# EPIGENETIC AND TRANSCRIPTIONAL DYNAMIC IN PERIODONTAL DISEASE

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## ABSTRACT

Eman Hefni: Epigenetics and Transcriptional Dynamin in Periodontal Disease
(Under the direction of Silvana Barros)

**Objectives**: Several studies have shown the involvement of epigenetics with periodontal disease. Since functional dissociation of Paracellular permeability is expected during bacterial infection, we hypothesize that the methylation of host oral epithelial DNA represents an important element in the disruption of barrier function and pathogenesis of periodontal diseases. With this *In vitro* study, we aimed to assess whether there is altered epithelial permeability measuring trans epithelial resistance after *Porphyromonas gingivalis* (*P. gingivalis)*, *Campylobacter rectus* (*C. rectus)* and *Fusobacterium nucleatum (F. nucleatum)* infection. Plakophilin2 (*PKP2*) methylation status and expression levels were also investigated. In addition, investigate the potential effects of DNA methyltransferase (DNMT) inhibitors on epithelial barrier function in response to infection with periodontal pathogen in human gingival epithelial cells.

**Methods**: Primary human gingival epithelial cells (HGEPs) were stimulated with *P. gingivalis*, strain, *C. rectus* and *F. nucleatum* (MOI 50) either in the presence or absence of DNMT inhibitors (10 μM of RG108 or EGCG). CellTiter-Blue® Cell Viability Assay (Promega) was used to determine an optimum cell density and maximum inhibitor concentration at which cell viability is maintained. Transepithelial electrical resistance (TER) at various time points were performed using an EVOM® electrical resistance system. DNA methylation was quantified

by PCR using EpiTect Methyl II PCR Primer Assays for *PKP2*. Immunofluorescence analysis was performed using *PKP2* antibody and analysis performed using Zeiss710 confocal microscope.

**Results**: Exposure of HGEPs to *P. gingivalis* resulted in decreased TER (P=<0.001) associated with increased cell permeability. Methylation assays showed increased methylation levels of the *PKP2* in comparison to non-infected controls (P=<0.001) and an associated *PKP2* down-regulation (P=<0.005). For infected cells treated with DNMT inhibitors, *PKP2* mRNA expression was increased (P=<0.001) and TER values similar to non-infected cells. Comparatively, immunofluorescent staining of the *PKP2* protein showed reduced protein expression in infected cells not treated with DNMT inhibitors.

**Conclusion**: DNA methylation levels of *PKP2* can affect epithelial barrier function potentially conferring increased susceptibility to infection. DNMT inhibitors can affect cell adhesion dissociation in response to infection minimizing the disturbance to the barrier function.

To my family and Anmar, I couldn't have done this

without your continued love and support.

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| | |
|---|---|
| 5-aza-CR | 5-azacytidine |
| 5-aza-CdR | 5-aza-2V-deoxycytidine |
| CDH1 | Cadherin 1 or *E-cadherin* |
| COX-2 | Cyclooxygenase-2 or PTGS2 |
| CpG | Cytosine-guanine dinucleotide |
| *C. rectus* | *Campylobacter rectus* |
| DNMT | DNA methyltransferases |
| DP | Desmoplakins |
| Dsc | Desmocollin |
| Dsg | desmoglein |
| EGCG | Epigallocatechin-3-gallate |
| *F. nucleatum* | *Fusobacterium nucleatum* |
| HGE | Human gingival epithelial cells |
| IFN-γ | Interferon gamma |
| IGF | Insulin growth factor |

| | |
|---|---|
| IL | Interleukin |
| MAPK | Mitogen activated protein kinase |
| MDR | Multi-Drug Resistance Gene |
| LPS | Lipopolysaccharide |
| MDCK | Madin-Darby canine kidney |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| OD | Optical Density |
| PG | plakoglobins |
| *P. gingivalis* | *Porphyromonas gingivalis* |
| *PKP2* | Plakophilins |
| *PTGS* | prostaglandin endoperoxide synthase |
| RG 108 | N-Phthaloyl-L-tryptophan |
| RT-PCR | Real-time Polymerase Chain Reaction |
| SNP | Single nucleotide polymorphism |
| TLR | Toll-like receptors |

# CHAPTER1: LITERATURE REVIEW

## I.1 Epigenetic, DNA Methylation – An Overview

The term "epigenetics" was first used by Conrad H. Waddington (1942), who defined it as "the branch of biology that studies the causal interaction between genes and their product, which bring the phenotype into being" (Barros S.P & Offenbacher S, 2009; Goldberg A.D, Allis C.D, & Bernstein E, 2007; Sato P.Y et al., 2009). However, the definition has gradually changed over the following years, and epigenetics is known today as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"(Wu C.T & Morris J.R, 2001). Epigenetics modification lead to events which chemically modify certain DNA regions, leading to alteration of the chromatin and ultimately silencing or activation of a gene (Barros S.P & Offenbacher S, 2009; Gomez R.S, Dutra W.O, & Moreira P.R, 2009; Larsson L, Castilho R.M, & Giannobile W.V, 2015). Epigenetics are influenced by age, environmental factors, such as smoking and diet, and microbial exposures (Barros S.P & Offenbacher S, 2014). Any disruption of the balance of epigenetic mechanism can cause several major pathologies, including cancer, syndromes involving chromosomal instabilities, and mental retardation (Table 1) (Egger G, Liang G, Aparicio A, & Jones PA, 2004).

Table 1.1 Epigenetic disease, the table is adopted from (Egger G et al., 2004)

Table 1 **Epigenetic diseases**

| Disease | Symptom | Aetiology | References |
|---|---|---|---|
| ATR-X syndrome | Intellectual disabilities, α-thalassaemia | Mutations in *ATRX* gene, hypomethylation of certain repeat and satellite sequences | 82 |
| Fragile X syndrome | Chromosome instability, intellectual disabilities | Expansion and methylation of CGG repeat in *FMR1* 5′ UTR, promoter methylation | 83 |
| ICF syndrome | Chromosome instability, immunodeficiency | *DNMT3b* mutations, DNA hypomethylation | 84 |
| Angelman's syndrome | Intellectual disabilities | Deregulation of one or more imprinted genes at 15q11–13 (maternal) | 85 |
| Prader–Willi syndrome | Obesity, intellectual disabilities | Deregulation of one or more imprinted genes at 15q11–13 (paternal) | 86 |
| BWS | Organ overgrowth | Deregulation of one or more imprinted genes at 11p15.5 (e.g. *IGF2*) | 87 |
| Rett syndrome | Intellectual disabilities | *MeCP2* mutations | 25,26 |
| α-Thalassaemia (one case) | Anaemia | Methylation of α2-globin CpG island, deletion of *HBA1* and *HBQ1* | 23 |
| Various cancers | Microsatellite instability | *De novo* methylation of *MLH1* | 29 |
| | Disruption of Rb, p53 pathway, uncontrolled proliferation | *De novo* methylation of various gene promoters | 4 |
| | Disruption of SWI–SNF chromatin remodelling complex | Mutations in *SNF5*, *BRG1*, *BRM* | 36 |
| | Overexpression of IGF2, silencing of *CDKN1C* | Loss of imprinting | 88, 89 |
| Leukaemia | Disturbed haematopoiesis | Chromosomal translocations involving HATs and HMTs | 62 |
| Rubinstein–Taybi syndrome | Intellectual disabilities | Mutation in CREB-binding protein (histone acetylation) | 90 |
| Coffin–Lowry syndrome | Intellectual disabilities | Mutation in *Rsk-2* (histone phosphorylation) | 90 |

ATR-X syndrome, α-thalassaemia, mental retardation syndrome, X linked; BWS, Beckwith–Wiedemann syndrome; CREB, cAMP-response-element-binding protein; HAT, histone acetyltransferase; HMT, histone methyltransferase; ICF, immunodeficiency, centromeric region instability and facial anomalies syndrome; UTR, untranslated region.

Various important epigenetic mechanisms include : DNA methylation, histone modifications ( methylation , acetylation , deacetylation) and non-coding RNA (Barros S.P & Offenbacher S, 2009; Egger G et al., 2004; Goldberg A.D et al., 2007; Lod, Johansson, Abrahamsson, & Larsson, 2014).

DNA methylation is associated with gene silencing and is the most studied of the epigenetic processes, especially in the field of cancer research (Barros S.P & Offenbacher S, 2009) (Goldberg A.D et al., 2007; Subramaniam D, Thombre R, Dhar A, & Anant S, 2014). DNA methylation occurs when a methyl group binds to a cytosine residue within the cytosine-guanine dinucleotide (CpG) regions (Barros S.P & Offenbacher S, 2009; Larsson L et al., 2015) . Cytosines in CpA, CpC, and CpT dinucleotides can also be methylated, but less frequently (Rakyan, Down, Balding, & Beck, 2011). Methylated CpG regions interfere with the access of transcription factors to the promoter region, thereby silencing the gene. The process of DNA methylation is catalyzed by a family of DNA methyltransferases (DNMT1, DNMT2, and DNMT3). DNMT1 maintain DNA methylation during cell division, it copies DNA methylation

marks from the original to the nascent strand during DNA replication (Tang M, Xu W, Wang Q, Xiao W, & Xu R, 2009). DNMT2 catalyzes methylation of small RNA molecules and has some residual *de novo* DNA methyltransferase activity. The DNMT3 family has three members: DNMT3A, DNMT3B, and DNMT3L. DNMT3A and DNMT3B are responsible for *de novo* methyltransferases that methylate DNA without a template. DNMT3L lacks DNA methyltransferase activity but it enhances DNMT3A and DNMT3B activity (Lee & Pausova, 2013).

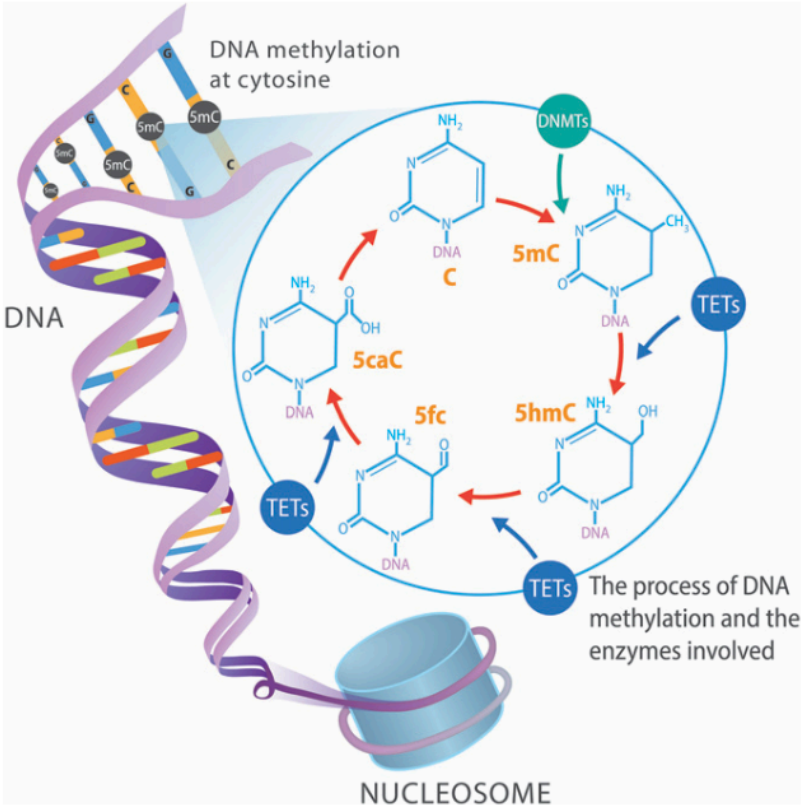



Figure 0.1 DNA methylation. (Adopted from (Larsson L et al., 2015)

DNA methylation is important for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, aging and carcinogenesis. Longitudinal Twin Study showed that there was divergence in DNA methylation patterns due to environmental rather than genetic influences (Wong C.Y et al., 2010). Another study showed loss of DNA methylation during aging (Gonzalo, 2010). Also, DNA methylation has been implicated in the development and progression of cancer mainly through hypermethylation of tumor suppressor genes and hypomethylation of oncogenes (Allis C.D & Jenuwein T, 2016).

Since many human diseases have an epigenetic etiology this has encourage the development of a new therapy "Epigenetic therapy". Many agent have been discovered to alter the DNA methylation and Histone modification and there are currently being tested in clinical trial.

Table 1.2 Epigenetic drugs, the table is adopted from (Egger G et al., 2004).

Table 2 **Epigenetic drugs**

| Target | Drug | Clinical trials |
|---|---|---|
| DNA methylation | 5-Azacytidine | Phase I/II/III |
| | 5-Aza-2′-deoxycytidine | Phase I/II/III |
| | FCDR | |
| | Zebularine | |
| | Procainamide | |
| | EGCG | Phase I |
| | Psammaplin A | |
| | Antisense oligomers | Phase I |
| Histone deacetylase | Many[55], including: | |
| | Phenylbutyric acid | Phase I/II |
| | SAHA | Phase I/II |
| | Depsipeptide | Phase I/II |
| | Valproic acid | Phase I/II |

EGCG, epigallocatechin-3-gallate; FCDR, 5-fluoro-2′-deoxycytidine; SAHA, suberoylanilide hydroxamic acid.

The Epigenetic drug used to target DNA methylation is DNA Methyltransferase Inhibitor: 5-azacytidine (5-aza-CR), 5-aza-2V-deoxycytidine (5-aza-CdR), zebularine, procaine, (-) epigallocatechin-3-gallate (EGCG), and RG108 (Stresemann C, Brueckner B, Musch T, Stopper H, & Lyko F, 2006).

