

# The interaction of *Porphyromonas gingivalis* with host epithelial cells and its relevance to periodontal disease

By:

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## Abstract

Periodontitis is one of the most prevalent bacterial diseases affecting man with up to 90% of the global population affected. Its severe form can lead to the tooth loss in 10-15% of the population worldwide. The disease is caused by a dysbiosis of the local microbiota and one organism that contributes to this alteration in the bacterial population is *Prophyromonas gingivalis*. This organism possesses a range of virulence factors that appear to contribute to its growth and survival at a periodontal site amongst which is its ability to invade oral epithelial cells. Such an invasion strategy provides a means of evasion of host defence mechanisms, persistence at a site and the opportunity for dissemination to other sites in the mouth. However, previous studies have demonstrated that invasion of the mammalian cells in a population by *P. gingivalis* is heterogenous, with some cells becoming heavily invaded while others harbour no or only a few bacteria. An understanding of this heterogeneity may throw light on the mechanisms involved and we hypothesised that the phase of the host cell cycle may explain this phenomenon.

In an attempt to study the factors influencing *P. gingivalis* invasion and the cell response to that invasion, a standard antibiotic protection assay was employed and an oral keratinocyte cell line, H357. The results showed that *P. gingivalis* NCTC 11834 invasion was significantly increased with increasing time of exposure to the cells and the cell density. This may reflect an increased host cell surface area available for bacterial attachment. No effect on invasion of *P. gingivalis* invasion was observed by the bacterial growth phase, H357 cell passage number or whether cells were pre-incubated with *P. gingivalis* lipopolysaccharide. Epithelial cells did, however, respond to the presence of *P. gingivalis* in a number of ways. For example, the mRNA expression of endothelin-1 and urokinase receptor were upregulated with increasing *P. gingivalis* infection time, suggesting that these proteins could act as inflammatory mediators and possibly as useful markers of the severity of periodontal disease or in the diagnosis and treatment of periodontitis.

Secondly, in an attempt to investigate the reason for the observed heterogeneous *P. gingivalis* invasion of H357 cell populations, the effect of cell cycle phase on *P. gingivalis* invasion was investigated. H357 cells were synchronized by serum starvation. On re-introduction of serum, characterisation of cell cycle phase distribution was performed by flow cytometry following staining with propidium iodide (PI) or by immunofluorescence using bromodeoxyuridine (BrdU), which specifically identifies cells in S-phase. The effect of cell cycle phases on *P. gingivalis* invasion was measured using the antibiotic protection assay, immunofluorescence and flow cytometry and these were correlated with gene and surface expression of the urokinase receptor and the  $\alpha$ 5-integrin subunit, which is thought to mediate *P. gingivalis* invasion. Results showed that the percentage invasion was enhanced with increasing serum re-introduction time, and positively correlated with the number of cells in S-phase. In addition, flow cytometry data showed that the highest association of fluorescent *P. gingivalis* was with PI positive S-phase cells. Moreover, BrdU positive S-phase cells were 3 times more likely to be invaded and contained 10 times more *P. gingivalis* than cells in other phases. Also,  $\alpha$ 5-integrin was more highly expressed in cells in S-phase than other phases, which could explain the mechanism underlying this enhanced invasion. Data presented here have suggested that *P. gingivalis* targeting of cells in S-phase could, *in vivo*, allow preferential invasion of the junctional epithelial cells which turns over rapidly.

The data presented in this thesis suggest that *P. gingivalis* invasion is greatly dependent on several factors attributed to the host, the bacteria itself, and to the environment which the bacteria reside in. The invasion occurs within a population of host cells in a heterogeneous fashion, and is dependent on the cell cycle phase, specifically S-phase. This novel finding, in addition to the previously reported mechanisms of *P. gingivalis* invasion, increases our understanding of this virulence trait and suggests that such a strategy is a highly organised process which the bacteria can follow to ensure its survival within the host. Furthermore, knowledge of these mechanisms could provide novel approaches to treatment of periodontal diseases.

