nitrosamines originating from tobacco smoking. These factors may be involved in the potential role of oral bacteria as factors involved in the process of orodigestive carcinogenesis.
6. **AIMS OF THE STUDY**

The main goal of this study was to investigate the role of *T. denticola* in maintaining chronic inflammation in periodontitis and to examine its role in carcinogenesis. To achieve the main goal, we divided the study into three specific aims:

1) To study *T. denticola* infection-induced apoptosis in periodontal tissue and its possible role in the amplification of the inflammatory processes mediated by endogenous danger signaling.

2) To investigate the presence and the role of *T. denticola* CTLP in various orodigestive carcinomas.

3) To determine the presence of *T. denticola* in mobile tongue squamous cell carcinoma (MTSCC) and its association with the clinicopathological characteristics of the patients.
7. MATERIAL AND METHODS

7.1 Study design

This study has been divided into three parts as shown in Figure 2. At first, we investigated the role of *T. denticola* in different conditions in nonmalignant oral tissue i.e. gingival tissue from periodontitis and gingivitis patients and healthy controls. We observed apoptosis and the markers associated with chronical inflammatory process in those tissues (I). Secondly, we investigated the presence of *T. denticola* and its CTLP in orodigestive carcinoma tissues and analyzed the immunomodulatory activity of *T. denticola* CTLP on a protein critical for creating a cancer microenvironment (II). Finally, we correlated the presence of *T. denticola*-CTLP in MTSCC tissues with the clinicopathological characteristics of the patients (III).
Figure 2. Study design used for the dissertation.
7.2 Material

7.2.1 Patients and tissue samples

Study I examined the gingival tissue from Finnish and Indonesian patients suffering from periodontitis (n=10, 9 Finnish and 1 Indonesian), gingivitis (n=5, 5 Indonesian), and also healthy control patients (n=10, 10 Finnish) with informed consent and with ethical approval given by the Local Ethical Committee of the Faculty of Dentistry Ref no.630a/KKEP/FGK-UGM/EC/2014, Universitas Gadjah Mada, Indonesia and the Ethical Committee of the Institutes of Dentistry, University of Helsinki (Järvensivu et al., 2004). The periodontitis tissue samples were obtained from patients with moderate to severe generalized adult type chronic periodontitis as diagnosed by a clinical assessment of pocket depths, loss of attachment, bone loss, and bleeding on probing. The patients had radiographic alveolar bone loss of 20%–50% in many teeth and loss of attachments between 4 to 6 mm. The patients did not receive any antimicrobial therapy or professional periodontal treatment of the sampling area prior to the operation (Rautemaa et al., 2004). Gingivitis was defined clinically as the occurrence of redness, swelling of gingiva, and bleeding on probing (Gölz et al., 2014). Gingivitis samples for this study were obtained from patients with gingival index <2 and probing depth ≤4 mm, without supporting tissue destruction and recovered during gingivectomy in the case of gingival enlargement in incisor, canine, or premolar sites.

We took a periodontitis-affected gingival tissue sample during flap surgery and gingivitis during gingivectomy. Healthy control tissue from clinically non-inflamed gingiva were taken during odontectomy of a fully embedded third molar. Although there were no clinically apparent signs of inflammation or pericoronitis, we cannot fully exclude the fact that some control tissues might have been histologically slightly inflamed, as shown by the presence of some inflammatory cell infiltrates in the lamina propria (Al-samadi et al., 2014). The tissue sections were evaluated by an oral pathologist. The periodontitis tissue samples contained the oral, sulcular, and junctional epithelium and lamina propria beneath the epithelium. Gingivitis tissue samples contained the oral and sulcular epithelium and the lamina propria, while healthy control tissues only contained oral
epithelium and the lamina propria since it is impossible to have the sulcular epithelium in this healthy tissue. Thus, in study I, we used sulcular epithelium and its lamina propria to compare between periodontitis and gingivitis; for the healthy tissues, we used its oral epithelium and its lamina propria. Additionally, 5 periodontitis-affected gingival samples contained dental plaque biofilm adjacent to the tissue and the immunoexpression of \textit{T. denticola} CTLP in this plaque was evaluated in addition to the gingival tissue. However, the biopsy samples were carefully selected according to specific criteria followed by an evaluation based on histological findings and the sample size fulfilled the minimal size to allow it to be statistically compared with One-way ANOVA or t-test. All of the patients involved in this study (I, II, III) are presented in Table 1.

Table 1. Patients involved in the study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Age; mean (years)</th>
<th>Sex (M/F)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>10</td>
<td>22-34; 27,4</td>
<td>4/6</td>
<td>I</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>5</td>
<td>19-23; 20,4</td>
<td>4/1</td>
<td>I</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>10</td>
<td>38-76; 56</td>
<td>5/5</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Tongue squamous cell carcinoma</td>
<td>60</td>
<td>23–95; 60,6</td>
<td>30/30</td>
<td>II, III</td>
</tr>
<tr>
<td>Tonsil squamous cell carcinoma</td>
<td>25</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Esophageal squamous cell carcinoma</td>
<td>3</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Gastric adenocarcinoma</td>
<td>32</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>6</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>54</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Mammary ductal carcinoma</td>
<td>1</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma</td>
<td>1</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Thyroid tumor tissues</td>
<td></td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Follicular thyroid carcinoma</td>
<td>7</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Papillary thyroid carcinoma</td>
<td>8</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Medullary thyroid carcinoma</td>
<td>7</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Follicular thyroid adenoma</td>
<td>1</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>Hyperplastic thyroid tissue</td>
<td>5</td>
<td>unspecified</td>
<td>unspecified</td>
<td>II</td>
</tr>
<tr>
<td>(struma nodules)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For study II, we used orodigestive carcinoma subjects consisted patients with mobile tongue squamous cell carcinoma, tonsil squamous cell carcinoma, esophageal squamous cell carcinoma, gastric adenocarcinoma, pancreatic adenocarcinoma, and
colon adenocarcinoma. Non-orodigestive tumor tissues including mammary ductal carcinoma, squamous cell carcinoma of the lung, and various types of thyroid tumor tissues consist of follicular thyroid carcinoma, papillary thyroid carcinoma, medullary thyroid carcinoma, follicular thyroid adenoma, hyperplastic thyroid tissue were also used for comparisons. The number of the orodigestive carcinoma and non-orodigestive tumor tissues varied, depending on the availability of samples taken during the time course of the study.

In study III, we focused on the MTSCC patients and used the same samples as examined previously by Keski-Säntti et al. (2007). The characteristic and clinicopathological data of MTSCC patients used in study III have been previously described in more detail (Keski-Säntti et al., 2007). In summary, clinicopathological data of 141 patients diagnosed with MTSCC and treated in University of Helsinki Central Hospital, Finland from 1992 until 2002 were reviewed. The inclusion criteria included a clinical diagnosis of stage T1/T2N0M0, original histopathological data and clinically followed-up data for a minimum of 24 months or until death available for review. Altogether, 73 patients fulfilled the inclusion criteria. Carcinoma tissues from 60 patients with MTSCC (24 patients of T1N0M0 and 36 patients of T2N0M0) were available for immunohistochemistry. All the patients were treated with curative intent. The neck lymph node status of the patients was assessed using different combinations of clinical and radiological approaches, i.e. the assessment consisted of palpation, computed tomography (CT), ultrasonography, magnetic resonance imaging and fine-needle aspiration biopsy (microscopically). After the histopathological evaluation, 40 patients’ tissue samples were classified as pT1 and 20 as pT2. All patients’ primary carcinomas were resected. Furthermore, 37 patients underwent elective neck dissection. The demographic and clinicopathological data of the 60 MTSCC patients are presented in Table 2.

The tissues from each patient (used in study II and III) were prepared in a tissue microarray block (TMA) with 1 mm diameter. Tissues and clinicopathological data of the patients were obtained from the archives of the Department of Pathology, Helsinki University Hospital. The study design complied with the Declaration of Helsinki. The use
of patient samples and follow-up information were approved by the Ethics Committee of Helsinki University Hospital (Dnro 166/E9/07) on 31st of May 2007 and by the National Supervisory Authority for Welfare and Health (Valvira) on 4th of June 2013 (Dnro 10041/06.01.03.01/2012).