Although DNA methylation has been identified as important components in various physiological and pathological processes, its role in periodontal pathogenesis healing has not yet fully investigated.

I.2 DNA Methylation and Its Effect on the Periodontium

Epigenetic events such as DNA methylation effect gene expression through selective activation or inactivation of genes. DNA methylation induce changes in immune mechanisms, cytokine profile, and thereby contribute to the pathogenesis of inflammatory diseases. Periodontitis is a multifactorial disease characterized by inflammation and destruction of the tooth supporting structures (Socransky SS, 1998). Periodontal diseases are caused by microbial biofilms. In classical studies, the increase in numbers and proportions of the "red complex" species (*Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*) in subgingival biofilms has been highly associated with the presence of periodontitis. Even though periodontitis has a microbial etiology, its progression can be influenced by other factors: systemic diseases, environmental factors, and genetic factors (Kornman K.S, 2008).

It was recognized that pathogens in the oral mucosa may cause epigenetic changes in the host. In patients with squamous cell carcinoma of the head and neck region, bacteria were shown to be associated with methylation of the Multi-Drug Resistance Gene 1 (MDR1) (Bebek G et al., 2012). Infection with *C.rectus* in pregnant mice induced hypermethylation of the murine placenta Igf2 which would affect the development of the newborn (Bobetsis Y.A et al., 2007). Persistent periodontal inflammation can also cause DNA methylation, which inactivates suppressors of cytokine signaling, resulting in exaggerated cytokine production. This lack of suppression renders an individual susceptible the development of a proinflammatory state, increasing the risk for periodontitis (Gomez R.S et al., 2009).

Several studies have investigated the DNA methylation of inflammatory cytokines in chronic periodontitis. *In vitro* study showed that DNA methylation differentially affects gingival cytokine secretion (IL1α, IL6, TNFα, CXCL1) from epithelial cell in response to *P. gingivalis* or

*F. nucleatum* (Drury J.L & Chung W.O, 2015). Analysis of the methylation level of IL-6 in the gingival biopsy of periodontitis patient was similar to the control (Stefani F.A et al., 2013). In contrast, peripheral blood sample from periodontitis patient and rheumatoid arthritis showed lower methylation level of IL-6 compared to the control (Ishida K et al., 2012). Interestingly, a group reported that higher frequency of hypomethylation of IL8 promoter region was identified in oral epithelial cells from chronic periodontitis patients (Oliveira N.F et al., 2009). Regarding TNF-α, gingival biopsy from periodontitis patient showed higher level of DNA methylation at two CpG sites in the TNF-α promotor region compared to the healthy patient. The mRNA expression of TNF-α increase after treatment with 5-aza (Zhang S et al., 2013). Two studies have been investigated the methylation level of interferon (INF- γ). (Zhang et al., 2010) found an increase transcription level of interferon INF- γ and lower DNA methylation in periodontitis patient compared to healthy patient. On the other hand, (Viana M.B et al., 2011) found no different in the methylation status of INF- γ and IL10 between gingival biopsy of healthy and periodontitis patient. Larsson L et al, (Larsson L, Thorbert Mros S, Rymo L, & Berglundh T, 2012) reported three CpG sites in the distal IL-10 promotor region were methylated in both gingival biopsy and peripheral blood sample in periodontitis patient. Also, (Barros S.P & Offenbacher S, 2014) analysis several thousand gene using microarray and found different in DNA methylation level between healthy and inflamed gingiva.

In addition to the methylation level of inflammatory cytokines another gene related to the inflammation have been studies in the periodontitis. The DNA methylation status of Toll – like receptor 2 (TLR2) and TLR4 was studies in gingival biopsy (De Oliveira N.F et al., 2011). TLR2 was methylated and unmethylated in most of the sample. While TLR4 was unmethylated in most sample in all group. Another study report TLR2 methylation in periodontitis group (de Faria

Amormino S. A et al., 2013). There was an increase in the DNA methylation pattern of E cadherin and cyclooxygenase2 (COX-2) for periodontitis and cancer patients (Loo W.T, Jin L, Cheung M.N, Wang M, & Chow L.W, 2010). In addition to that chronic periodontal disease is associated with increased DNA methylation of the prostaglandin endoperoxide synthase 2 (*PTGS2*) promoter, the gene coding for cyclooxygenase (COX-2), resulting in lower level of COX-2 expression (Zhang S et al., 2010). In the periodontal ligament of aged individuals, the decreased expression of collagen α1 gene is associated with DNA hypermethylation at most CpG sites in the proximal and distal regions of the promoter (Ohi T, Uehara Y, Takatsu M, Watanabe M, & Ono T, 2006).

However, relatively little is known about the role epigenetics plays in the progression and development of periodontal disease.

I.3 Epithelial Barrier function

The epithelium of the periodontal pocket or gingival sulcus is the first line of defense against bacterial invasion of periodontal pathogens. Disruptions of gingival epithelial cell barrier allow bacteria and bacterial products to enter into the underlying tissues, activate immune responses and cause bone resorption.

The integrity of epithelial tissue is maintained by several cell-to-cell adhesions and junctions, including tight junctions, adherent junctions, gap junctions and desmosomal junctions (Groeger S.E & Meyle J, 2015). Desmosomal and adherens junction act as anchoring adhesion structure in various tissues including oral epithelial (Nagafuchi A, 2001).

Adherent junction regulates the activity of the entire junctional complex. It is a cell junction where anchoring protein (cadherin and integrins) attached to actin filaments. Cadherin is calcium dependent adhesion molecule directly bind to beta-catenin or plakoglobin, which in turn bind to the α catenin and at the end bind to actin. These adherent junctions are important for epithelial morphogenesis and formation of mature of cell-cell contacts.

Desmosomal junctions are cell-cell adhesive structures that maintain the structural integrity of tissue. Usually it is abundant in tissues that experience mechanical stress such as the epithelium and heart. The components of the desmosome are Desmocollin (Dsc) and desmoglein (Dsg). The transmembrane cadherins bind a group of cytoplasmic proteins termed desmosomal plaque proteins that include plakoglobins (PG), desmoplakins (DP), and plakophilins (PKP). The plaque proteins in turn recruit the intermediate filament cytoskeleton (Keratin) to the transmembrane desmosomal cadherins and assemble the electron dense desmosomal plaque (Stokes D.L, 2007).

Several studies report the effect of periodontal pathogen on the expression of epithelial junctions. Dickinson et al showed that the anaerobic periodontal pathogen *P. gingivalis* was able to invade the oral epithelium and contaminated epithelial cells, while *actinomycetemcomitans* and *F. nucleatum* remained extracellularly. Also, when they measured the transepithelial electrical resistance none of the bacterial species disrupted barrier function (Dickinson B.C et al., 2011). Moreover, *P. gingivalis* is able to invade the deeper structures of connective tissues and decrease the transepithelial resistance through decreases in the amounts of immunoreactive occludin, *E-cadherin*, and 1-integrin which were related to a disruption of cell-cell junctions in MDCK cells (S. V. Katz J, Wu J.H, Michalek S.M, Balkovetzd D.F, 2000). Groeger S et al. have shown a distinct effect on epithelial barrier after infection with *P. gingivalis* due to the virulence factor gingipain (Groeger S, Doman E, Chakraborty T, & Meyle J, 2010). In this study, we will focus mainly on *E-cadherin* and Plakophilin expression after bacteria stimulation (figure 1.2).

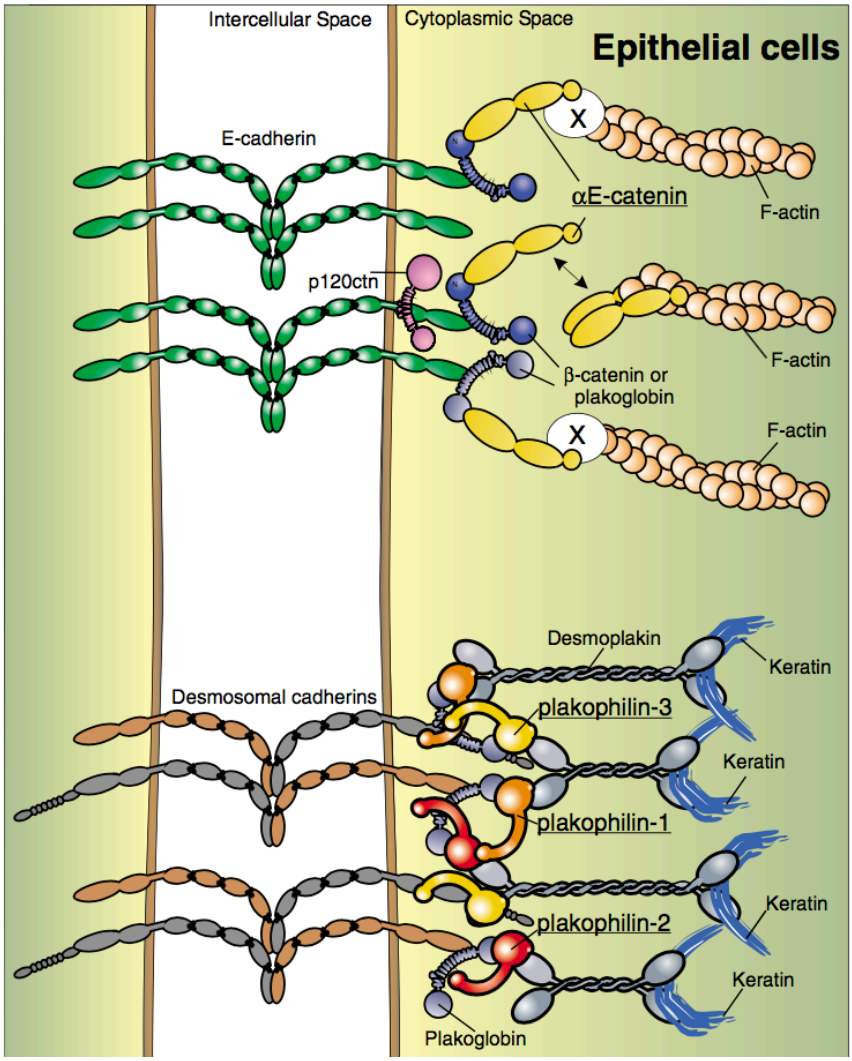



Figure 0.2 Epithelial cell adherent and desmosomal junction (adopted from Goossens S et al (2007)

I.4 *E-cadherin*

*E-cadherin (CDH1)* is a calcium-dependent cell adhesion molecule (Van Roy F & Berx G, 2008). It is considered Type I classic cadherin that mediate a strong cell-cell adhesion (Halbleib J.M & Nelson W.J, 2006). The *E-cadherin* molecule has extracellular, transmembrane and intracellular domains. The cell–cell adhesion function resides in the extracellular domain. The intracellular domain is linked to the actin cytoskeleton via α, β and p120 catenins (Canas-Marques & Schnitt, 2016). Decreased *E-cadherin* gene transcription results in a loss of cell–cell adhesion and increased cell migration (Thiery, 2002). B-catenin protein has an important role in cell adhesion, it also functions in the Wnt signal transduction pathway (Miller & Moon, 1996). In spinous layers of the oral gingival epithelium and sulcular epithelium, *E-cadherin* act as the main transmembranous molecule of adherens junctions (Hatakeyama S et al., 2006). *E-cadherin* work as a tumor suppressor gene (Christofori G & Semb H, 1999).

*E-cadherin* silencing or reduction of expression has been associated with different mechanisms: germ line mutations (Sarrio et al., 2003), single nucleotide polymorphisms (Li, Pan, Guo, & Li, 2014), frame shift and splice site mutations (Droufakou S, 2001), gene deletion, and epigenetic events such as DNA Methylation (Grady W et al., 2000).

Mutations *E-cadherin* (CDH1) are associated with gastric, breast, and colorectal, thyroid, ovarian cancers (Berx G, Becker KF, Höfler H, & FV, 1998; Canas-Marques & Schnitt, 2016; Pharoah, Guilford, & Caldas, 2001). DNA methylation and repression of promoter activity directly regulate *E-cadherin* transcription. Methylation of the *E-cadherin* promoter is associated with reduced *E-cadherin* expression, and with disease progression and metastasis.

*E-cadherin* degradation via an intercellular pathway happen when *C. albicans* invades mucosal tissues (Villar C.C, Kashleva H, Nobile C.J, Mitchell A.P, & Dongari-Bagtzoglou A,

2007). Recent study by Abe-Yutori showed decrease in *E-cadherin* expression in human gingival epithelial cells by *P. gingivalis* - lipopolysaccharide and they were able to show that $H_2O_2$, a major ROS, induced a decrease in *E-cadherin* expression (Abe-Yutori, Chikazawa, Shibasaki, & Murakami, 2016). Another study showed that *P. gingivalis* bacterial protease are capable of degrading epithelial junction transmembrane proteins (occludin, E-cadherin, and 1-integrin) in Madin-Darby canine kidney (MDCK) cells (Katz, Sambandam, Wu, Michalek, & Balkovetz, 2000). Rubinstein et al. (2013) show that *F. nucleatum*, Promotes colorectal carcinogenesis via binding of the adhesion molecule, *FadA*, to E-cadherin, results in downstream activation of NF-Kb (Rubinstein et al., 2013).