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## Abbreviations

®	Trade mark
α	Alpha
β	Beta
γ	Gamma
μ	Micro
AAP	American Academy of Periodontology
APC	Anaphase promoting complex
BHI	Brain heart infusion
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
°C	Centigrade
CD	Cluster of Differentiation
CDKs	Cyclin-dependent kinases
cDNA	Complementary Deoxyribonucleic Acid
CKIs	Cyclin-dependent kinase inhibitors
CO <sub>2</sub>	Carbon dioxide
CPS	Capsular polysaccharide
CR3	Complement receptor 3
C <sub>T</sub>	Cycle Threshold
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
ERK	Extracellular signal-regulated kinases
ET-1	Endothelin-1
FA	Fastidious anaerobe
FACs	Fluorescence Activated Cell Sorting
FAK	Focal adhesion kinase
FISH	Fluorescence <i>in situ</i> hybridization
FITC	Fluorescein-isothiocyanate
GCF	Gingival crevicular fluid
GECs	Gingival epithelial cells
GPI	Glucosylphosphatidylinositol
GTP	Guanosine 5'-Triphosphate
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
Hag	Haemagglutinin
HGK	Human gingival keratinocytes
HIV	Human Immuno-deficiency virus
ICAM	Intercellular adhesion molecules
IFN	Interferons
IL	Interleukin
JAK/STAT	Janus Kinase/Signal Transducer and Activator of transcription
JNK	Jun N-terminal Kinase
KGM	Keratinocyte growth medium

Kgp	Lysine-specific proteinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MMPs	Matrix metalloproteases
MOI	Multiplicity of Infection
mRNA	Messenger ribonucleic Acid
NF- $\kappa$ B	Nuclear factor-Kappa-B
OMM	Oral mucosal model
OPG	Osteoprotegerin
PAMPs	Pathogen-associated molecular patterns
PARs	Protein associated receptors
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDLCs	Periodontal ligament cells
PI3K	Phosphoinositide 3-kinase
PMNs	Polymorphonuclear cells
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
Rgp	Arginine-specific proteinases
RANKL	Receptor activator of nuclear factor-kappa B ligand
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SCC	Squamous cell carcinoma
SD	Standard Deviation
SFM	Serum free medium
T3SS	Type 3 secretion system
TGF- $\beta$	Transforming growth factor-beta
Th	T helper
TIMP	Tissue inhibitor of matrix metalloproteases
TNF	Tumour necrosis factor
TLRs	Toll-like receptors
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

## Table of Contents

<b>Abstract .....</b>	<b>ii</b>
<b>Acknowledgments .....</b>	<b>iv</b>
<b>Abbreviations .....</b>	<b>v</b>
<b>Table of Contents.....</b>	<b>vii</b>
<b>List of Figures .....</b>	<b>xi</b>
<b>List of Tables .....</b>	<b>xiv</b>
<b>Chapter 1 Literature review.....</b>	<b>1</b>
1.1 General introduction .....	2
1.2 Periodontal diseases.....	4
1.2.1 Clinical signs of periodontitis.....	4
1.2.2 Types of periodontitis.....	5
1.2.3 Features of chronic and aggressive periodontitis .....	5
1.2.4 Genetic factors .....	7
1.2.5 Environmental risk factors.....	7
1.2.6 Pathogenesis of periodontal disease .....	9
1.2.7 Role of periodontal pathogens in periodontitis .....	10
1.2.8 Role of the host response in periodontitis .....	17
1.2.9 Treatment of periodontal diseases .....	24
1.3 <i>Porphyromonas gingivalis</i> .....	26
1.3.1 <i>P. gingivalis</i> structure .....	27
1.3.2 Growth demands of <i>P. gingivalis</i> .....	27
1.3.3 Virulence factors.....	29
1.3.3.1 Capsule.....	29
1.3.3.2 Lipopolysaccharides (LPS).....	30
1.3.3.3 Fimbriae.....	31
1.3.3.4 Gingipains.....	32
1.3.3.5 Other enzymes.....	33
1.3.3.6 Hemagglutinins.....	33
1.3.3.7 Outer membrane proteins.....	34
1.3.3.8 Outer membrane vesicles.....	34
1.4 Bacterial invasion .....	35