Table 2. Clinicopathological characteristic of MTSCC patients

<table>
<thead>
<tr>
<th>Clinicopathological variable</th>
<th>N of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 60</td>
<td>32 (53)</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>28 (47)</td>
</tr>
<tr>
<td>Range</td>
<td>23-95</td>
</tr>
<tr>
<td>Median</td>
<td>60</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30 (50)</td>
</tr>
<tr>
<td>Female</td>
<td>30 (50)</td>
</tr>
<tr>
<td><strong>Clinical T class (mm)</strong></td>
<td></td>
</tr>
<tr>
<td>cT1 (≤ 20)</td>
<td>24 (40)</td>
</tr>
<tr>
<td>cT2 (21-40)</td>
<td>36 (60)</td>
</tr>
<tr>
<td><strong>Pathological T class (mm)</strong></td>
<td></td>
</tr>
<tr>
<td>pT1 (≤ 20)</td>
<td>40 (67)</td>
</tr>
<tr>
<td>pT2 (21-40)</td>
<td>20 (33)</td>
</tr>
<tr>
<td><strong>Pathological node positivity</strong></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>22 (37)</td>
</tr>
<tr>
<td>N+</td>
<td>15 (25)</td>
</tr>
<tr>
<td><strong>Stage (TNM)</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>34 (57)</td>
</tr>
<tr>
<td>II</td>
<td>11 (18)</td>
</tr>
<tr>
<td>III</td>
<td>12 (20)</td>
</tr>
<tr>
<td>IV</td>
<td>3 (5)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19 (32)</td>
</tr>
<tr>
<td>II</td>
<td>30 (50)</td>
</tr>
<tr>
<td>III</td>
<td>11 (18)</td>
</tr>
<tr>
<td><strong>Tumor diameter (mm)</strong></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>3-35</td>
</tr>
<tr>
<td>Mean</td>
<td>15.38</td>
</tr>
<tr>
<td><strong>Invasion depth (mm)</strong></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>18 (30)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>42 (70)</td>
</tr>
<tr>
<td>Range</td>
<td>0.5-20</td>
</tr>
<tr>
<td>Mean</td>
<td>7.17</td>
</tr>
</tbody>
</table>

T, tumor; N, nodal; c, clinical; p, pathological.
The original histological tissue sections of orodigestive carcinoma tissue and non-orodigestive tumor tissues (breast, lung, thyroid carcinoma, thyroid adenoma, and hyperplastic thyroid) were re-evaluated by an experienced pathologist. Clinical data of the patients was retrieved from medical records and the mortality data collected from Statistics Finland, the national agency for population statistics. Survival time was calculated from (i) primary surgery to death for any reason (overall survival, OS), (ii) primary surgery to death from MTSCC (disease-specific survival, DSS) and (iii) primary surgery to local, locoregional or distant recurrence (disease-free survival, DFS).

7.3 Methods

7.3.1 Immunohistochemistry (study I, II, III)

All formalin-fixed paraffin-embedded tissue (study I) and tissue microarray-block slides (study II, III) were cut into 4 μm sections. Tissue sections were deparaffinized automatically and antigens were retrieved in citrate buffer (pH 6) in a microwave oven (MicroMED T/T Mega Histoprocessing Labstation; Milestone Srl, Sorisole, Italy).

The subsequent staining method of \( T. denticola \) CTLP was as follows; endogenous peroxidase activity was inhibited with 3% \( \text{H}_2\text{O}_2 \) in methanol for 30 min; non-specific staining was inhibited with 1:50 normal goat serum in 2% BSA-PBS for 1 hour at room temperature (RT) before incubation with 1:3000 (study I) or 1:1500 (study II and III) primary rabbit polyclonal antibodies against \( T. denticola \) CTLP for 30 min at 37°C followed by overnight incubation at 4°C (Marttila et al., 2014). Since we had different tissue types and staining protocols used in study I (non-cancer and in non-TMA form) compared with study II and III (cancer and in TMA form), we performed different optimization of the \( T. denticola \) CTLP antibody concentrations. We decided that 1:3000 would be the optimal working dilution for tissues to be used in study I and 1:1500 for application in studies II and III based on the best results obtained during the optimization procedure.

Normal serum was used as provided in the Vectastain® kit (Vector Laboratories, Burlingame, CA, USA) for rabbit. Biotinylated secondary antibody (Vectastain®) was used.
at 1:200 dilutions in 0.1% BSA-PBS followed by incubation with the AB-Complex for 30 min at 37°C. The tissue was incubated in 0.006% H2O2 substrate and 0.023% 3,3’-diaminobenzidine tetrahydrochloride (DAB) chromogen for 10 min at RT (study I) or 3-amino-9-ethylcarbazole (AEC) with 0.03% hydrogen peroxidase to reveal a peroxidase-binding site for 15 min (study II, III) and the specimen was then washed in running tap water for 10 min and counterstained with Mayer’s hematoxylin for 4 min. For study II and III, the slides were mounted with Glycergel (DAKO Glostrup, Denmark) whereas in study I we used Pertex (HistoLab, Gothenburg, Sweden) after dehydration. Negative control staining was performed with non-immune species specific rabbit IgG (Vector Laboratories, Burlingame, CA, USA) and by omitting the primary antibody. Gingival tissue of periodontitis verified *T. denticola* proved positive by PCR was used as positive tissue control, while ductal carcinoma of breast cancer, squamous cell carcinoma of the lung, and various types of thyroid tumor tissues were used in comparison with the non-orodigestive tumor tissues.

The other immunohistochemistry staining procedures used in study I was performed according to a previous protocol (Al-Samadi et al., 2014) whereas those applied in study III were conducted according to another protocol (Häyry et al., 2010; Mäkinen et al., 2012; Mäkinen et al., 2015). Briefly, after antigen retrieval, the slides were incubated in 3% H2O2 in PBS for 30 minutes then incubated in 10% appropriate normal serum in 0.1% BSA-PBS 1 hour RT. After that, the sections were incubated in primary antibody diluted in 0.1% BSA-PBS overnight at 4°C. On the next day, the slides were incubated respectively with biotin-conjugated secondary antibody, avidin–biotin complex (1h RT) and the color was developed with 0.006% hydrogen peroxide substrate and DAB chromogen for 10 min RT. The tissue were counterstained with Mayer’s hematoxylin and mounted with Pertex mounting media after dehydration.

### 7.3.2 Immunofluorescence

After deparaffinization and antigen retrieval, as described above, the slides were incubated in 10% goat normal serum at RT for 1 h and in mixed primary antibodies of 4.1 μg/ml monoclonal mouse anti-human of CD68 (Dako, Glostrup, Denmark) and 1
μg/ml monoclonal rabbit anti-human of caspase-3 (Cell Signaling Technology Inc., Danvers, MA, USA), 2 μg/ml polyclonal rabbit anti-human of TLR-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 0.1 μg/ml monoclonal mouse anti-human of MCT (AbD Serotec, Oxford, UK) or 2 μg/ml monoclonal mouse anti-human of CD8 (Dako) at +4°C overnight. Fluorescein-conjugated goat anti-rabbit IgG Alexa Fluoro 568 (Life Technologies, Eugene, OR, USA) and fluorescein-conjugated goat anti-mouse IgG Alexa Fluoro 488 (Invitrogen Molecular Probe, Eugene, OR, USA) were used as secondary antibodies for 1h at RT, then incubated in 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, MO, USA) for 10 min and mounted with Prolong® gold antifade reagent (Life Technologies, Carlsbad, USA). Non-immune mouse IgG1, goat IgG, and rabbit IgG at the same concentrations were used in the negative staining controls. In this study, we used cleaved caspase-3 as an active form of caspase-3 to investigate the apoptotic cells present in the tissue. All of the antibodies used in this study are shown in Table 3.
Table 3. Antibodies used in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Concentration (μg/mL) or dilution</th>
<th>Methods</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGB1</td>
<td>Abanova, Taipei, Taiwan</td>
<td>1</td>
<td>IHC</td>
<td>I</td>
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<tr>
<td>T. denticola CTLP</td>
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<td>1:3000</td>
<td>IHC</td>
<td>I</td>
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<tr>
<td>C. albicans 158</td>
<td>Biodesign International, Saco, ME</td>
<td>0.9</td>
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<td>I</td>
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<td>TLR-4</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
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<td>I</td>
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<td>I</td>
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<td>I</td>
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<td>I</td>
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<td>MMP-9</td>
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<td>TLR-2</td>
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<td>TLR-9</td>
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<td>Snail</td>
<td>ab17731, Abcam Cambridge, UK</td>
<td>1:2000</td>
<td>IHC</td>
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</table>


7.3.3 TUNEL-assay (study I)

We confirmed apoptosis by detecting the presence of cells with death-associated DNA fragmentation in situ by Terminal TdT-mediated dUTP-biotin nick end labeling (TUNEL) as this has been considered to be a reliable indicator of apoptosis (Gamonal et al., 2001).