I.5 *PKP2*

The Plakophilins (PKP) proteins are a family of desmosomal plaque proteins that link cadherin to the keratin. They are members of the $p120^{ctn}$ family of armadillo related proteins (Hatzfeld M, 2007). Plakophilins have been shown to be important for desmosome formation, clustering, and maturation through interactions with the desmosomal cadherins, desmoplakin, intermediate filaments, and perhaps actin. Any disturbance of plakophilins functions lead to inherited diseases and cancer pathogenesis (Bass-Zubek A.E, Godsel L.M, Delmar M, & Green K.J, 2009). Plakophilins are located at the membrane, in the cytoplasm, and in the nucleus (Koetsier J.L, Amargo E.V, Todorovic V, Green K.J, & Godsel L.M, 2014; Schmidt A et al., 1997). They consist of 4 closely related gene products, PKP 1–4, which contain arm-repeat (armadillo) domains and located at the membrane, in the cytoplasm, and in the nucleus of in all cell types with desmosomal junctions (Koetsier J.L et al., 2014; Schmidt A et al., 1997). PKP1 regulates cell migration and mediates increases in desmosomal protein content. *PKP2* plays an important role in desmosome assembly and mainly expressed in the basal cell layer (Bass-Zubek A.E et al., 2009; Hatzfeld M, 2007). PKP3 is essential for desmosome dependent adhesion and signaling pathways. PKP4 is a component of desmosomal adhesion plaques that regulates junctional plaque organization and cadherin function (Takahashi H et al., 2012). Up to date no data are available if these proteins are integrated into any signaling pathways as shown for other junctional plaque proteins. (Schwarz J et al., 2006)

Mutations in *PKP2* cause a severe inherited heart disease arrhythmogenic right ventricular cardiomyopathy (ARVC) (Hall C, Li S, Li H, Creason V, & Wahl J.K, 2009; Trenkwalder T, Deisenhofer I, Hadamitzky M, Schunkert H, & Reinhard W, 2015). Studies showed that elevated *PKP2* levels are associated with tumor progression of oropharyngeal

cancers (Papagerakis S, AH, Depondt J, Gehanno P, & Forest N, 2003), prostate, colorectal, pancreatic (Schwarz J et al., 2006) and invasive bladder cancer (Takahashi H et al., 2012) but tumor suppressive effects of *PKP2* have not been reported for any cancer cells.

*PKP2* is considered a novel activator of the Epidermal Growth Factor Receptor (EGFR) signaling pathway and a potential new drug target for inhibiting tumor growth (Arimoto K et al., 2014). *PKP2* loss results in a loss of desmoplakin (DP) assembly competence and its failure to accumulate normally at borders (Bass-Zubek A.E et al., 2008). Recent study showed that loss of *PKP2* lead to loss of desmoplakin (DP) which lead to promotes TGF-β1/p38 MAPK- dependent fibrotic gene expression in cardiomyocytes (Dubash A.D et al., 2016)

One of the studies in our lab showed decrease in *PKP2* in periodontitis patients. *P. gingivalis* specifically degrades *PKP2* protein through cysteine proteases, not serine proteases, or intracellular proteasomal or lysosomal degradation pathways. Although *in vitro* stimulations of *P. gingivalis* increase the overall *PKP2* DNA methylation level, periodontitis gingival biopsies do not display differential DNA methylation patterns compared to the healthy biopsies (Yu, 2015). Furthermore, a recent gene wide association study showed a significant association between an SNP variant of *PKP2* (rs6488099) that is located on chromosome 12 locus and periodontal disease (p=1.1x10-8|) (Figure 1.3) (Offenbacher S et al., 2016). From all of this data, further study is needed to understand the role of *PKP2* in periodontal disease. In this study, we aim to investigate the effect of *P. gingivalis* and *C. rectus* and *F. nucleatum* on epithelial barrier function and if it is inducing epigenetic changes to the *PKP2 – E cadherin*.

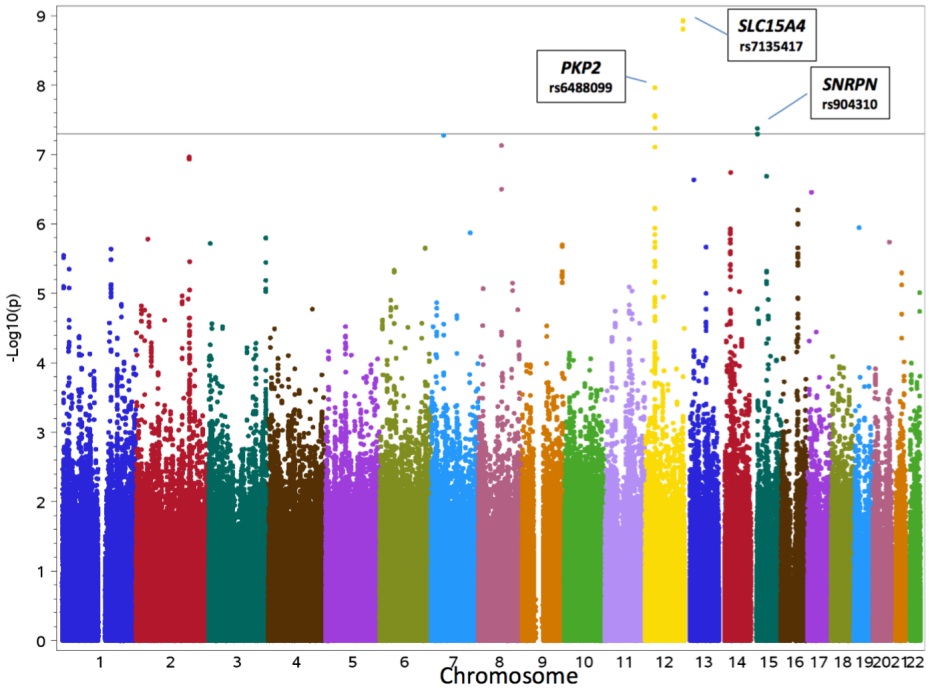



Figure 0.3 Manhattan blot showing *PKP2* SNP on chromosome #12 (adopted from (Offenbacher et al., 2016)

# CHAPTER II: EPIGENETIC AND TRANSCRIPTIONAL DYNAMIC IN PERIODONTAL DISEASE

## II.1 INTRODUCTION

Epigenetics is known today as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"(Wu C.T & Morris J.R, 2001). Epigenetics modification lead to events which chemically modify certain DNA regions, leading to alteration of the chromatin and ultimately silencing or activation of a gene (Barros S.P & Offenbacher S, 2009; Gomez R.S et al., 2009; Larsson L et al., 2015). Epigenetics are influenced by age, environmental factors, such as smoking and diet, and microbial exposures (Barros S.P & Offenbacher S, 2014). Any disruption of the balance of epigenetic mechanism can cause several major pathologies, including cancer, syndromes involving chromosomal instabilities, and mental retardation (Table 1) (Egger G et al., 2004). Various important epigenetic mechanisms include : DNA methylation, histone modifications ( methylation , acetylation , deacetylation) and non-coding RNA (Barros S.P & Offenbacher S, 2009; Egger G et al., 2004; Goldberg A.D et al., 2007; Lod et al., 2014). DNA methylation is associated with gene silencing and is the most studied of the epigenetic processes, especially in the field of cancer research (Barros S.P & Offenbacher S, 2009) (Goldberg A.D et al., 2007; Subramaniam D et al., 2014). DNA methylation induce changes in immune mechanisms, cytokine profile, and thereby contribute to the pathogenesis of inflammatory disease. Epigenetic changes unlike genetic changes, it can be reversed by pharmacological intervention such as 5-aza (DNMT inhibitor)

which is FDA approved for treatment of myelodysplastic syndrome (Kaminskas E, Farrell A.T, Wang YC, Sridhara R, & Pazdur R, 2005)

Also, recently it has been recognized that periodontal pathogens in the oral mucosa may lead to epigenetic changes in the host tissues. In studies involving patients presenting with squamous cell carcinoma of the head and neck region, bacteria were shown to be associated with methylation of the Multi-Drug Resistance Gene 1 (MDR1) .Our group was the first to show in mouse model, that maternal infection with specific periodontal pathogen, *C.rectus*, could induce hypermethylation of Igf2 investigated in the murine placenta, with associated downregulation of the specific gene and fetal growth restriction, with potential interference in the development of the offspring (Bobetsis Y.A et al., 2007).

Persistent periodontal inflammation can also cause DNA methylation, which inactivates suppressors of cytokine signaling, resulting in exaggerated cytokine production, this lack of suppression renders a susceptible individual the development of a proinflammatory state, increasing the risk for periodontitis (Gomez R.S et al., 2009). Several studies have investigated the DNA methylation of inflammatory cytokines and other genes related to the inflammation in chronic periodontitis such as hypermethylation of TNF-a (Zhang et al., 2010), TLR2 (de Faria Amormino S. A et al., 2013), *E-Cadherin*, COX-2 (Loo W.T, Jin L, Cheung M.N, Wang M, & Chow L.W, 2010), *PTGS2* (Zhang S et al., 2010) in periodontitis patient. *In* vitro study showed that DNA methylation differentially affects gingival cytokine secretion (IL1α, IL6, TNFα, CXCL1) from epithelial cell in response to *P. gingivalis* or *F. nucleatum* (Drury J.L & Chung W.O, 2015).

However, the role that epigenetics plays in the progression and development of periodontal disease is still under investigation (Barros S.P & Offenbacher S, 2014) .

The oral epithelium plays an important role in host defense against pathogens either by providing a physical barrier against bacterial challenge and invasion or activator of the innate immune responses. The integrity of epithelial tissue is maintained by several cell-to-cell junctions, including tight junctions, adherent junctions, gap junctions and desmosomal junctions. Furthermore, a recent gene wide association study showed a significant association between an SNP variant of *PKP2* (rs6488099) that is located on chromosome 12 locus and periodontal disease ($p=1.1x10^{-8}$) (Offenbacher S et al., 2016).

The Plakophilins (PKP) proteins are a family of desmosomal plaque proteins that link cadherin to the keratin. They are members of the $p120^{ctn}$ family of armadillo related proteins (Hatzfeld M, 2007). Plakophilins have been shown to be important for desmosome formation, clustering, and maturation through interactions with the desmosomal cadherins, desmoplakin, intermediate filaments, and perhaps actin. Any disturbance of plakophilins functions lead to inherited diseases and cancer pathogenesis (Bass-Zubek A.E et al., 2009). Plakophilins are located at the membrane, in the cytoplasm, and in the nucleus of in all cell types with desmosomal junctions (Koetsier J.L et al., 2014; Schmidt A et al., 1997). They consist of 4 closely related gene products, PKP 1–4, which contain arm-repeat (armadillo) domains and are localized to nuclei and cell desmosomes. PKP1 regulates cell migration and mediates increases in desmosomal protein content. *PKP2* plays an important role in desmosome assembly and mainly expressed in the basal cell layer (Bass-Zubek A.E et al., 2009; Hatzfeld M, 2007). Mutations in *PKP2* cause a severe inherited heart disease arrhythmogenic right ventricular cardiomyopathy (ARVC) (Hall C et al., 2009; Trenkwalder T et al., 2015). Studies showed that

elevated *PKP2* levels are associated with tumor progression of oropharyngeal cancer (Papagerakis S et al., 2003), colorectal, pancreatic (Schwarz J et al., 2006) and invasive bladder cancers (Takahashi H et al., 2012), but tumor suppressive effects of *PKP2* have not been discussed for any cancer cells. Up to date no data are available if these proteins are integrated into any signaling pathway as shown for other junctional plaque proteins. (Schwarz J et al., 2006).

In this study, we aim to investigate the effect of *Periodontal pathogen* on oral epithelial barrier function and if it is inducing epigenetic changes to the *PKP2* or *E-cadherin* and what is the potential effects of DNA methyltransferase (DNMT) inhibitors on epithelial barrier function.

## II.2 AIMS

I. To determine whether infection of human gingival epithelial (HGE) cells with *P. gingivalis, C. rectus* and *F. nucleatum* are associated with altered epithelial barrier permeability.

II. To determine the methylation status of *PKP2 and E-cadherin* after infection of HGE cell with *P. gingivalis, C. rectus* and *F. nucleatum*.

III. Investigate the potential effects of DNA methyltransferase (DNMT-1) inhibitors on epithelial barrier function in response to infection with periodontal pathogen in human gingival epithelial cells.

## II.3 HYPOTHESES

It is hypothesized that infection of HGE cells with periodontal pathogen are able to induce epigenetic changes which, alter the epithelial barrier function and if there is epigenetic change we can prevent it using targeted epigenetic therapy.

## II.4 MATERIALS AND METHODS:

### II.4.1 Cell and Bacterial Culture

Human gingival epithelial cells (HGE) were purchased from CellnTec and maintained according to the manufacturer's instructions. All cultures would be maintained in a 5% $CO_2$, 100% humidity, 37°C incubator and used between passages 3 and 6.

*Porphyromonas gingivalis (P. gingivalis)* A7436 and *Fusobacterium nucleatum (F. nucleatum) 25586 were* cultured anaerobically at 37ºC in Wilkins-Chalgren anaerobe broth medium (Thermo Scientific) for 3-4 days. Cell number measured via the optical density. When the optical density reaches 0.5, the concentration of *P. gingivalis or F. nucleatum* is equal to $10^9$ CFU/ml.

*Campylobacter rectus (C. rectus)* strain 314 was cultured anaerobically in Enriched Tryptic Soy Agar (ETSA) plate (Anaerobic System) for 4-5 days. When the optical density reaches 1.0, the concentration of *C. rectus* is equal to $10^9$ CFU/ml. Upon the *In vitro* stimulation, bacteria were pelleted at a speed of 6000g for 15mins, washed with 1X PBS twice, and reconstituted in the cell culture medium. For the heat kill *P. gingivalis* it was inactivated for 30 minutes at 60°C before infection of HGE.

II.4.2 Cell viability

Human gingival epithelial cells (HGE) were plated in 96-well plates at densities ranging from 10,000 to 30,000 cells $cm^{-2}$. The cells were allowed to attach for 24h, and then either RG108 (N-Phthalyl-L-tryptophan) was added at 1, 5, 10,15 μM or ECGC (Epigallocatechin gallate) was added at 10, 25, 50 μM for 24h. CellTiter-Blue® Cell Viability Assay (Promega) was used to determine an optimum cell density and maximum inhibitor concentration at which cell viability is maintained.

II.4.3 Transepithelial electrical resistance (TER)

HGE were seeded in a 12mm Trans well with 0.4um Pore Polyester Membrane Insert (Costar). After 24 hours, the cells were attached to the membrane surface of the insert, which is oriented so that and the apical part of the cell is oriented upwards and the basal parts of the cells are towards the membrane surface of the insert. Transepithelial electrical resistance (TER) was measured using EVOM2 Epithelial Voltammeter at different time point: 2h, 4h, 6h, 8h and 24h. Background resistance from cell free membranes will be subtracted from initial resistance values.

II.4.4 RNA Isolation and Quantitative Real-Time Reverse Transcription PCR

RNA was isolated from HGE cells using RNeasy Mini kit (Qiagen, Valencia, CA). The RNA was quantified using the Nano Drop (Thermo Scientific, Wilmington, DE). For each sample, a volume of 300 ng of RNA was used to generate complementary DNA (cDNA) through reverse transcription reactions using the using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen TM, Life Technologies TM, Grand Island, NY). Quantitative real-time PCR (qRT-PCR) was performed using 2µL of synthesized cDNA, 7.5µL of Taqman® Universal PCR Master Mix, .75µL of Taqman® Gene Expression Assay Mix for *PKP2* or CDH1 gene and 5.5µL of ddH2O in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Carlsbad, CA). mRNA expression level was normalized using GAPDH as a housekeeping gene.

II.4.5 DNA Isolation, Selective Digestion and Quantitative Real-Time PCR

Genomic DNA was isolated from HGE using a DNeasy Mini Kit (Qiagen, Valencia, CA). The DNA was quantified using the NanoDrop (Thermo Scientific, Wilmington, DE). 500

ng of genomic DNA isolated from HGE cells was digested using EpiTect Methyl II DNA Restriction Kit (Qiagen Cat# 335452). DNA was aliquot into four equal portions. Each portion was subjected to a different reaction: Mock digest (no digestion), methylation sensitive digest (digestion of unmethylated and partially methylated DNA only), methylation dependent digest (digestion of methylated DNA only), and double digest (digestion of both methylated and unmethylated DNA). DNA digestion was allowed to take place at 37°C for 6 hours to overnight. Following the digestion inactivate the enzymes at 65° C for 20 minutes. Digested DNA was then quantified by qPCR using EpiTect Methyl II PCR Primer Assay for Human *PKP2* (CpG Island 103034) and Human *CDH1* (cadherin 1, type 1, *E-cadherin*) (CpG Island 105415). Quantitative Real time -PCR (qRT-PCR) was performed using 12.5µL of RT SYBR Green qPCR Master Mix, 1µL of PCR primer mix for *PKP2* or *CDH1* gene, 1µL of each digested DNA and 10.5µL of dd$H_2O$ in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Carlsbad, CA). Data analysis was done using integrated Excel-based templates provided by the manufacturer, which provide gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. Unmethylated (UM) represents the fraction of input genomic DNA non-methylated CpG sites in the amplified region of a gene. Methylated (M) represents the fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene. A relative methylation fold change to controls can be reported. The promotor region of *PKP2* gene was evaluated in present study (UniGene No: Hs.164384; NCBI ID: 5318). The location of CpG island is Chr12: 33049301 – 33050098 (Figure II.1). The promotor region of *E-cadherin* gene was evaluated in present study (UniGene No: Hs.461086; NCBI ID: 999). The location of CpG island is: Chr16: 68770974 – 68772344 (Figure II.2)


Figure 0.1 *PKP2* (CpG Island 103034): Chr12: 33049301 – 33050098

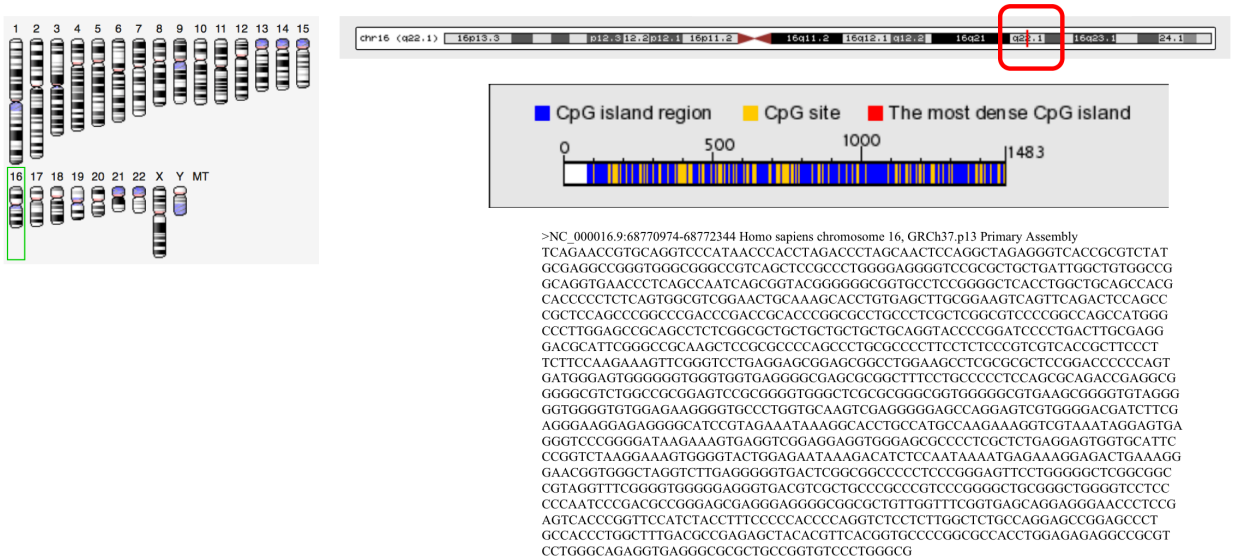



Figure 0.2CDH1 (CpG Island 105415): Chr16: 68770974 – 68772344

II.4.6 Immunofluorescence staining (IF)

HGE cells will be seeded in 35mm glass culture dishes (MatTek). At the end of the stimulation, cells were fixed with 4% paraformaldehyde for 10 minutes, Permeablized with 0.1% Triton X-100 in PBS for 30 mins, and blocked with 1% BSA/PBS/0.1% Triton X-100 at RT for 1h. Primary antibody Anti *PKP2* (1:400 dilutions, Santa Cruz, sc-393711).

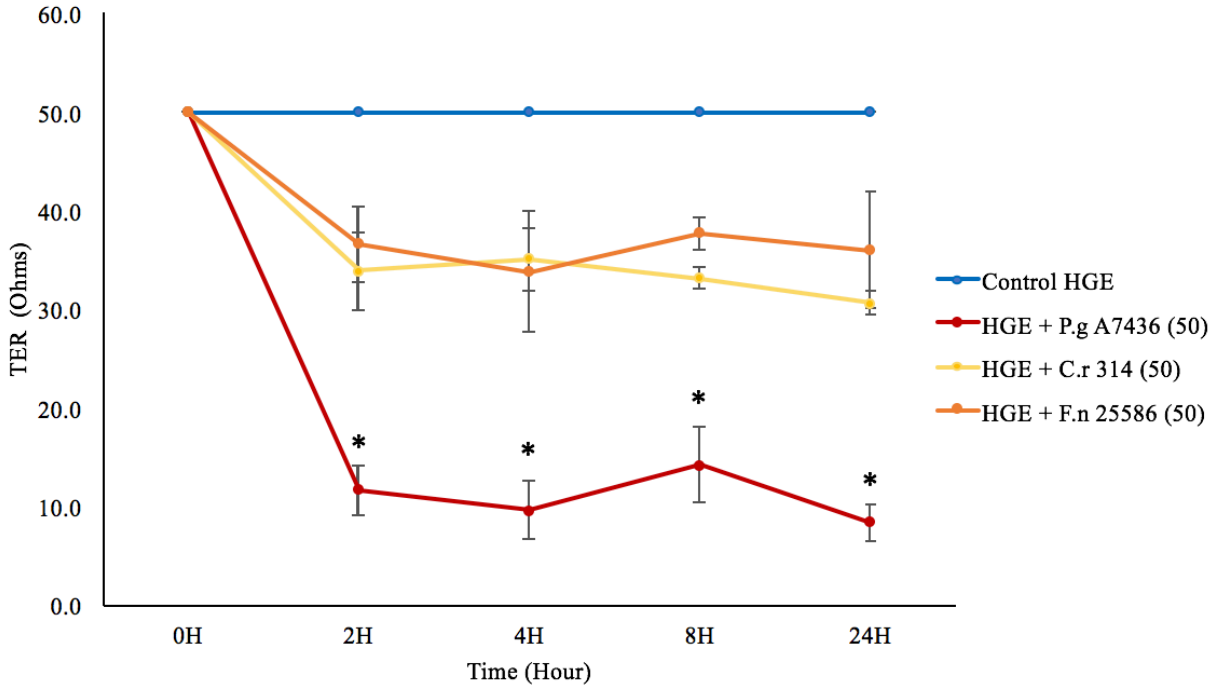
II.4.7 Statistical Analysis

Changes of TER were tested using the ANCOVA. The one-way ANOVA test was applied for comparison in viability, mRNA expression and DNA methylation among various groups. The threshold for statistical significance was set at a p-value $<0.005$. All analyses were performed with SAS 9.2 (SAS Institute, Cary, NC, USA).

## II.5 RESULTS:

II.5.1 Effect of *P. gingivalis*, *C. rectus* and *F. nucleatum* infection on epithelial barrier function:

The permeability of the epithelial barrier is commonly assessed by measuring TER. It is a measure of the barrier to small ions (predominantly Na and Cl) in an experimentally applied electrical field in the bathing medium. Assessment of the TER after infection HGE cells with different periodontal pathogen provided information on the ability of this pathogen to alter the epithelial barrier function. Changes in TER after infection of HGE with *P. gingivalis, C. rectus* and *F. nucleatum* were seen (Figure II.3). At the first 2 hour, an acute drop of TER can be observed after apical infection of HGE with *P. gingivalis* and *C. rectus* and *F. nucleatum* but it was statistically significant with *P. gingivalis* (p-value <0.0001). Exposure of HGE to *P. gingivalis* decreased the TER in a time dependent manner and it was statistically significant over 24 h (p-value <0.0001). *For C. rectus* and *F. nucleatum* It can be seen that after 2 h the system equilibrated but it did not go back 100% to the normal barrier function. These data indicate that *P. gingivalis* was able to invade HGE and disrupt the barrier function.




*P value <.0001 compare to the control

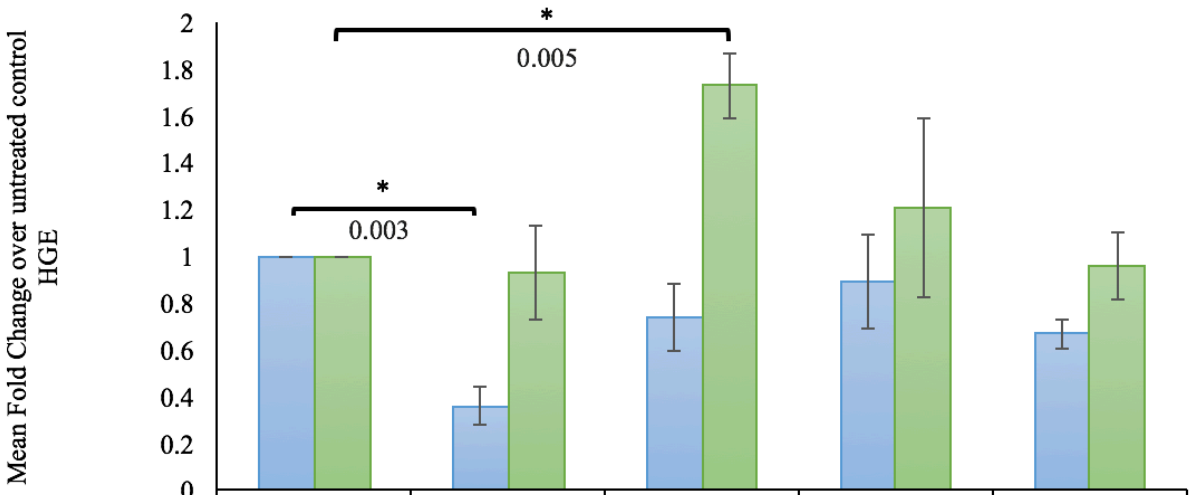
Figure 0.3 The adjusted mean TER of human gingival epithelial (HGE) after apically infected with P. gingivalis A7436, *C. rectus* 314 and *F. nucleatum* 25586 for 2h, 4h, 8h and 24h

The results are expressed in mean of TER and standard deviations, the baseline TER of was adjusted to the control.