TUNEL method assessed the presences of death-associated DNA fragmentation in situ and was performed using the DeadEnd Colorimetric TUNEL System kit (Promega® Corporation, Fitchburg, WI, USA) according to the manufacturer’s protocol. Shortly, after deparaffinization and rehydration, the slides were pretreated by immersion in 0.85% NaCl and PBS for each 5 min. Apoptosis detection was undertaken first by immersing the slides in 4% formaldehyde in PBS for 15 min, followed by permeabilization with 20 μg/ml proteinase K solution in RT for 10 min, repeating the fixation for 5 min, and equilibrating in equilibration buffer at RT for 10 min. Next, the tissues were labelled with the TdT reaction mix and then covered with plastic coverslips (to ensure an even distribution of the mix) and incubated at 37°C for 1 h. Light exposure was avoided during this step. The reaction was stopped by immersing the slides in 2X SSC in dH2O for 15 min at RT. The endogenous peroxidases were blocked with 0.3% H2O2 in PBS for 5 min at RT. Streptavidin HRP solution 1:500 in PBS was then added to the slides which were then incubated at RT for 30 min. The color was developed by adding fresh DAB solution for 10 min. The slides were washed in PBS 5 min between the steps. The slides were then rinsed in dH2O and mounted with Glycergel mounting media.

7.3.4 Electrophoresis, immunoblot, and zymography (study II)

Some key proteins related to the cancer microenvironment and growth including MMP-8, MMP-9, TIMP-1, TIMP-2, α-1-AC, and C1q were investigated using gel electrophoresis, immunoblotting, and zymographic methods to reveal the potential role of *T. denticola* CTLP in cancer.

SDS-PAGE, Western blotting, and zymography were performed in the degradation assay. SDS-PAGE and Western blotting analysis were used to detect *T. denticola*’s CTLP capability to degrade immunomodulatory component (proMMP-8, proMMP-9, TIMP-1, TIMP-2, alpha-1-antichymotrypsin or α-1-AC, C1q) and *T. denticola*’s CTLP capability to activate proMMP-8 to degrade type I and II collagen. The same amount (400 ng) of 75 kDa human recombinant proMMP-8, of 92 kDa proMMP-9, 400 ng of 28 kDa TIMP-1, of 21 kDa TIMP-2 (R and D System, Oxon, UK), α-
1-AC (Sigma-Aldrich, MO, USA), and C1q (Abcam, Cambridge, UK) were incubated with 80 ng purified \textit{T. denticola} CTLP in 37°C for 0, 20, 40, 60 minutes. ProMMP-8 and -9 were also incubated for 60 minutes (37°C) with 1 mM organomercurial activator APMA (aminophenylmercuric acetate) (Sigma-Aldrich, MO, USA) and 25 ng human tumor-associated trypsin-2 as the positive control. The reaction was stopped by adding Laemmli’s sample buffer (Laemmli 1970) and the protein in the sample mixture was denatured by boiling for 5 minutes in 95°C hot plate and then run on 7–10% SDS-polyacrylamide gels. To visualize the size of the protein sample, the gel was then stained with Coomassie brilliant blue (Serva Electrophoresis, Heidelberg, Germany) staining. In addition, Western blotting was performed to analyse the conversion of proMMP-8 and -9 by \textit{T. denticola} CTLP. The gel was transferred to nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany). Nonspecific binding was blocked with 5% non-fat dry milk (Difco) for 90 min at 37°C. The membranes were incubated with rabbit polyclonal antibodies (1:1000 dilution) against MMPs for 3 h at 37°C followed by addition of peroxidase-conjugated goat anti-rabbit immunoglobulins (1:200-dilution; DAKO A/S, Glostrup, Denmark) for 1 h at 22°C. After washing, the blot was developed with a solution of 60 mg/ml diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl, pH 8.0, and 0.003% H2O2.

Native soluble type I or II collagen incubated with buffer or 5 μg/ml of proMMP-8 or 10 μg/ml of \textit{T. denticola} CTLP or \textit{T. denticola} CTLP-treated proMMP-8 at 22°C for 18 h. The incubations were stopped by the addition of Laemmli’s sample buffer containing 40 mM EDTA and then by immediate heating at 100°C. The specific degradation products of collagen were separated by SDS-PAGE on 10% cross-linked gels and stained with Coomassie brilliant blue (Sorsa et al., 1992).

Type I gelatin-zymographic analysis was used to investigate the effect of \textit{T. denticola} CTLP on human 92 kDa proMMP-9. \textit{T. denticola} CTLP with buffer or with 11 μM proMMP-9 was incubated for 0, 20, 40, and 60 minutes at 37°C. Gelatinase activity was assayed by zymography in 1.5-mm-thick 7.5–10% SDS-polyacrylamide gels impregnated with 1 mg/ml gelatin. The degradation of gelatin was visualized under longwave UV light. Zymography was also conducted using unlabeled gelatin as a substrate. Samples were electrophoresed without reduction. The gels were then incubated in 50 mMTris-HCl buffers, pH 7.8, containing 150 mM NaCl, 5 mM CaCl2, 1 μM ZnCl2, for 10 h at 37°C and stained with Coomassie brilliant blue (Sorsa et al., 1997).
7.3.5 Microscopy and image analysis

Immunostained sections were analysed under a Leica DM6000 B/M light microscope connected to a digital camera (DFC420 and DFC365FX, Leica Microsystems, Wetzlar, Germany). The image from three different microscope fields at 400x magnification were analysed with the ImageJ program (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA) to automatically calculate the number of positive cells. The subcellular localization of the HMGB1 was observed and highlighted using a colocalization highlighter plug-in.

The immunostained tissues were evaluated by two independent observers with a single blind method. A strong interobserver agreement ($\kappa = 0.818$, $p < 0.0005$) of *T. denticola* CTLP immunopositivity scoring was revealed by Cohen kappa analysis. The percentage of the positive *T. denticola* CTLP, c-Myc, Ki-67, BMI, and SNAIL on the cancer cells was graded as in previous studies (Häyry et al., 2010; Mäkinen et al., 2012; Mäkinen et al., 2015) as 0 (negative), 1 (low, up to 29%), 2 (moderate, 30-49%), 3 (high, 50-80%), and 4 (strong, over 80%). The MMP-8, MMP-9, and TLR-7 were graded as 0 (negative), 1 (low, up to 10%), 2 (moderate, 11-50%), 3 (high, 51-90%), and 4 (strong, over 90%). TLR-9 positivity was scored by the intensity of positively stained cancer cells with the grade 0 (negative), 1 (mild), 2 (moderate), and 3 (strong). We used the highest score from 3 different spot tissue areas from each patient placed in the TMA. In the survival analysis, *T. denticola* CTLP immunopositivity was classified into 2 categories i.e. low immunopositivity (containing scores 0-2) and high immunopositivity (containing scores 3-4).

7.3.6 Statistics

In study I, the analyses were performed by IBM SPSS Statistic 21 software. Spearman’s correlation analysis was used to convince if the age different is not as a confounding factor for the level of apoptosis. The mean difference between HMGB1 expression in the cytoplasm and nucleus in each group was analyzed with a paired t-test and the mean difference between groups was analyzed using an independent t-test. The mean difference between the groups of caspase-3 in CD68 positive cells was analyzed using one-way ANOVA followed by a post-hoc Tukey test. P values less than or equal to 0.05 were considered as statistically significant.
In study III, Cohen kappa coefficient was used to analyse inter-observer scoring agreement in the immunostaining evaluation. The association between the immunopositivity score of *T. denticola* CTLP with clinicopathological variables (age, sex, cancer location, grade, cancer size, node positivity, invasion depth, margin of resection, and diameter of cancer) and with other prognostic markers (MMP-8, MMP-9, TLR-7, TLR-9, c-Myc, Ki-67, BMI, and SNAIL) were assessed with the Spearman test. The Kaplan-Meier estimator was used to analyze the relationship between the immunopositivity of *T. denticola* CTLP with the survival rate of MTSCC patients and compared with the log rank test.
8. RESULTS

8.1 Immunoexpression of *T. denticola* CTLP in periodontitis

*T. denticola* CTLP was examined in periodontitis-affected tissue samples. Its presence in subgingival plaque (attached to the gingival tissue samples seen in immunohistochemical staining in periodontitis tissue) through the epithelium to lamina propria emphasized the invasiveness capacity of *T. denticola* CTLP (Figure 3). *T. denticola* CTLP positivity was localized extracellularly in gingival plaque in all 5 samples containing the dental plaque biofilm. In the epithelium, *T. denticola* CTLP was shown to exist intracellularly in epithelial cells; in some areas, its demonstration showed a specific pattern from superficial to the deeper layer of epithelium, a pattern which is not found in healthy and gingivitis tissue samples. In lamina propria, *T. denticola* CTLP appeared to be present extracellularly in granular deposits in the extracellular matrix (Figure 3).
Figure 3. Immunoexpression of *T. denticola* CTLP in normal, gingivitis, periodontitis and orodigestive carcinoma tissues. Negative control staining is shown in (A). *T. denticola* CTLP presence in (B) healthy, (C) gingivitis, (D) dental plaque biofilm adjacent to the gingival tissue of periodontitis is shown in between the black dotted line and (E) in periodontitis tissue, where extracellular immunoexpression of *T. denticola* CTLP is shown in the lamina propria of periodontitis gingival tissue, the area shown in black rectangle and magnified in the upper right corner. The presence of *T. denticola* CTLP in orodigestive tissues is shown in morphologically normal epithelium adjacent to the carcinoma site (F); squamous cell carcinoma of (G) mobile tongue, (H) tonsil, (I) esophagus; and adenocarcinoma of (J) gastric, (K) pancreas, (L) colon. Positivity is indicated by a brown color with DAB (A-E), and red with AEC (F-L). Scale bar 50 μm relevant for all panels.
8.2 Expression of apoptosis, danger signal HMGB1, TLR4, and pro-inflammatory cytokines markers in healthy, gingivitis, and periodontitis

**Apoptosis marker caspase-3**

Our results revealed that caspase-3 positive cells were rarely present in healthy tissues, but increased gradually in gingivitis, with an even greater intensity in periodontitis (Figure 4). In healthy control tissues, caspase-3 staining was positive in only a few connective tissue cells, which were undergoing apoptosis during normal tissue regeneration. In gingivitis and periodontitis, caspase-3 was expressed more intensely in lamina propria cells and to a lesser extent in the epithelium. The majority of the apoptotic cells were observed in the sulcular epithelium and in the underlying lamina propria. The increased apoptosis was also observed in the oral epithelial area. The pattern of caspase-3 expression was similar to the TUNEL labeling, confirming the high degree of apoptotic cells present in periodontitis (Figure 4). In order to investigate the expression of caspase-3 in macrophages, double immunofluorescence staining of caspase-3 in combination with CD68 as a macrophage marker was used. The percentage of CD68 positive cells that expressed caspase-3 significantly increased significantly in periodontitis (74±21.71%) as compared with that in gingivitis (26±23.02%) (p=0.0051) or healthy controls (18± 14.76%) (p=0.000003) (Figure 5).
Figure 4. Double immunofluorescence staining of the expression of caspase-3 (red) and CD68 (green) and TUNEL staining confirming the presence of the late stage of apoptotic cells in healthy, gingivitis, and periodontitis epithelium and lamina propria tissue. The basal lamina separating the epithelium and lamina propria is marked with a white or black dotted line. Scale bar 50 μm relevant for all panels.

Danger signal HMGB1

In our immunohistochemical analysis, we found a higher expression of cytoplasmic HMGB1 in periodontitis both in the epithelium and in connective tissue as compared to gingivitis and healthy controls. We also found some extracellular HMGB1 in periodontitis tissue. HMGB1 expression in epithelium showed a different staining pattern between each of the three groups (Figure 6). In healthy control epithelium, HMGB1 was expressed mostly in the basal layer (epithelium layer attached to basal membrane), and mainly in a condensed manner in the perinuclear area. Its expression was gradually less intense from the basal to superficial layers (upper epithelial layer). In gingivitis, HMGB1 was mostly expressed in a diffuse manner throughout the cytoplasm. In periodontitis, diffused staining of HMGB1 was found in the cytoplasm in all layers of the epithelium and it was strongly expressed in the nucleus and cytoplasm in all layers of sulcular epithelium.
In connective tissue, HMGB1 was expressed in the nucleus and cytoplasm of some fibroblasts, endothelial cells, and inflammatory cells. In the connective tissue of the healthy controls, only a few cells were HMGB1 positive (1091.52±134.59 cells/mm²). In gingivitis, more cells (1402.41±812.57 cells/mm²) expressed HMGB1 as compared to healthy controls. Numerous HMGB1 positive cells were found in periodontitis (2598.33±732.19 cells/mm²), which might result from the evident presence of the inflammatory cells in the periodontitis tissue.

The number of HMGB1 positive cells in connective tissue was significantly increased in periodontitis as compared to specimens from gingivitis subjects (p=0.032) and healthy controls (p= 0.00014), but there was no difference between healthy controls and gingivitis (p=0.31). The localization of HMGB1 expression in the nucleus was not significantly different between the groups (p>0.05), whereas the cytoplasmic expression of HMGB1 was significantly increased in periodontitis group (2160.08±517.79 cells/mm²) compared with the healthy (675.19±182.38 cells/mm²) (p=0.000007) and gingivitis (871.03±641.01 cells/mm²) (p=0.004) groups (Figure 5).
Figure 5. The diagrams show the positivity of CD68-caspase-3 (A), total numbers of HMGB1 positive cells (B), and subcellular localization of HMGB1 (C) in the lamina propria of healthy, gingivitis, and periodontitis tissues.

**HMGB1 receptor TLR4**

There was a major increase of TLR-4-positive cells in periodontitis but there was no noticeable difference between healthy and gingivitis samples. TLR-4 expression in epithelium of healthy control was almost the same as its expression in gingivitis which was tended to show a milder intensity compared to periodontitis (Figure 7). In periodontitis, TLR-4 expression was increased in the epithelium (Figure 7). This increase occurred in a parallel manner to that of HMGB1 expression. In the oral epithelium of periodontitis samples, all layers expressed TLR-4 with almost similar intensities (data not shown). In the sulcular epithelium, all layers expressed TLR-4 also at similar intensities but the intensity was slightly weaker when compared with the oral epithelium. In lamina propria, we observed that TLR-4 was expressed by macrophages, rarely by mast cells, but often by T-cell/ Natural Killer cells (Figure 7).
Figure 7. Immunohistochemical staining of TLR4 in healthy (A), gingivitis (B), and periodontitis tissue (C) and double immunofluorescence staining of TLR4 (red) with inflammatory cells markers (green) of CD68 (D), CD8 (E), and mast cell tryptase (F) in periodontitis tissues. Scale bar 50 μm relevant for all panels.