II.5.2 mRNA Expression of *PKP2* and E-cadherin after infection with *P. gingivalis*, *C. rectus* and *F. nucleatum* infection:

The impact of *P. gingivalis, C. rectus* and *F. nucleatum* infection on mRNA expression of *PKP2* and *E-cadherin* gene in HGE was evaluated. Infection duration for 24 h was tested. mRNA Expression of *PKP2* in response to infection with live *P. gingivalis* show a statistically significant down-regulation (p-value <0.0001) (Figure II.4). While infection with *C. rectus* and *F. nucleatum* showed a slight down-regulation but it was not statistically significant. The fold changes for the HGE infected with live *P. gingivalis,* heat killed *P. gingivalis, C. rectus* and *F. nucleatum* were 0.36±0.08, 0.74±0.14,0.89±0.2 and 0.67±0.06 respectively. Results suggest that decrease epithelial *PKP2* mRNA is associated with live *P. gingivalis.*

For *E-cadherin*, the mRNA Expression of *PKP2* in response to infection with live *P. gingivalis,* and *F. nucleatum* shows no different compare to the control (p-value >0.05). On the other hand, Infection with heat kill *P. gingivalis* and *C. rectus* shows upregulation of *E-cadherin* but it only was significant for the heat kill *P. gingivalis* (p-value <0.005).



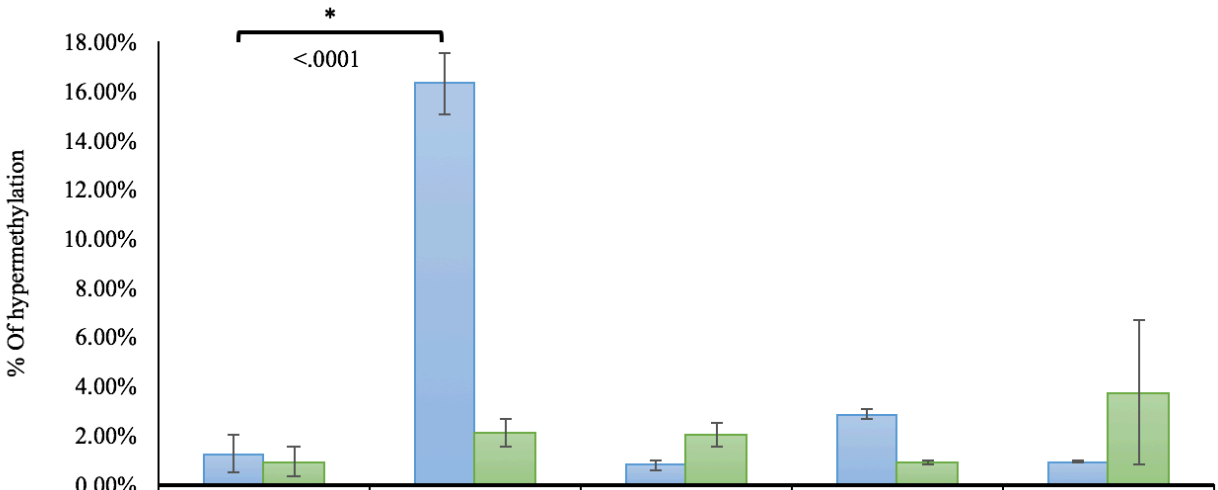

| | Control HGE | HGE + P.g A7436 (50) | HGE + HK P.g A7436 (50) | HGE + C.r 314 (100) | HGE + F.n 25586 (50) |
|---|---|---|---|---|---|
| PKP2 | 1 | 0.36 | 0.74 | 0.89 | 0.67 |
| E-CADHERIN | 1 | 0.93 | 1.73 | 1.21 | 0.96 |

Figure 0.4 mRNA Level of *PKP2* and *E-cadherin* after infection with live and heat kill *P. gingivalis* A7436, *C. rectus* 314 and *F. nucleatum* 25586 at MOI 50 for 24h

II.5.3 Effect of *P. gingivalis*, *C. rectus* and *F. nucleatum* infection on *PKP2* and *E-cadherin* Global DNA methylation

We want to check the role of the bacteria (*P. gingivalis*, *C. rectus* and *F. nucleatum*) in inducing epigenetic changes as DNA methylation. In HGE infected with live *P. gingivalis* there was a statistically significant increase in the hypermethylation level of the *PKP2* promotor (p-value<.0001) compared to the control non-infected HGE samples (Figure II.5). On the other hand, infection of HGE with *C. rectus or F. nucleatum* showed no increase in the methylation level of the *PKP2*. The percentages of hypermethylation in the control non-infected, HGE infected with live *P. gingivalis* and heat killed *P. gingivalis, C. rectus* and *F. nucleatum* were 1.24± 0.01, 0.77±0.002, 16.32±0.01, 2.83± 0.002 and 0.9 ±.0003 respectively. For E- cadherin,

there was no increase in the methylation level after infection with *P. gingivalis*, *C. rectus* or *F. nucleatum.* The percentages of hypermethylation in the control non-infected, HGE infected with live *P. gingivalis* and heat killed *P. gingivalis, C. rectus* and *F. nucleatum* were 0.09± 0.006, 2.00±0.005, 2.1±0.006, 0.9± 0.001 and 3.7 ±.02 respectively. Results suggest that live *P. gingivalis* is able to cause a DNA methylation and effect gene expression of *PKP2* which was confirm by mRNA expression of *PKP2*.




| | Control HGE | HGE + P.g A7436 (50) | HGE + HK Pg A7436 (50) | HGE + C.r 314 (50) | HGE + F.n (50) |
|---|---|---|---|---|---|
| PKP2 | 1.24% | 16.30% | 0.77% | 2.83% | 0.90% |
| E- cadherin | 0.9% | 2.10% | 0.020 | 0.9% | 3.7% |

*P value <.0001

Figure 0.5 Mean percentage of DNA hypermethylation for *PKP2* / *E-cadherin* gene in response to infection with P.g A7436, *C. rectus* 314 and *F. nucleatum* 25586 at MOI 50 for 24h

II.5.4 DNMT-1 inhibitors concentration optimization

Experimental optimization was completed to ensure cell viability and health (Figure II.6) shows the results of HGE exposure to a range of concentration of RG108 and EGCG for an optimal seeding density of 20 000 cells $cm^{-2}$. The stars indicate the maximum inhibitor concentration in which viability was statistically significant affected compared to untreated cultures ($p > 0.05$). The arrow indicates the maximum inhibitor concentration used in all subsequent experiments, 10 µM for RG108 and EGCG.

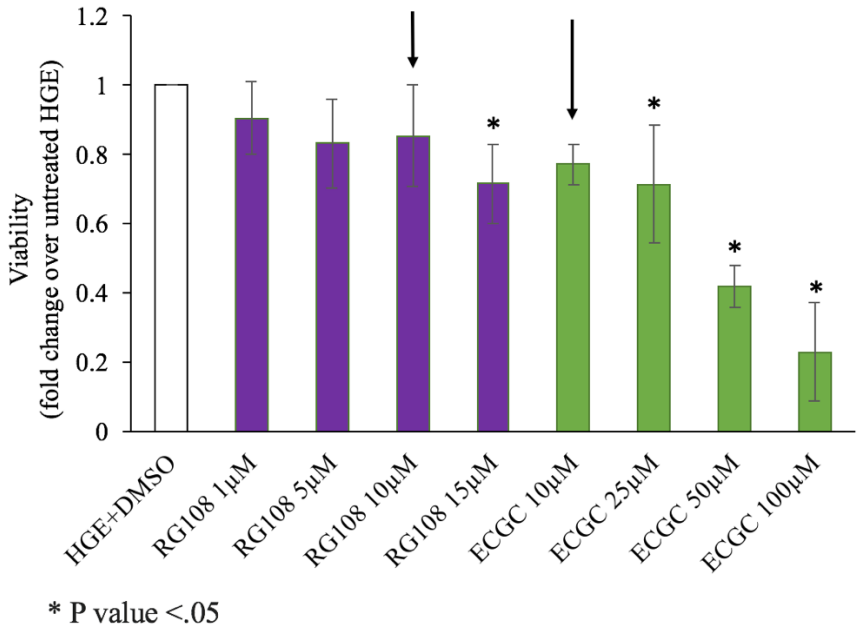



Figure 0.6 HGE viability following Inhibitor Treatment

**II.5.5 Effects of DNA Methylation Inhibitors on Epithelial barrier function**

HGE expression of *PKP2* was assessed to determine if DNMT-1 inhibitors were affecting the methylation status on the responsiveness to HGE to bacterial challenges. We repeat the TER experiment after treatment with either RG108 or EGCG alone (Figure II.7). TER result showed improve in the TER of the pretreated HGE with DNMT-1 inhibitors compare to the HGE

infection with *P. gingivalis* (p-value <0.005). Also, the mRNA expression of *PKP2* was significantly increase (p-value <0.001) when HGE were pretreated with DNMT-1 inhibitors then exposed to *P. gingivalis* (Figure II.8). The fold changes for the HGE pretreated with RG108 or EGCG then exposed to live *P. gingivalis* were 2.17±0.14 and, 1.51±0.05 respectively. In addition to the increase in the *PKP2* expression, pretreatment of HGE with DNMT-1 inhibitors followed by *P. gingivalis* stimulation result in statistical significant decrease in the methylation level of *PKP2* compare to the HGE infected with live *P. gingivalis.* The percentage of hypermethylation for the HGE pretreated with RG108 or EGCG then exposed to *P. gingivalis* were reduce *to* 1.5±0.001, 0.4±0.003 respectively (Figure II .9). These results suggest a protective effect of DNMT-1 inhibitors on the epithelial barrier function against *P. gingivalis*.

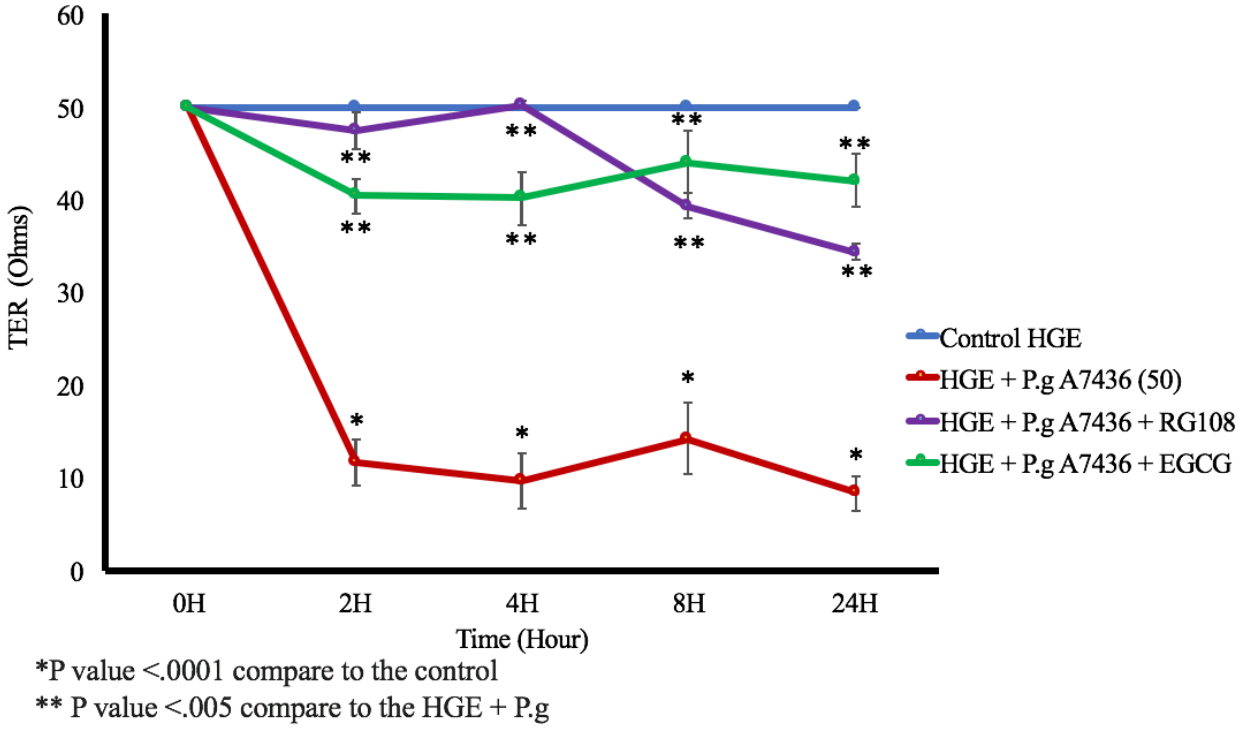



Figure 0.7 Time response of TER of human gingival epithelial (HGE) after pretreat the HGE with RG108 or EGCG then exposed to *P. gingivalis* A7436 at MOI 50 for 2h, 4h, 8h and 24h

The results are expressed in mean % and standard deviations, the baseline TER of was adjusted to the control.

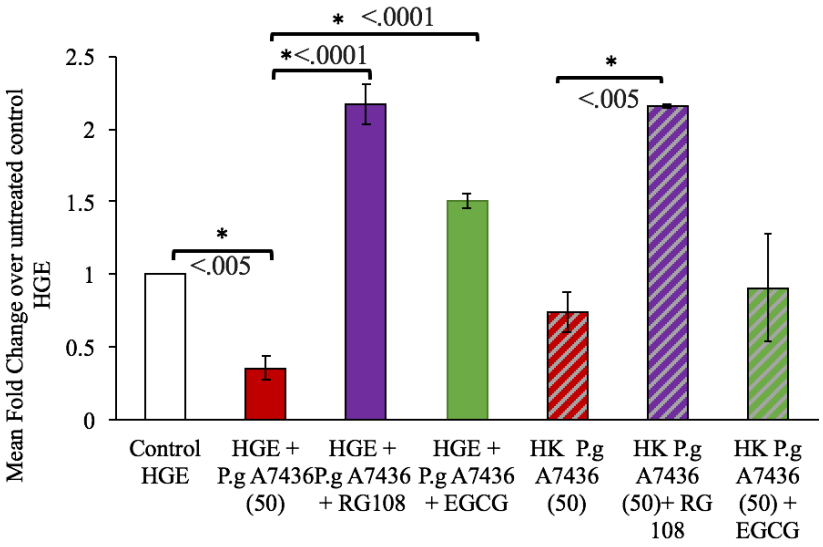



Figure 0.8 mRNA Level of *PKP2* after pretreat the HGE with RG108 or EGCG then exposed to *P. gingivalis*.