Pro-inflammatory cytokines IL-8 and IL-1β

The expressions of IL-8 and IL-1β were increased in periodontitis tissue as compared to healthy and gingivitis (Figure 8). In healthy controls, the epithelium was negative or weakly stained with IL-8 and IL-1β. In gingivitis, the intense staining of IL-8 and IL-1β in epithelium was increased as was the number of the positive cells in the connective tissue. In periodontitis, epithelial staining of IL-8 and IL-1β was stronger as compared to gingivitis and in the connective tissue, the numbers of positive cells were highly increased as was the extracellular expression of IL-8 and IL-1β.
Figure 8. Immunohistochemical staining of IL-8 and IL-1β in healthy, gingivitis, and periodontitis tissue. Scale bar 50 μm relevant for all panels. NC=negative control.
8.3 Immunoexpression of *T. denticola* CTLP in orodigestive carcinoma tissues

Our immunohistochemistry staining results revealed the presence of *T. denticola* CTLP in orodigestive carcinoma tissue including MTSCC (19 from 29 tissue samples), tonsillar (20 from 25 tissue samples), and esophageal squamous cell carcinoma (20 from 25 tissue samples) and also in gastric adenocarcinoma (21 from 32 tissue samples), pancreatic (6 from 6 tissue samples), and colon cancers (25 from 54 tissue samples) (Figure 3). *T. denticola* CTLP is immunoexpressed in carcinoma cells and occasionally also in the adjacent normal epithelial cells in the cytoplasm of the cell. Interestingly, *T. denticola* CTLP was not detected in non-orodigestive carcinoma tissue including mammary ductal carcinoma and squamous cell carcinoma of the lung. In thyroid tumor tissue, only minimum and weak immunoexpression was found in follicular thyroid carcinoma (1 from 7) and follicular thyroid adenoma (1 from 1), but it was negative in the other examined thyroid tumor samples including papillary thyroid carcinoma (n=8), medullary thyroid carcinoma (n=7), and hyperplastic thyroid tissue (n=5). Furthermore, staining using non-immune species specific rabbit IgG at the same concentration and the omission of primary antibodies gave negative results (Figure 3).

8.4 *T. denticola* CTLP activity on immunomodulatory component involved in cancer microenvironment

Pretreatment of proMMP-8 and proMMP-9 with *T. denticola* CTLP resulted in 75 kDa proMMP-8 degradation into 60 kDa as well as 92 kDa proMMP-9 into the 80-82 kDa active form. *T. denticola* CTLP partly fragmented 28 kDa TIMP-1 into an inactive form after 30 minutes' incubation, it was completely degraded after 60 minutes. The effect of *T. denticola* CTLP on 21 kDa TIMP-2 degradation was observed after 20 minutes incubation. These results highlight the ability of *T. denticola* CTLP to activate MMP-8 and -9 and inactivate their inhibitors (TIMP-1 and TIMP-2) as analyzed by SDS-PAGE and confirmed by western blotting. Zymographic analysis confirmed the results that MMP-8 treated *T. denticola* CTLP significantly amplified the degradation of type I and II collagens as compared with MMP-8 alone. Type I gelatin-zymographic analysis revealed that *T. denticola* CTLP is a gelatinolytic protease that can convert inactive gelatinolytic proMMP-9 (92 kDa) into its active form (82 kDa). In addition to its effect on MMPs and TIMPs, *T. denticola* CTLP is also capable of
degrading 65 kDa α-1-AC and 68 kDa C1q into smaller fragments that thus may inactivate these components.

8.5 Association of *T. denticola* CTLP with the clinicopathological data of MTSCC patients

This study represents a homogeneous group of patients with mobile tongue squamous cell carcinoma, with early-stage (T1/T2N0M0) of disease and a primary presentation who had undergone similar surgery with curative intent.

The immunoexpression of *T. denticola* CTLP, classified based on the demographic and clinicopathological feature of MTSCC patients, is shown in Table 4 and on cancer-related markers immunoexpression in Table 5. The association between *T. denticola* CTLP immunoexpression with the clinicopathological characteristics and the cancer-related markers of the MTSCC patients is shown in Table 6. Higher *T. denticola* CTLP has a significant positive association with a deeper invasion depth (Rs= 0.331, p= 0.01) and a wider diameter (Rs= 0.302, p= 0.019). *T. denticola* CTLP did not show any significant association with age (p= 0.281), sex (p= 0.669), carcinoma grade (p= 0.213), nodal positivity (p= 0.750), and carcinoma localization in the tongue (p= 0.285) (upper surface, tip, edge, or lower surface). However, it is worthwhile noting that most cases (51 from 60 patients) were localized in the edge of tongue with most of them (21 from 51 patients) displaying strong expression, 13 patients displaying high expression with the rest of them displaying moderate (7 from 51), low (8 from 51) or negative (2 from 51) expression of *T. denticola* CTLP (Table 4).
Table 4. Demographics, clinicopathological features, and *T. denticola* CTLP immunoexpression of mobile tongue squamous cell carcinoma patients. *T. denticola* CTLP immunoexpression was scored as negative (0), low positive (1), mild positive (2), moderate positive (3), and high positive (4).

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>T. denticola CTLP immunoexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative 1 2 3 4</td>
</tr>
<tr>
<td>All cases</td>
<td>60</td>
<td>3 9 9 16 23</td>
</tr>
<tr>
<td>Age, years (mean)</td>
<td></td>
<td>67.33 57.44 51.33 60.94 64.26</td>
</tr>
<tr>
<td>≤60</td>
<td>32</td>
<td>1 6 7 10 8</td>
</tr>
<tr>
<td>&gt;60</td>
<td>28</td>
<td>2 3 2 6 15</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>Male 2 2 8 8 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female 0 5 5 7 13</td>
</tr>
<tr>
<td>Clinical T class (mm)</td>
<td></td>
<td>cT1 (≤20) 1 5 5 7 6</td>
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<tr>
<td></td>
<td></td>
<td>cT2 (21-40) 2 4 4 9 17</td>
</tr>
<tr>
<td>Pathological T class (mm)</td>
<td></td>
<td>pT1 (≤20) 4 1 8 6 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pT2 (21-40) 2 1 3 4 10</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>Upper surface 1 0 0 0 0 1</td>
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<tr>
<td></td>
<td></td>
<td>Tip 2 0 0 1 1 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Edge 51 2 8 7 13 21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lower surface 6 1 1 1 2 1</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td>I 19 0 1 4 5 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II 30 1 6 4 10 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III 11 2 2 1 1 5</td>
</tr>
<tr>
<td>Node positivity (pN)1</td>
<td></td>
<td>N0 22 0 4 5 4 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N+ 15 2 1 1 4 7</td>
</tr>
<tr>
<td>Invasion depth, mm (mean)</td>
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<td>60 6 5.97 5.98 6 9.07</td>
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<tr>
<td>Tumor diameter, mm (mean)</td>
<td></td>
<td>60 17.67 9.89 14.33 14.44 18.3</td>
</tr>
</tbody>
</table>

1 37 from 60 patients had elective neck dissection.

We compared the immunoexpression of *T. denticola* CTLP with some other cancer-related markers and found a significant positive association with TLR-7 (Rs = 0.297, p=0.021), TLR-9 (Rs=0.320, p=0.013), cytoplasmic c-Myc (Rs= 0.276, p=0.033. However, there were no
significant association (p>0.05) between the immunoexpression of *T. denticola* CTLP with MMP-8, MMP-9, TLR-2, TLR-7, TLR-9, Bmi-1, Snail, and Ki-67 (Table 6).

The median survival time of the 60 patients involved in this study was 8.35 years. The 5-year overall survival rate of these patients was 62% and 5-year disease-free survival rate was 66%. In the Kaplan-Meier survival analysis, there was no difference between low and high expression of *T. denticola* CTLP in overall, disease-specific, and disease-free survival (Figure 9). In patients at and under 60 years old at diagnosis, we observed that a high expression of *T. denticola* CTLP was a poor marker of recurrence (Log Rank, p= 0.045) (Figure 9).

Table 5. Tumor-related markers and *T. denticola* CTLP immunoexpression of mobile tongue squamous cell carcinoma patients. *T. denticola* CTLP immunoexpression was scored as negative (0), low positive (1), mild positive (2), moderate positive (3), and high positive (4).

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
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<tr>
<td></td>
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<td>Negative</td>
</tr>
<tr>
<td>MMP-8, %</td>
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</tr>
<tr>
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<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2 = 11-50% (moderate)</td>
<td>6</td>
<td>0</td>
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<tr>
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<td>1</td>
</tr>
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<td>4 = over 90% (strong)</td>
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<td>1</td>
</tr>
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<td>1</td>
</tr>
<tr>
<td>3 = 51–90% (high)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>4 = over 90% (strong)</td>
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</tr>
<tr>
<td>TLR-2 cytoplasmic, intensity</td>
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<tr>
<td>0 = negative</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1 = mild</td>
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</tr>
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<td>2 = moderate</td>
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<td>1</td>
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<tr>
<td>3 = strong</td>
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<td>1</td>
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<tr>
<td>TLR-2 nuclear, %</td>
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<td>0</td>
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<tr>
<td>1 = up to 10% (low)</td>
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<td>1</td>
</tr>
<tr>
<td>2 = 11-50% (moderate)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3 = 51–80% (high)</td>
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<td>------------------------</td>
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<tr>
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</tr>
<tr>
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<td>10</td>
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<td>2</td>
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</tr>
<tr>
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<td>13</td>
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<td>9</td>
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<tr>
<td>Bmi-1, %</td>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
<td>1 = up to 29% (low)</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>2 = 30-49 % (moderate)</td>
<td></td>
<td>9</td>
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<tr>
<td>3 = 50–80% (high)</td>
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<td>4</td>
</tr>
<tr>
<td>Snail, %</td>
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<td>0</td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>2 = 30-49 % (moderate)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>3 = 50–80% (high)</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>4 = over 80% (strong)</td>
<td></td>
<td>40</td>
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59
Table 6. Association of *T. denticola* CTLP immunoexpression with clinicopathological features and tumor-related markers.

<table>
<thead>
<tr>
<th>Variables</th>
<th>p-value (p&lt;0.05)</th>
<th>Correlation Coefficient Rs</th>
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</tr>
<tr>
<td>Sex</td>
<td>0.669</td>
<td>0.056</td>
</tr>
<tr>
<td>Location</td>
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<td>-0.14</td>
</tr>
<tr>
<td>Grade</td>
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<td>-0.163</td>
</tr>
<tr>
<td>Node positivity (pN)</td>
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<td>0.054</td>
</tr>
<tr>
<td>Invasion depth, mm (mean)</td>
<td>0.01*</td>
<td>0.331</td>
</tr>
<tr>
<td>Tumor diameter, mm (mean)</td>
<td>0.019*</td>
<td>0.302</td>
</tr>
<tr>
<td>MMP-8</td>
<td>0.065</td>
<td>0.240</td>
</tr>
<tr>
<td>MMP-9</td>
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<td>0.202</td>
</tr>
<tr>
<td>TLR-2 cytoplasmic</td>
<td>0.877</td>
<td>-0.021</td>
</tr>
<tr>
<td>TLR-2 nuclear</td>
<td>0.530</td>
<td>0.084</td>
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<tr>
<td>TLR-4</td>
<td>0.731</td>
<td>-0.045</td>
</tr>
<tr>
<td>TLR-7</td>
<td>0.021*</td>
<td>0.297</td>
</tr>
<tr>
<td>TLR-9</td>
<td>0.013*</td>
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<tr>
<td>c-Myc cytoplasmic</td>
<td>0.033*</td>
<td>0.276</td>
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<td>Ki-67</td>
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<td>-0.017</td>
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<td>Bmi-1</td>
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<td>0.009</td>
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<tr>
<td>Snail</td>
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Spearman analysis, * p ≤ 0.05.
Figure 9. Kaplan-Meier analysis for survival of early-stage MTSCC patients with either low or high *T. denticola* CTLP immunoexpression in all patients and in younger age patients (<60 years old). A significant difference was found in the disease-free survival of younger patients (p<0.05).
9. DISCUSSION

9.1 *T. denticola* may play a role in chronicity of inflammation in periodontitis

The immunohistochemical findings about the subgingival plaque in periodontitis-affected tissue were in line with and supported a previous study that *T. denticola* has most often been reported isolated from subgingival plaque in the periodontitis site (Byrne et al., 2009). Furthermore, our results also indicated that *T. denticola* CTLP also existed within the tissue i.e. evidence of *T. denticola* infection in periodontitis gingival tissue (Figure 3). It has been shown that CTLP facilitates its adherence and invasion to the epithelial cells preceding the invasion of deeper tissue (Inagaki et al., 2016; Sela 2001). This ability to penetrate into epithelial cells suggests that the microorganism has crossed the first barrier, subsequently CTLP assists the penetration of *T. denticola* into the deeper layer of epithelium through the basement membrane to the lamina propria by degrading intracellular adhesion and other host cell matrix proteins (Dashper et al., 2011).

The high expression of caspase-3 and TUNEL-positive cells in the periodontitis samples in our study indicated that most cells undergo apoptosis. These findings are supported by previous studies (Pradeep et al., 2014; Gamonal et al., 2001). The majority of the apoptotic cells in the sulcular epithelial and its underlying lamina propria that were found in this study may be explained as follow. Sulcular and pocket epithelium are known as the most exposed gingival area with the periodontal pathogen accumulated in dental plaque biofilm and it is also suggested that this is the area into which the periodontal pathogens are invading the tissue (Ji et al., 2015). The increased apoptosis in the oral epithelial cells, which was further from the infection site, may indicated that the immune defense mechanism present in the oral epithelial area may actively participate in the periodontal inflammation. This suggests that the apoptosis in periodontitis may be a result either from a direct effect of the pathogens or an indirect effect mediated through the host immune response. Increasing information indicates that infection of tissues by some pathogenic bacteria can indeed trigger host cell apoptosis (Lancellotti et al., 2006; Zychlinsky and Sansonetti, 1997). Apoptosis has a critical role during the inflammation process and in the resolution from inflammation, however infection-induced apoptosis can also promote proinflammatory processes (Zychlinsky and Sansonetti, 1997).
The overall higher apoptosis in lamina propria compared to epithelium showed in this study may be evidence that the inflammatory cells and fibroblasts within the lamina propria were rapidly going into apoptosis due to the influence of pathogens and their products such as proteinases. These metabolites are known to promote apoptosis in fibroblasts. There are also leukotoxins which demonstrated to induce apoptosis in neutrophil cells, T-cells, natural killer cells, and macrophages. (Pöllänen et al., 2012). The increase in the numbers of apoptotic epithelial and macrophage in periodontitis may be directly induced by T. denticola as it has been reported that T. denticola stimulates the apoptosis of epithelial (Leung et al., 2002), endothelial, and macrophage cells (Jun et al., 2017). Apoptotic cells were excessive in the tissue, meanwhile many of the macrophages also had undergone apoptosis in periodontitis. This situation could indicate that there was a deficiency in the number of active macrophages with excessive amounts of apoptotic cells, resulting in a defective clearance function. Deficient or delayed clearance of apoptotic cells will lead the apoptotic cells lose their membrane integrity and the death process switches to secondary necrosis (Elliott and Ravichandran, 2010).

HMGB1 translocation from nucleus can occur during cell activation and cell death by either necrosis or apoptosis (Bell et al., 2006; Abdulahad et al., 2013). Apoptotic cells also can activate macrophages to release HMGB1 (Qin et al., 2006). If professional phagocytes such as macrophages and neutrophils fail to remove apoptotic cells, the cells will undergo secondary necrosis leading to late HMGB1 release that could subsequently act as a proinflammatory mediator (Jiang et al., 2007; Chen et al., 2013; Al-Samadi et al., 2015). The extracellular HMGB1 that was found in periodontitis tissue in this study may suggest that the increase in the numbers of apoptotic cells in periodontitis leads to changes in HMGB1 expression not only via the release of HMGB1 by the apoptotic cells themselves but also through phagocytic clearance of apoptotic cells by macrophages that are activated to release HMGB1. The massive cellular apoptosis in periodontitis and the increase in the numbers of apoptotic macrophages, i.e. the tissue’s professional phagocytes, may overload the capacity of the clearance system to remove apoptotic cells. In addition, HMGB1 can also inhibit macrophage-mediated efferocytosis, which will lead to excessive accumulation of late apoptosis events and secondary necrosis (Friggeri et al., 2010). Therefore, there is an accumulation of HMGB1 as proinflammatory mediator in periodontitis, resulted from
passively released from necrotic cells and secondary necrotic cells, as well as from activated macrophages and other inflammatory cells.

HMGB1 binding to TLR-4 is known to generate an inflammatory response. HMGB1-TLR-4 signaling contributes to the secretion of proinflammatory mediators by several cells such as epithelial cells, monocytes-macrophage, and leukocytes. (Park et al., 2004). This signal will activate epithelial cells to produce IL-6 and IL-8 (Shimizu et al., 2016); monocytes-macrophage to release IL-1β, IL-8, and TNF-α (Andersson et al., 2000; Yu et al., 2006); lymphocytes to release cytokines and also proliferate as well as contributing to the polarization of these cells (Messmer et al., 2004; Li et al., 2013; Kang et al., 2014). However, the effect of HMGB1-TLR-4 signaling on mast cell activity remains unclear.