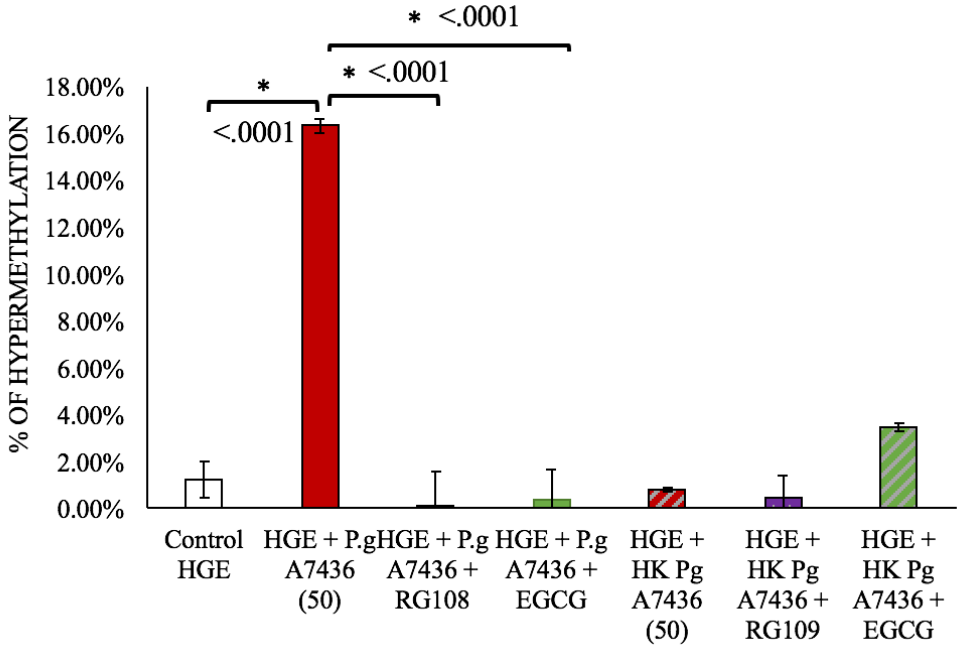



Figure 0.9 Mean percentage of DNA hypermethylation for *PKP2* after pretreated the HGE with RG108 or EGCG then exposed to *P. gingivalis*.

II.5.6 Immunofluorescence studies.

Control non-infected HGE cells showed uniform staining for *PKP2* (Figure II. 10) while HGE infected with *P. gingivalis* or *F. nucleatum* showed decrease in immunofluorescence labeling intensity. While, pretreatment with DNMT-1 inhibitors followed by *P. gingivalis stimulation* showed increase in immunofluorescence labeling intensity of *PKP2*.

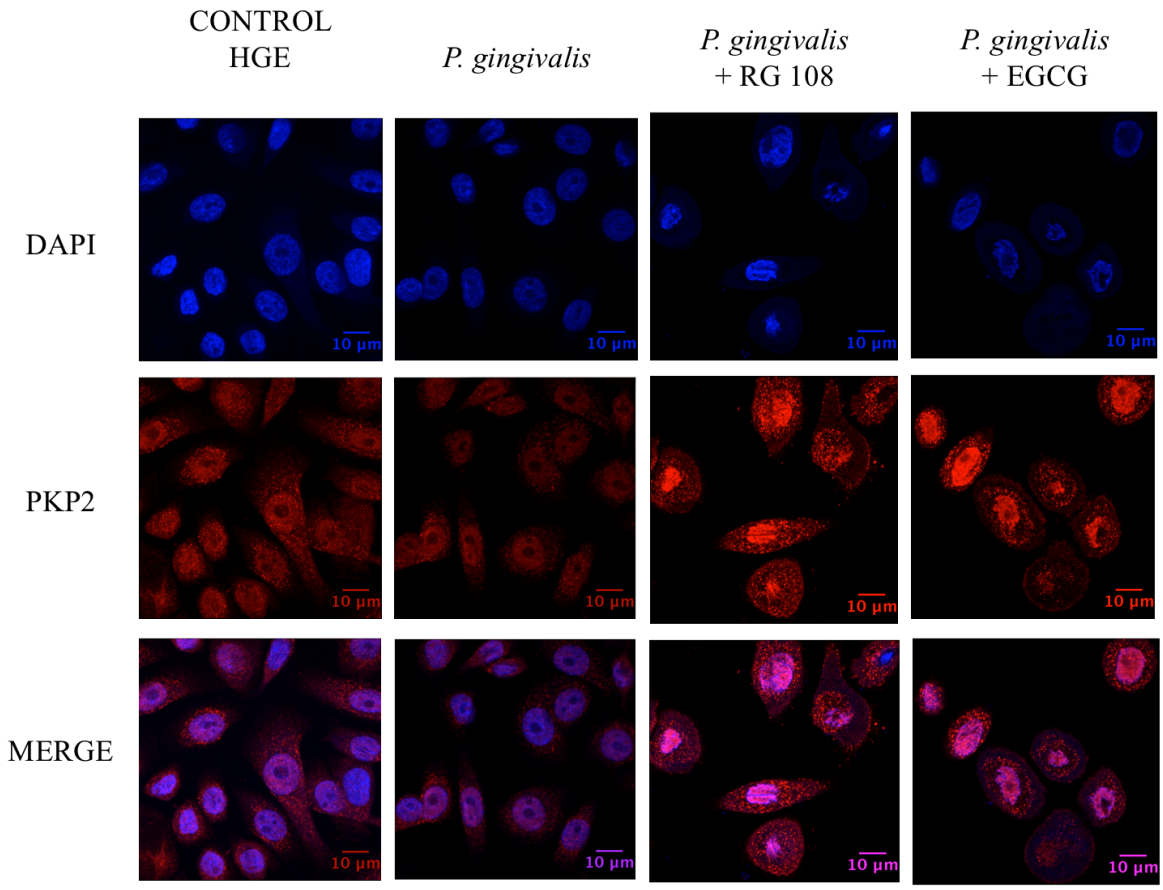



Figure 0.10 Immunofluorescent staining of *PKP2*

## II.6 DISCUSSION

The Oral epithelium plays an important role in host defense against pathogens either by providing a barrier against bacterial challenge and invasion or activator of the innate immune responses. In the present study, we investigated the effects of the periodontal pathogen *P. gingivalis* on *PKP2*, which are important desmosomal protein involved in cell adhesion and in maintaining the integrity of the oral epithelium. Also, we want to check if the epithelial barrier function affected through *PKP2* that may be under epigenetic control. Our approach involved treating HGE with known inhibitors of methylation and then exposing both treated and untreated cells *P. gingivalis*. Differences in response indicate the potential for epigenetic control. First, we measured the TER of HGE which we believe it would give us an estimation of the integrity of epithelial barrier function especially *PKP2*. Since Sato P.Y et al, showed that loss of Plakophilin-2 expression result in decreased sodium current and slower conduction velocity in cultured cardiac myocytes (Sato P.Y et al., 2009) and the TER is a measure of the barrier to small ions (predominantly Na and Cl) in an experimentally applied electrical field in the bathing medium (Fanning A.S, Mitic L.L, & Anderson J.M, 1999). In the present study, we have shown a statistically significant decrease in the TER of HGE cells within 2 h after exposure to *P. gingivalis*. The 2h initial invasion time for *P. gingivalis* was consistent with previous study which report 90 min for *P. gingivalis* invasion (Chung W.O et al., 2001; Lamont RJ et al., 1995). The change in TER after infection with *C. rectus* and *F. nucleatum* was not significant. The result was consistent with Dickinson et al finding where he showed that *P. gingivalis* was able to invade the oral epithelium and contaminated epithelial cells, while *actinomycetemcomitans* and *F. nucleatum* remained extracellularly (Dickinson B.C et al., 2011) . On the other hand, when they measured the transepithelial electrical resistance none of the bacterial species disrupted

barrier function (Dickinson B.C et al., 2011*)*. Katz et al demonstrated that *P. gingivalis* is able to invade the deeper structures of connective tissues and decreasing the transepithelial resistance in MDCK cells (Katz J, Sambandam V, WU J.H, Michalek S.M, & Balkovetz D.F, 2000). Also, Groeger S et al demonstrated that due to the virulence factor gingipain, *P. gingivalis W83* At an MOI of $10^4$ showed a decrease in the TER after 2 h (Groeger S et al., 2010).

Epigenetic events such as DNA methylation effect gene expression through selective activation or inactivation of genes. DNA methylation induce changes in immune mechanisms, cytokine profile, and thereby contribute to the pathogenesis of inflammatory diseases. Periodontitis is a multifactorial disease that engages many mechanisms, several papers have reported that DNA methylation is involved in regulating key genes in periodontal disease (Barros S.P & Offenbacher S, 2014; Gomez R.S et al., 2009; Larsson L et al., 2015). Zhang et all, showed higher level of DNA methylation at two CpG sites in the TNF-α promotor region TNF-α, of gingival biopsy from periodontitis patient compared to the healthy patient. The mRNA expression of TNF-α increase after treatment with 5-aza (Zhang S et al., 2013). Two studies have been investigated the methylation level of interferon (INF- γ). Zhang et all, (Zhang et al., 2010) found an increase transcription level of interferon INF- γ and lower DNA methylation in periodontitis patient compared to healthy patient. On the other hand, Viana et all, (Viana M.B et al., 2011) found no different in the methylation status of INF- γ and IL10 between gingival biopsy of healthy and periodontitis patient. Other evidence report methylation level of IL8 (Oliveira N.F et al., 2009), IL-10 (Larsson L et al., 2012), Toll like receptor 2 (TLR2) (de Faria Amormino S. A et al., 2013); De Oliveira N.F et al. (2011), *E-cadherin* and cyclooxygenase2 (COX-2) (Loo W.T et al., 2010), prostaglandin endoperoxide synthase 2 (*PTGS2*) (Zhang S et al., 2010) and collagen α1(Ohi T et al., 2006) in periodontal disease. We found a statistically

significant increase in DNA methylation status of *PKP2* gene in HGE infected with live *P. gingivalis* and decrease in mRNA expression. Heat-killed *P. gingivalis* have no effect on *PKP2*, which suggests that *PKP2* protein is affected by *P. gingivalis* products, which are likely to be proteases. *F. nucleatum* decrease *PKP2* mRNA expression but it did not increase the methylation of *PKP2*, which suggest that *F. nucleatum* affecting HGE through different mechanism than *P. gingivalis*. *PKP2* is an important protein for desmosome assembly and epithelial cell integrity. *PKP2* loss results in a loss of desmoplakin (DP) assembly competence and its failure to accumulate normally at borders (Bass-Zubek A.E et al., 2008). Recent study showed that loss of *PKP2* lead to loss of desmoplakin (DP) which lead to promotes TGF-β1/p38 MAPK- dependent fibrotic gene expression in cardiomyocytes (Dubash A.D et al., 2016).

In the present study, the CpG analyzed in the promotor region of PKP2 gene are situated close to important transcription site, like NF-kb, SP-1, FOXp3, P53 (Figure 2.11). NF-kb play a central role in inflammation through it is ability to induce the transcription of pro-inflammatory gene (Barnes P.J & Karin M, 1997). Sp-1 regulates the expression of a large number of genes involved in a variety of processes such as cell growth, apoptosis, differentiation and immune responses (Kadonaga J.T, Masiarz F.R, & Tjian R, 1987). These data suggest that *PKP2* may play an important role in maintaining epithelial integrity and may play an important protective role in the pathogenesis of periodontal disease.

For *E-cadherin* there was no decrease in the expression of *E-cadherin* after infection with different periodontal pathogen. We did not expected a decrease the expression as decrease *E-cadherin* expression is characteristic of cancer (Han Y.W, 2014; Kowalski P.J, Rubin M.A, & Kleer C.G, 2003; Rubinstein et al., 2013). Our finding is consistent with Belibasakis work were he analysis the expression of gingival epithelial junction in response to subgingival biofilms (red

complex) and in his finding there was no decrease in the expression of *E-cadherin* (Belibasakis G.N, Kast J.I, Thurnheer T, Akdis C.A, & Bostanci N, 2015). There are only two study report decrease in *E-cadherin* expression in human gingival epithelial cells by *P. gingivalis* (Abe-Yutori et al., 2016; Katz et al., 2000). In both study, they treat the epithelial cell with different strain of *P. gingivalis* for 48h and they observe the expression of *E-cadherin* with immunofluorescent staining. Furthermore, pretreatment of HGE with DNMT-1 inhibitors (RG108-EGCG) followed by *P. gingivalis* stimulation result in improve barrier function, since there was no change in the TER increase the mRNA expression of *PKP2* and decrease the methylation level compared to *P. gingivalis* infection. Therefore, we could suggest that DNMT-1 inhibitors can improve the epithelial barrier function. Recent study by Wan et all, reported that EGCG reduced bacterial translocation through the enhancement of the intestinal epithelial barrier function by inducing secretion of antimicrobial peptides. Also, they conclude that EGCG can be used to prevent intestinal disorders or bacterial infection in animals/humans (Wan M.L, Ling K.H, Wang M.F, & El-Nezami H, 2016). Both DNMT-1 inhibitors (RG108, EGCG) we used in this study are non-nuclease with no genotoxicity compared to 5-aza (Stresemann C et al., 2006). Unfortunately, there is limited research has been done using DNMT-1 inhibitors as treatments for periodontitis. These drugs are able to target epigenetic modification can be used to personalized treatment and preventive periodontal disease, the challenge with this approach is to specifically target the epigenetic marks which have a negative influence on the gene, leaving alone the beneficial one that help maintain health.