The increase of IL-8 and IL-1β in periodontitis in our study may be associated with the increase in the severity of periodontal diseases as shown by Hou et al. (2003). The increase of IL-8 and IL-1β could contribute to exacerbate the inflammation and tissue destruction by the following possible explanation. Both IL-8 and IL-1β contribute to the subsequent neutrophil recruitment and promotion of T-helper17 (Th17) polarization (van de Veerdonk et al., 2011). Furthermore, these cytokines will activate other host immune cells to produce MMPs and bone resorptive-mediators which evoke tissue catabolism triggering tissue destruction in periodontitis (Boch et al., 2001). In addition, IL-1β will also induce influence fibroblasts to decrease the synthesis of collagen which is another way of promoting tissue destruction (Boch et al., 2001).

This study may indicates that there is an association between the presence of *T. denticola*, more extensive apoptosis, HMGB1, HMGB1 receptor and the increase in the level of inflammation in periodontitis. However, this proposal is based only on an interpretation of immunohistological results from a relatively small sample size and it will need to be confirmed with a larger study sample.

### 9.2 *T. denticola* CTLP may contribute to initiating and promoting orodigestive carcinoma

The presence of *T. denticola* CTLP in the orodigestive carcinoma tissues may indicate its possible role in these carcinomas. Currently, there is very limited available data about the presence and the role of *T. denticola* in malignancy. Narikiyo et al. (2004), Shin et al. (2017),
and Peters et. al. (2018) have investigated this topic. Narikiyo et al. (2004), reported a more frequent presence of *T. denticola* together with other streptococci periodontopathogens in esophageal carcinoma in comparison with healthy tissues obtained from patients living in several regions of the world. Shin et al. (2017) reported a similar result i.e. a selective increase of *T. denticola* in primary OSCC, while Peters et al. (2018) reported the association of *T. denticola* with the higher risk of esophageal carcinoma. In our study, *T. denticola* CTLP was also present in the majority of MTSCC, esophageal squamous cell carcinoma, as well as other orodigestive carcinoma tissues but not in non-orodigestive tumor tissue.

The presence of *T. denticola* CTLP in MTSCC could be associated with the increasing number of *T. denticola* in periodontitis that may further induce the transition of the tissue from chronically inflamed non-malignant tissue (periodontitis) to malignant tissue (oral carcinoma). This finding is in line with another study reporting the selective increase of *T. denticola* in oral carcinoma detected by 16S rRNA sequencing (Shin et al., 2017). The finding of *T. denticola* CTLP in the organs of the digestive system may indicate that *T. denticola* primarily has originated as a pathogen in the oral cavity but could be transmitted through the gastrointestinal tract due to the ingestion of the spirochete with food and saliva. In pancreatic cancer, *T. denticola* could reach the target site by the duct connected to the bowel or gain access via the blood circulation since it has been detected in atheromatous plaques in patients with chronic periodontitis (Cavrini et al., 2005; Chukkapalli et al., 2014). A negative immunoexpression of *T. denticola* CTLP was found in lung and breast carcinomas. It could be hypothesized that the microenvironments in lung and breast carcinoma tissues were not optimal to allow *T. denticola* to adhere and grow. Another possible reason is that the concentration of *T. denticola* might be too low to be detected with immunostaining methods, thus a qPCR method might be needed to reveal its presence in these carcinoma tissues.

The presence of *T. denticola* CTLP in the cancerous tissue may reflect their involvement in the initiation or progression of the disease, however the mechanism underlying the etiology remains unclear and needs to be established. A study showed that *T. denticola* is resistant to human β-defensin-1 (hβD-1) and hβD-2, an antimicrobial peptides expressed by epithelial cells in their surface (Brissette and Lukehart 2002). *T. denticola* has also been reported to be able to modify an aerobic environment into a micro-anaerobic condition to survive and grow.
These abilities may help *T. denticola* to adapt to orodigestive tissue and persist in the infected tissue and thus to stimulate continuous inflammation which is conducive to cancer progression.

*T. denticola* exists as a part of a complex with other oral bacterial species. *T. denticola* binds specifically to and coexists with *P. gingivalis*, *T. forsythia*, and *F. nucleatum* in the tissue; these are bacteria that have been associated with various types of cancers (Nagy et al., 1998; Ahn et al., 2012a; Michaud et al., 2013; Gallimidi et al., 2015). *T. denticola* CTLP is known to mediate this coaggregation (Daspher et al., 2011). This indicated that the presence of *T. denticola* CTLP in orodigestive carcinoma tissues may also be accompanied by infection by other oral bacterial species especially *P. gingivalis* and *F. nucleatum* and they may interact with each other and act in synergy, and thus play a role in carcinoma progression.

The results of *T. denticola* CTLP in the activation of MMP-8 and MMP-9 together with the degradation potential of their inhibitors, TIMP-1 and TIMP-2, support the role of *T. denticola* in stimulating carcinogenesis and the invasiveness of carcinoma. *T. denticola* has been reported to stimulate inflammatory cells to upregulate MMP-8 and MMP-9 expressions (Ding et al., 1996). MMPs modulate the cancer microenvironment, and by thus they regulate the signaling pathway that controls cancer growth, inflammation, angiogenesis, tissue invasion, and metastasis (Kessenbrock et al., 2010).

The inactivation of TIMPs as protease inhibitors will liberate MMPs and these enzymes can promote carcinogenesis and have been associated with poor survival in several human cancer types (Jiang et al., 2002). Moreover, TIMPs also have MMP-independent effects including influences on cell growth and differentiation, cell migration, anti-angiogenesis as well as anti- and pro-apoptosis properties (Brew and Nagase, 2010). However, there are other studies reporting paradox phenomena associated with the roles of TIMPs in cancer, which may either suppress or promote carcinogenesis depending on the microenvironment (Jiang et al., 2002).

Our study showed that *T. denticola* CTLP inactivating (by degrading) inflammatory factors, acute phase protein α-1-AC and complement component C1q. As α-1-AC is a protein that inhibits the activity of certain proteases which are intended to protect the tissues from damage, thus α-1-AC inactivation may indirectly facilitate cancer invasion. Inactive and
deficient α-1-AC will increase the role of neutrophil elastase to activate MMPs, inhibit apoptosis, and activate cancer-promoting genes (Sun and Yang, 2004). C1q plays a key role in immune complex recognition in activating the classical pathway (Kishore and Reid, 2000). However, recent reports have demonstrated that C1q is expressed in cancer microenvironments and act to promote cancer growth by favoring cell adhesion, migration, proliferation, angiogenesis, and metastasis (Bulla et al., 2016). Hong et al. (2009) reported that C1q could induce apoptosis in prostate cancer cells and C1q downregulation has enhanced hyperplasia and cancer formation. Another study also revealed that C1q binding to cultured human gingival fibroblasts eliminated mitogen-induced proliferation of the cells through mechanisms that involved the activation of the intracellular p38 mitogen-activated protein kinase (MAPK) stress pathway (Bordin et al., 2003). Inactivation of C1q by T. denticola CTLP, as shown in this study, may decrease the presence of C1q in the orodigestive carcinoma tissue, a process that will inhibit cancer cells apoptosis, increase mitosis, and induce cancer formation.

These results reveal for the first time a plausible molecular mechanism to account for the role of T. denticola in carcinoma. It remains unclear whether T. denticola infection initiates the carcinoma progression or does it infect the tissue after carcinoma transformation. Hence, further studies are required to clarify it.

9.3 T. denticola CTLP associates with the clinicopathological data of MTSCC patients

A significant association between the presence of T. denticola with invasion depth and diameter of the cancer detected in this study highlighted the potential contribution of T. denticola to the invasiveness and development of MTSCC. We propose that there is a plausible mechanism to explain this finding. T. denticola via its CTLP is capable of degrading different types of proteins (Uitto et al., 1995) and thus it could destroy the matrix and disrupt the junction between the cells and even the basement membrane allowing the cancer cells to invade into the deeper tissue and undergo metastasis via the lymphatic or blood vessels. T. denticola also reported promotes epithelial cell proliferation and survival by activating extracellular signal-regulated kinase 1/2 (ERK1/2) (Leung et al., 2002). This proliferation capacity could enlarge the cancer size.
The association of the *T. denticola* CTLP immunopositivity with other cancer-related markers linked to MTSCC showed varying results. Our study showed that c-Myc has a significant positive correlation with *T. denticola* CTLP immunopositivity. C-Myc has an effect to increase cellular proliferation and metastatic capability (Miller et al., 2012). This may suggest that c-Myc expression in MTSCC may play a synergistic role with *T. denticola* CTLP to increase cellular proliferation that could enlarge the cancer size and the metastatic capability, thus contributing to cancer progression and metastasis.