A) ATC**CG**GAC**CG**TCCTGGGCCAGCAGATCCTGGGACAACTGGACAGCTCCAGCCTGG**CG**CTGCCCT

NF-κB

C**CG**AGGCCAAGCTGAAGCTGG**CG**GGGAGCAG**CG**GC**CGCGGCG**GCCAGACAGTCAAGAGCCTG

**CG**GATCCAGGAGCAGGTGCAGCAGACCCT**CG**CC**CG**GAAGGGC**CG**CAGCTC**CG**TGGGCAA**CG**G

NF-κB Sp1

TGAGTGGAGCC**CG**C**CG**CTCCC**CGG****CG**C**CG**CCCCCTGCCCCACACC**CG**GTCACCTCCTCCTATCC

Sp1

**CG**CCCTCACCC**CGCG**TGCTGGGAGTG**CG**GGACCC**CG**TGTTCTGCCCACC TGTTGCT**CG**C

B)

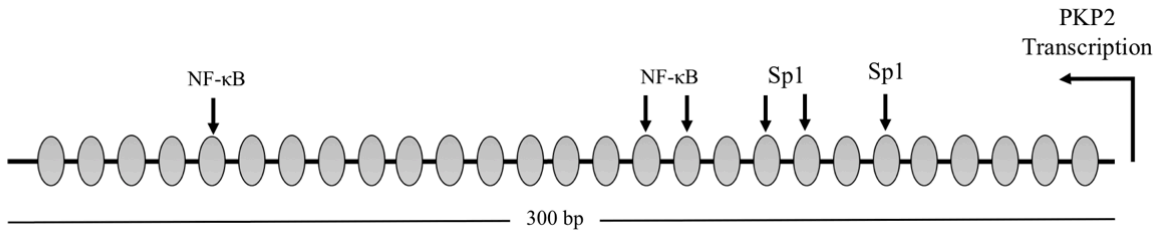



*Figure 0.11* Human *PKP2* GENE A) Genomic sequence of the *PKP2* 300 bp from promoter region. Sites for potential transcriptional factor binding are over and underlined. CpG potential methylation sites are in boldface. B) Schematic representation of the human *PKP2* promoter. The circles are representative of the CpG dinucleotides evaluated and the binding sites for different transcription factors are indicated.

## II.7 SUPPLEMENTARY

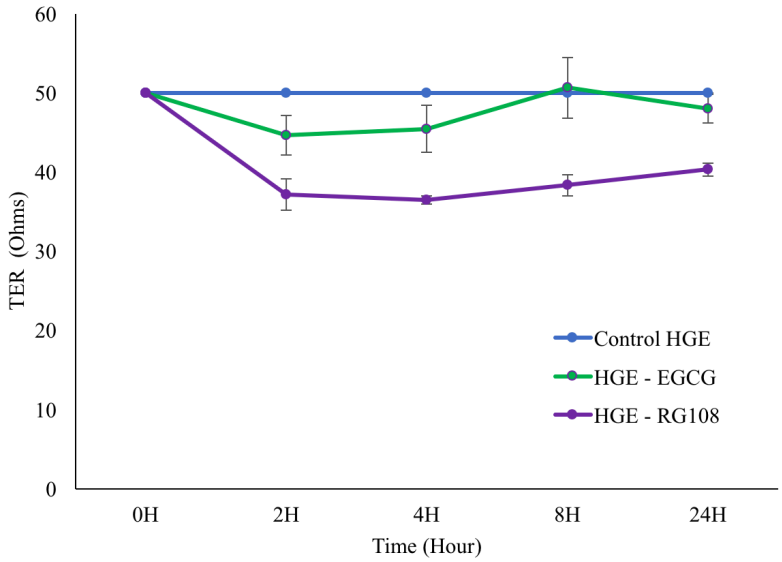
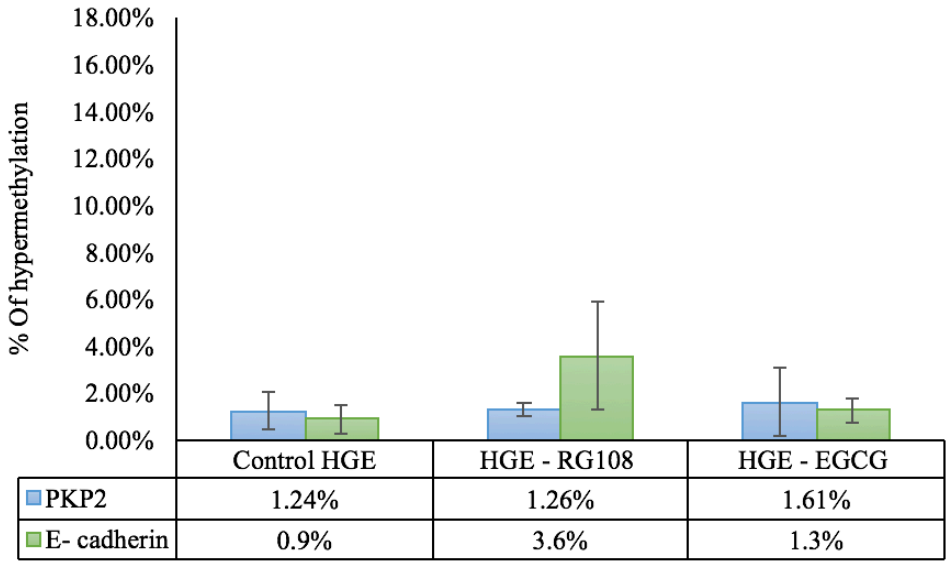



Figure 0.12 The adjusted mean of TER of human gingival epithelial (HGE) after pretreat the HGE with RG108 or EGCG.




| | Control HGE | HGE - RG108 | HGE - EGCG |
|---|---|---|---|
| PKP2 | 1.24% | 1.26% | 1.61% |
| E- cadherin | 0.9% | 3.6% | 1.3% |

Figure 0.13 Mean percentage of DNA hypermethylation for *PKP2* after pretreated the HGE with RG108 or EGCG

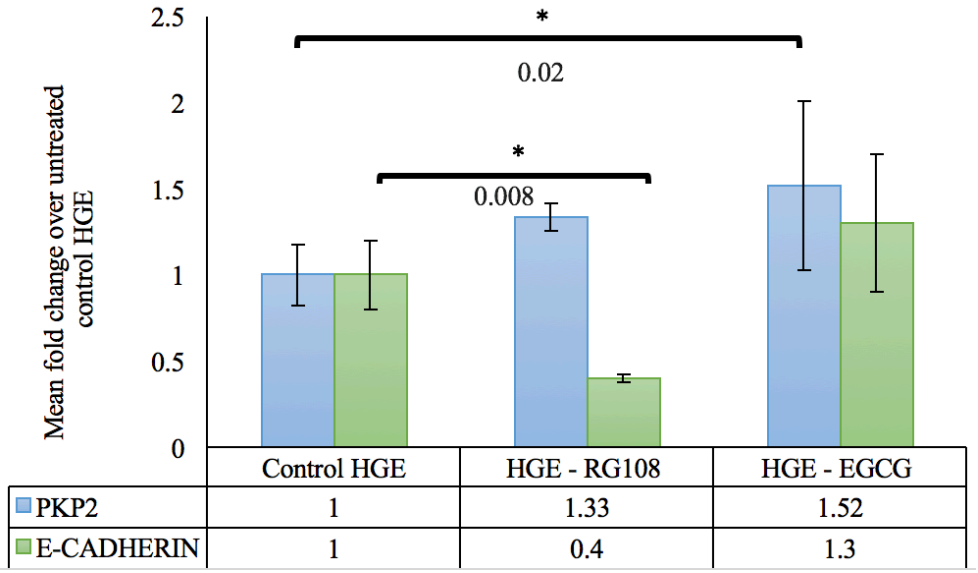



Figure 0.14 mRNA Level of *PKP2* and *E-cadherin* after pretreat the HGE with RG108 or EGCG

## REFERENCES


Abe-Yutori, M., Chikazawa, T., Shibasaki, K., & Murakami, S. (2016). Decreased expression of E-cadherin by Porphyromonas gingivalis-lipopolysaccharide attenuates epithelial barrier function. *J Periodontal Res*. doi:10.1111/jre.12367

Allis C.D, & Jenuwein T. (2016). The molecular hallmarks of epigenetic control. *Nat Rev Genet, 17*(8), 487-500. doi:10.1038/nrg.2016.59

Arimoto K, Burkart C, Yan M, Ran D, Weng S, & Zhang D.E. (2014). Plakophilin-2 promotes tumor development by enhancing ligand-dependent and independent epidermal growth factor receptor dimerization and activation. *Mol Cell Biol, 34*(20), 3843-3854. doi:10.1128/MCB.00758-14

Barnes P.J, & Karin M. (1997). Nuclear Factor-κB — A Pivotal Transcription Factor in Chronic Inflammatory Diseases. *N Engl J Med 1997, 336*, 1066-1071.

Barros S.P, & Offenbacher S. (2009). Epigenetics: connecting environment and genotype to phenotype and disease. *J Dent Res, 88*(5), 400-408. doi:10.1177/0022034509335868

Barros S.P, & Offenbacher S. (2014). Modifiable risk factors inperiodontal disease: Epigenetic regulation of gene expression in the inflammatory response. *Periodontology 2000, 64*, 95-110.

Bass-Zubek A.E, Godsel L.M, Delmar M, & Green K.J. (2009). Plakophilins: multifunctional scaffolds for adhesion and signaling. *Curr Opin Cell Biol, 21*(5), 708-716. doi:10.1016/j.ceb.2009.07.002

Bass-Zubek A.E, Hobbs R. P, Amargo E. V, Garcia N. J, Hsieh S. N, Chen X, . . . Green K.J. (2008). Plakophilin 2: a critical scaffold for PKC alpha that regulates intercellular junction assembly. *J Cell Biol, 181*(4), 605-613. doi:10.1083/jcb.200712133

Bebek G, Bennett K.L, Funchain P, Campbell R, Seth R, Scharpf J, . . . Eng C. (2012). Microbiomic subprofiles and MDR1 promoter methylation in head and neck squamous cell carcinoma. *Hum Mol Genet, 21*(7), 1557-1565. doi:10.1093/hmg/ddr593

Belibasakis G.N, Kast J.I, Thurnheer T, Akdis C.A, & Bostanci N. (2015). The expression of gingival epithelial junctions in response to subgingival biofilms. *Virulence, 6*(7), 704-709. doi:10.1080/21505594.2015.1081731

Berx G, Becker KF, Höfler H, & FV, R. (1998). Mutations of the Human E-Cadherin (CDH1) Gene. *HUMAN MUTATION, 12*(226-237).

Bobetsis Y.A, Barros S.B, Lin D.M, Weidman J.R, Dolinoy D.C, Jirtle R.L, . . . Offenbacher S. (2007). Bacterial Infection Promotes DNA Hypermethylation. *J Dent Res, 86*(2), 169-174.

Canas-Marques, R., & Schnitt, S. J. (2016). E-cadherin immunohistochemistry in breast pathology: uses and pitfalls. *Histopathology, 68*(1), 57-69. doi:10.1111/his.12869

Christofori G, & Semb H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci, 24*, 73-76.

Chung W.O, Park Y, Lamont R.J, McNab R, Barbieri B, & Demuth D.R. (2001). Signaling system in Porphyromonas gingivalis based on a LuxS protein. *J Bacteriol, 183*(13), 3903-3909. doi:10.1128/JB.183.13.3903-3909.2001

de Faria Amormino S. A, Arao T.C, Saraiva A.M, Gomez R.S, Dutra W.O, da Costa J.E, . . . Moreira P.R. (2013). Hypermethylation and low transcription of TLR2 gene in chronic periodontitis. *Hum Immunol, 74*(9), 1231-1236. doi:10.1016/j.humimm.2013.04.037

De Oliveira N.F, Andia D.C, Planello A.C, Pasetto S, Marques M.R, Nociti F.H, . . . De Souza A.P. (2011). TLR2 and TLR4 gene promoter methylation status during chronic periodontitis. *J Clin Periodontol, 38*(11), 975-983. doi:10.1111/j.1600-051X.2011.01765.x

Dickinson B.C, Moffatt C.E, Hagerty D, Whitmore S.E, Brown T.A, Graves D.T, & Lamont R.J. (2011). Interaction of oral bacteria with gingival epithelial cell multilayers. *Mol Oral Microbiol, 26*(3), 210-220. doi:10.1111/j.2041-1014.2011.00609.x

Droufakou S, D. V., Roylance R, Hanby A, Tomlinson I, Hart I. (2001). . Multiple ways of silencing E-cadherin gene expression in lobular carcinoma of the breast. *Int J Cancer, 92*, 404-408.

Drury J.L, & Chung W.O. (2015). DNA methylation differentially regulates cytokine secretion in gingival epithelia in response to bacterial challenges. *Pathog Dis, 73*(2), 1-6. doi:10.1093/femspd/ftu005

Dubash A.D, Kam C.Y, Aguado B.A, Patel D.M, Delmar M, Shea L.D, & Green K.J. (2016). Plakophilin-2 loss promotes TGF-beta1/p38 MAPK-dependent fibrotic gene expression in cardiomyocytes. *J Cell Biol, 212*(4), 425-438. doi:10.1083/jcb.201507018

Egger G, Liang G, Aparicio A, & Jones PA. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *nature, 429*, 457-463.

Fanning A.S, Mitic L.L, & Anderson J.M. (1999). Transmembrane Proteins in the Tight Junction Barrier. *J Am Soc, 10*, 1337-1345.

Goldberg A.D, Allis C.D, & Bernstein E. (2007). Epigenetics: a landscape takes shape. *Cell, 128*(4), 635-638. doi:10.1016/j.cell.2007.02.006

Gomez R.S, Dutra W.O, & Moreira P.R. (2009). Epigenetics and periodontal disease: future perspectives. *Inflamm Res, 58*(10), 625-629. doi:10.1007/s00011-009-0041-7

Grady W, Willis J, Guilford P, Dubnbier AK, Toro TT, Lynch H, . . . S, M. (2000). Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. *nature genetics, 26*, 16-17.

Groeger S, Doman E, Chakraborty T, & Meyle J. (2010). Effects of Porphyromonas gingivalis infection on human gingival epithelial barrier function in vitro. *Eur J Oral Sci, 118*(6), 582-589. doi:10.1111/j.1600-0722.2010.00782.x

Halbleib J.M, & Nelson W.J. (2006). Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev, 20*(23), 3199-3214. doi:10.1101/gad.1486806

Hall C, Li S, Li H, Creason V, & Wahl J.K. (2009). Arrhythmogenic right ventricular cardiomyopathy plakophilin-2 mutations disrupt desmosome assembly and stability. *Cell Commun Adhes, 16*(1-3), 15-27. doi:10.1080/15419060903009329

Han Y.W. (2014). Commentary: Oral bacteria as drivers for colorectal cancer. *J Periodontol, 85*(9), 1155-1157. doi:10.1902/jop.2014.140039

Hatakeyama S, Yaegashi T, Oikawa Y, Fujiwara H, Mikami T, Takeda Y, & Satoh M. (2006). Expression pattern of adhesion molecules in junctional epithelium differs from that in other gingival epithelia. *J Periodontal Res, 41*(4), 322-328. doi:10.1111/j.1600-0765.2006.00875.x

Hatzfeld M. (2007). Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? *Biochim Biophys Acta, 1773*(1), 69-77. doi:10.1016/j.bbamcr.2006.04.009

Ishida K, Kobayashi T, Ito S, Komatsu Y, Yokoyama T, Okada M, . . . Yoshie H. (2012). Interleukin-6 gene promoter methylation in rheumatoid arthritis and chronic periodontitis. *J Periodontol, 83*(7), 917-925. doi:10.1902/jop.2011.110356

Kadonaga J.T, Masiarz F.R, & Tjian R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell, 51*(6), 1079-1090.

Kaminskas E, Farrell A.T, Wang YC, Sridhara R, & Pazdur R. (2005). FDA Drug Approval Summary: Azacitidine (5-azacytidine, Vidaza™) for Injectable Suspension. *The Oncologist, 10*, 176-182.

Katz J, Sambandam V, WU J.H, Michalek S.M, & Balkovetz D.F. (2000). Characterization of Porphyromonas gingivalis-Induced Degradation of Epithelial Cell Junctional Complexes. *INFECTION AND IMMUNITY, 68*, 1441–1449.

Katz, J., Sambandam, V., Wu, J. H., Michalek, S. M., & Balkovetz, D. F. (2000). Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. *Infect Immun, 68*(3), 1441-1449.

Katz J, S. V., Wu J.H, Michalek S.M, Balkovetzd D.F. (2000). Characterization of Porphyromonas gingivalis-Induced Degradation of Epithelial Cell Junctional Complexes. *INFECT. IMMUN., 68*(3), 1441-1449.

Koetsier J.L, Amargo E.V, Todorovic V, Green K.J, & Godsel L.M. (2014). Plakophilin 2 affects cell migration by modulating focal adhesion dynamics and integrin protein expression. *J Invest Dermatol, 134*(1), 112-122. doi:10.1038/jid.2013.266

Kornman K.S. (2008). Mapping the pathogenesis of periodontitis: a new look. *J Periodontol, 79*(8 Suppl), 1560-1568. doi:10.1902/jop.2008.080213

Kowalski P.J, Rubin M.A, & Kleer C.G. (2003). E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Research, 5*(6), 217-222. doi:10.1186/bcr651)

Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, & Weinberg A. (1995). Porphyromonas gingivalis in- vasion of gingival epithelial cells. . *INFECTION AND IMMUNITY, 63* (3878-3885.).

Larsson L, Castilho R.M, & Giannobile W.V. (2015). Epigenetics and its role in periodontal diseases: a state-of-the-art review. *J Periodontol, 86*(4), 556-568. doi:10.1902/jop.2014.140559

Larsson L, Thorbert Mros S, Rymo L, & Berglundh T. (2012). Influence of epigenetic modifications of the interleukin-10 promoter on IL10 gene expression. *Eur J Oral Sci, 120*(1), 14-20. doi:10.1111/j.1600-0722.2011.00917.x

Li, G., Pan, T., Guo, D., & Li, L. C. (2014). Regulatory Variants and Disease: The E-Cadherin -160C/A SNP as an Example. *Mol Biol Int, 2014*, 967565. doi:10.1155/2014/967565

Lod, S., Johansson, T., Abrahamsson, K. H., & Larsson, L. (2014). The influence of epigenetics in relation to oral health. *Int J Dent Hyg, 12*(1), 48-54. doi:10.1111/idh.12030

Loo W.T, Jin L, Cheung M.N, Wang M, & Chow L.W. (2010). Epigenetic change in E-cadherin and COX-2 to predict chronic periodontitis. *J Transl Med, 8*, 110. doi:10.1186/1479-5876-8-110

Miller, J. R., & Moon, R. T. (1996). Signal transduction through beta-catenin and specification of cell fate during embryogenesis. *Genes Dev, 10*(20), 2527-2539.

**Nagafuchi A**. (2001). Molecular architecture of adherens junctions. *Current Opinion in Cell Biology, 13*, 600–603.

Offenbacher S, Divaris K, Barros S.P, Moss K.L, Marchesan J.T, Morelli T, . . . North K.E. (2016). Genome-wide association study of biologically informed periodontal complex

traits offers novel insights into the genetic basis of periodontal disease. *Hum Mol Genet, 25*(10), 2113-2129. doi:10.1093/hmg/ddw069

Ohi T, Uehara Y, Takatsu M, Watanabe M, & Ono T. (2006). Hypermethylation of CpGs in the Promoter of the COL1A1 Gene in the Aged Periodontal Ligament. *J Dent Res, 85*(3), 245-250.

Oliveira N.F, Damm G.R, Andia D.C, Salmon C, Nociti F.H Jr, Line S.R, & de Souza A.P. (2009). DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. *J Clin Periodontol, 36*(9), 719-725. doi:10.1111/j.1600-051X.2009.01446.x

Papagerakis S, AH, S., Depondt J, Gehanno P, & Forest N. (2003). Immunohistochemical localization of plakophilins (PKP1, PKP2, PKP3, and p0071) in primary oropharyngeal tumors: correlation with clinical parameters. *Human Pathology, 34*(6), 565-572. doi:10.1016/s0046-8177(03)00174-6

Pharoah, P. D. P., Guilford, P., & Caldas, C. (2001). Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. *Gastroenterology, 121*(6), 1348-1353. doi:10.1053/gast.2001.29611

Rakyan, V. K., Down, T. A., Balding, D. J., & Beck, S. (2011). Epigenome-wide association studies for common human diseases. *Nat Rev Genet, 12*(8), 529-541. doi:10.1038/nrg3000

Rubinstein, M. R., Wang, X., Liu, W., Hao, Y., Cai, G., & Han, Y. W. (2013). Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. *Cell Host Microbe, 14*(2), 195-206. doi:10.1016/j.chom.2013.07.012

Sarrio, D., Moreno-Bueno, G., Hardisson, D., Sanchez-Estevez, C., Guo, M., Herman, J. G., . . . Palacios, J. (2003). Epigenetic and genetic alterations of APC and CDH1 genes in lobular breast cancer: relationships with abnormal E-cadherin and catenin expression and microsatellite instability. *Int J Cancer, 106*(2), 208-215. doi:10.1002/ijc.11197

Sato P.Y, Musa H, Coombs W, Guerrero Serna G, Patino G. A, Taffet S. M, . . . Delmar M. (2009). Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes. *Circ Res, 105*(6), 523-526. doi:10.1161/CIRCRESAHA.109.201418

Schmidt A, Langbein L, Rode M, Prätzel S, Zimbelmann R, & Franke W.W. (1997). Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components. *Cell Tissue Res., 290*, 481–499.

Schwarz J, Ayim A, Schmidt A, Jager S, Koch S, Baumann R, . . . Moll R. (2006). Differential expression of desmosomal plakophilins in various types of carcinomas: correlation with

cell type and differentiation. *Hum Pathol, 37*(5), 613-622. doi:10.1016/j.humpath.2006.01.013

Socransky SS, H. A., Cugini MA, Smith C, Kent Jr. (1998). Microbial Complexes in Subgingival Plaque. *Journal of Clinical Periodontology, 25*, 134-144.

Stefani F.A, Viana M.B, Dupim A.C, Brito J.A, Gomez R.S, da Costa J.E, & Moreira P.R. (2013). Expression, polymorphism and methylation pattern of interleukin-6 in periodontal tissues. *Immunobiology, 218*(7), 1012-1017. doi:10.1016/j.imbio.2012.12.001

Stokes D.L. (2007). Desmosomes from a structural perspective. *Curr Opin Cell Biol, 19*(5), 565-571. doi:10.1016/j.ceb.2007.09.003

Stresemann C, Brueckner B, Musch T, Stopper H, & Lyko F. (2006). Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res, 66*(5), 2794-2800. doi:10.1158/0008-5472.CAN-05-2821

Subramaniam D, Thombre R, Dhar A, & Anant S. (2014). DNA methyltransferases: a novel target for prevention and therapy. *Front Oncol, 4*, 80. doi:10.3389/fonc.2014.00080

Takahashi H, Nakatsuji H, Takahashi M, Avirmed S, Fukawa T, Takemura M, . . . Kanayama H. (2012). Up-regulation of plakophilin-2 and Down-regulation of plakophilin-3 are correlated with invasiveness in bladder cancer. *Urology, 79*(1), 240 e241-248. doi:10.1016/j.urology.2011.08.049

Tang M, Xu W, Wang Q, Xiao W, & Xu R. (2009). Potential of DNMT and its Epigenetic Regulation for Lung Cancer Therapy. *Current Genomics, 10*, 336-352.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer, 2*(6), 442-454. doi:10.1038/nrc822

Trenkwalder T, Deisenhofer I, Hadamitzky M, Schunkert H, & Reinhard W. (2015). Novel frame-shift mutation in PKP2 associated with arrhythmogenic right ventricular cardiomyopathy: a case report. *BMC Med Genet, 16*, 117. doi:10.1186/s12881-015-0263-1

Van Roy F, & Berx G. (2008). The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci, 65*(23), 3756-3788. doi:10.1007/s00018-008-8281-1

Viana M.B, Cardoso F.P, Diniz M.G, Costa F.O, da Costa J.E, Gomez R.S, & Moreira P.R. (2011). Methylation pattern of IFN-gamma and IL-10 genes in periodontal tissues. *Immunobiology, 216*(8), 936-941. doi:10.1016/j.imbio.2011.01.006

Villar C.C, Kashleva H, Nobile C.J, Mitchell A.P, & Dongari-Bagtzoglou A. (2007). Mucosal tissue invasion by Candida albicans is associated with E-cadherin degradation, mediated

by transcription factor Rim101p and protease Sap5p. *Infect Immun, 75*(5), 2126-2135. doi:10.1128/IAI.00054-07

Wan M.L, Ling K.H, Wang M.F, & El-Nezami H. (2016). Green tea polyphenol epigallocatechin-3-gallate improves epithelial barrier function by inducing the production of antimicrobial peptide pBD-1 and pBD-2 in monolayers of porcine intestinal epithelial IPEC-J2 cells. *Mol Nutr Food Res, 60*(5), 1048-1058. doi:10.1002/mnfr.201500992

Wong C.Y, Caspi A, Williams B, Craig I.W, Houts R, Ambler A, . . . Mill J. (2010). A longitudinal study of epigenetic variation in twins. *Epigenetics, 5*(6), 516-526. doi:10.4161/epi.5.6.12226

Wu C.T, & Morris J.R. (2001). Genes, genetics, and epigenetics: a correspondence. *Science, 293*(5532), 1103–1105.

Yu, N., Barros S.P, Zhang S, Phillips S.T, Dillow K, Kim S, Offenbacher S. (2015). Impaired Function of Epithelial Plakophillin-2 is Associated with Periodontitis. .

Zhang S, Barros S.P, Moretti A.J, Yu N, Zhou J, Preisser J.S, . . . Offenbacher S. (2013). Epigenetic regulation of TNFA expression in periodontal disease. *J Periodontol, 84*(11), 1606-1616. doi:10.1902/jop.2013.120294

Zhang S, Barros S.P, Niculescu M.D, Moretti A.J, Preisser J.S, & Offenbacher S. (2010). Alteration of PTGS2 promoter methylation in chronic periodontitis. *J Dent Res, 89*(2), 133-137. doi:10.1177/0022034509356512

Zhang, S., Crivello A, Offenbacher S, Moretti A, Paquette D.W, & Barros S.P. (2010). Interferon-gamma promoter hypomethylation and increased expression in chronic periodontitis. *J Clin Periodontol, 37*(11), 953-961. doi:10.1111/j.1600-051X.2010.01616.x