In this study, we also found a significant positive correlation between *T. denticola* CTLP immunopositivity with TLR-7 and TLR-9. TLR-7 and TLR-9 as cytoplasmic or endosomal receptor are located inside the cell and could be activated by intracellular pathogen such as *T. denticola*. The activation of TLR-7 and TLR-9 induces a proliferative and prosurvival effect on cancer cells (Jego et al., 2006; Cherfils-Vicini et al., 2010). TLR-9 expression was reported to have a correlation with cancer size in MTSCC (Ruan et al., 2011). This correlation supports our finding that TLR-9 and *T. denticola* CTLP both may be contribute to cancer cell proliferation. In addition, TLR-9 seems to be crucial for a TLR-9 agonist to induce cellular invasion in breast and prostate cancers (Ilvesaro et al., 2007, 2008; Merrell et al., 2006). Furthermore, TLR-9 promotes the invasion of cancer cells by activating MMP-13 and MMP-9 (Merrell et al., 2006; Kauppila et al., 2015) while *T. denticola* CTLP may exert the same effect via MMP-8 and MMP-9. However, to date, the roles of TLR-7, -9 and other TLRs in cancer have been reported to have contrasting outcomes, depending on the cancer microenvironments (Rich et al., 2014). The significant positive correlation between *T. denticola* CTLP immunopositivity with c-Myc expression found in this study suggests that c-Myc may have a synergistic role with *T. denticola* CTLP contributing a risk factor for MTSCC metastasis. Another study reported that in colon cancer, c-Myc could induce survival and motility that contributed to cancer progression and metastasis (Conacci-Sorrell et al., 2014), while in MTSCC, Vora et al. (2007) reported that there was a negative correlation between cytoplasmic c-Myc and cancer stage.

Our results indicate a role for *T. denticola* CTLP as a prognostic marker to predict relapse time of MTSCC in younger patients (less and at 60 years of age at the time of diagnosis). This could be explained by the ability of *T. denticola* CTLP to break down various proteins allowing the bacteria to penetrate into deeper parts of the tissue, and thus it may remain in the
adjacent (deep) tissue even after surgery. In addition, without any antimicrobial treatment, the bacteria left in the remaining tissue could initiate or stimulate the growth of the cancer.

Recently, it has been reported that the incidence of MTSCC in younger patients is increasing (Patel et al., 2011; Hussein et al., 2017). The age of the patients may contribute to the relapse time and survival rate of MTSCC (Jeon et al., 2017). The younger patients have been found to have lower survival rate than the older patients and have a higher recurrence rate (Zhang et al., 2017), including regional, locoregional recurrence and distant metastasis, with the highest risk of distant metastasis (Jeon et al., 2017). However, the underlying factors that determine this difference between young and old patients is largely unknown. The etiology of the cancer itself may contribute to the survival and recurrence of this patients group. The usual causative factors for MTSCC (i.e. alcohol and tobacco) may play a minimal role in the etiology of the younger patients (Harris et al., 2009). Although the younger patients may have tobacco and alcohol risk factor, it was reported that the association risk is low and the exposure time is relatively short (Llewellyn et al., 2004). Therefore, in addition to individual genetic factors, the presence and activities of certain microbes may play a more significant role.

There are other factors that may possibly contribute to the relapse of MTSCC including the stage, surgical methods (flap repair), mode of infiltration (distinction with surrounding tissue), the presence of an epithelial-to-mesenchymal transition (EMT), depth of invasion, history of smoking and alcohol consumption (Scheidt et al., 2012; Wang et al., 2013; Mohri et al., 2016). However, these factors were not reviewed in our study.

The strength of this study is that we have focused on potentially the most important virulence factor of *T. denticola* i.e. CTLP. We also utilized a highly specific polyclonal antibody against *T. denticola* CTLP. The *in situ* localisation of this enzyme in the periodontitis and orodigestive carcinoma tissues may provide insights into its effect in biological systems.

However, this study has several limitations. First, we did not perform a qPCR method to confirm the immunoexpression of *T. denticola* CTLP in the tissue due to the limited availability of the tissue source. Therefore, it is possible that low expressions of *T. denticola* CTLP in the tissue might not be detected with only an immunostaining method. Second, certain patient-related information such as high-risk characteristics of the MTSCC patients
including their HPV status, history of oral and systemic health, smoking and alcohol consumption were not available. This type of patient-related information/characteristics could have been enlightening and useful when interpreting the results emerging from this study. Finally, this study evaluated a relatively small sample size of patients and furthermore the lack of functional cell culture experiments can also be considered as a study limitation.

Therefore, further investigations to overcome these study limitations are needed; these would include: 1) an examination of larger patient samples to represent better the general population, 2) confirming the results obtained here by other different methods such as qPCR, 3) performing in vitro cell studies to trace and clearly elucidate the mechanism of the pathogenic pathway, 4) undertaking in vivo intervention trials in experimental animals.
10. CONCLUSIONS

Our study focused on the role of \textit{T. denticola} and particularly its major virulence factor, CTLP, in the chronicity of periodontitis inflammation via their effect on the endogenous danger signals and in orodigestive carcinoma via immunomodulation. Moreover, this study also elucidated \textit{T. denticola} association with the clinicopathological characteristics of mobile tongue SCC patients (Figure 10). The main conclusions emerging from this study are:

I. \textit{T. denticola} is present in dental plaque biofilm and in gingival tissue of periodontitis. Its presence in periodontitis was accompanied by an increased expression of apoptotic cell markers, endogenous danger signal (HMGB1), the HMGB1 receptor (TLR-4), and proinflammatory cytokines. This result highlights the possibility that infection-induced cellular apoptosis in periodontitis may progress to secondary necrosis and lead to elevated HMGB1 production. The apoptosis-secondary necrosis-HMGB1-TLR-4 pathway activates several cells in periodontal tissue and thus promotes the release of proinflammatory cytokines which may create a continuous cycle of inflammation. This may represent one of the possible reasons for prolonged inflammation in periodontitis.

II. \textit{T. denticola} chymotrypsin-like proteinase is present in orodigestive carcinoma tissues. The \textit{in vitro} study result revealed the capability of \textit{T. denticola} CTLP to dysregulate the functions of the host regulatory proteins by activating the MMP-8 and -9 enzymes; these changes provide the carcinoma cells with the means to invade deep into the tissue and to inactivate the MMP inhibitors (TIMP-1 and TIMP-2) as well as diminishing the effectiveness of the host’s immune system. This indicates that \textit{T. denticola}, via its CTLP, may promote orodigestive carcinogenesis.

III. The immunoexpression of \textit{T. denticola} CTLP in MTSCC associates with the invasion depth and carcinoma size which represent the carcinoma invasiveness and its aggressiveness. The immunoexpression is also associated with the expression of cancer-related markers TLR-7, TLR-9, and c-Myc thus highlighting the synergistic nature of these mechanisms in cancer invasiveness and aggressiveness. Moreover, the presence of \textit{T. denticola} showed an association with an early relapse in this carcinoma, especially in patients at and under 60 years old.
To summarize, we revealed the potential role of *T. denticola*, an oral pathogen involved in periodontitis, in the development of orodigestive carcinoma. We found an association between *T. denticola* CTLP immunoexpression and the chronicity of inflammation in periodontitis. Moreover, we elucidated the molecular mechanism explaining how *T. denticola* CTLP can regulate the critical proteins in cancer microenvironment. With respect to the clinical situation, we found an association of *T. denticola* CTLP immunoexpression with the invasiveness and aggressiveness of MTSCC, and this predicted an earlier relapse in patients at and under 60 years old. More functional analyses using cell cultures are at a preliminary stage; these are intended to clarify the direct effect of *T. denticola* on carcinogenesis.
Figure 10. The role of *T. denticola* in exacerbating inflammation in periodontitis and in carcinoma progression. The numbers are referred to the publications.
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12. REFERENCES


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