1.4.1 Mechanisms of <i>P. gingivalis</i> adhesion and invasion.....	35
1.4.1.1 Adhesion.....	35
1.4.1.2 Invasion.....	36
1.4.1.2.1 Lipid raft mediated internalization.....	40
1.4.1.2.2 Clathrin-mediated endocytosis.....	40
1.4.2 Invasion benefits to the bacteria. ....	41
1.4.3 Adhesion and invasion assays. ....	42
1.4.3.1 Adhesion assays.....	42
1.4.3.2 Invasion assays.....	43
1.4.4 Evidence of bacterial coaggregation/interactions and their subsequent effects on adhesion and invasion of host-related cells. ....	44
1.5 Invaded host cells .....	45
1.5.1 Types of cells used in invasion assays and range of invasion. ....	45
1.5.2 Host cell response to <i>P. gingivalis</i> challenge. ....	46
1.6 The mammalian cell cycle.....	51
1.6.1 General overview.....	51
1.6.2 Cell mitosis.....	53
1.6.3 Cell cytokinesis .....	54
1.6.4 Cell cycle regulation.....	54
1.6.4.1 Cyclins and cyclin-dependant kinases (CDKs).....	55
1.6.4.2 Cell cycle checkpoints.....	56
1.6.5 Bacterial interaction with cell cycle. ....	57
1.6.6 Periodontal pathogens-cell cycle interactions .....	59
1.7 Hypothesis, aims and objectives.....	61
<b>Chapter 2 Materials and Methods .....</b>	<b>63</b>
2.1 Materials .....	64
2.2 Epithelial cell culture conditions .....	64
2.3 General bacterial growth and culture conditions.....	65
2.4 <i>P. gingivalis</i> invasion of H357 cell monolayer (Antibiotic protection assay). ....	66
2.5 Analysis of <i>P. gingivalis</i> invasion using DNA extraction.....	69
2.5.1 <i>P. gingivalis</i> DNA extraction. ....	69
2.5.2 Calculating the number of invaded <i>P. gingivalis</i> according to its DNA concentration. ....	70



2.6 Incubation time and the invasion efficiency of <i>P. gingivalis</i> .	73
2.7 <i>P. gingivalis</i> growth phases.	73
2.7.1 Analysis of <i>P. gingivalis</i> growth curve.	73
2.7.2 Effect of growth phases on <i>P. gingivalis</i> invasion efficiency.	73
2.8 Effect of different lysis buffers on <i>P. gingivalis</i> invasion efficiency with H357 monolayer cell culture.	73
2.9 Effect of cell seeding density on <i>P. gingivalis</i> invasion.	74
2.10 Effect of cell passage on <i>P. gingivalis</i> invasion	74
2.11 <i>P. gingivalis</i> invasion of H357 cells pretreated with <i>P. gingivalis</i> Lipopolysaccharide (LPS).	74
2.12 Comparison of <i>P. gingivalis</i> invasion of adherent H357 cells to those in suspension.	75
2.13 Gene expression of H357 cells exposed to <i>P. gingivalis</i> .	76
2.14 RNA extraction of H357 cell monolayers following exposure to <i>P. gingivalis</i> NCTC 11834.	76
2.15 Reverse transcriptase-Polymerase chain reaction (RT-PCR).	77
2.16 Real time- Polymerase chain reaction (qPCR).	77
2.17 Cell cycle analysis.	79
2.18 Cell cycle characterisation of H357 oral keratinocytes.	80
2.19 Effect of cell cycle phases on the bacterial association.	80
2.20 Effect of cell cycle synchronization on the <i>P. gingivalis</i> invasion efficiency.	81
2.21 Effect of serum synchronization on cell surface proteins.	81
2.21.1 Effect of serum synchronization on cellular gene expression.	81
2.21.2 Effect of serum synchronization on cell surface protein expression.	82
2.22 Immunofluorescence of serum synchronized cells using BrdU staining	83
2.23 Cell-bacterial co-localization:	84
2.23.1 Immunocytochemical staining of <i>P. gingivalis</i> invaded H357 cells.	84
2.23.2 Double immunofluorescence of oral keratinocytes and invasive <i>P. gingivalis</i> .	87
2.24 Statistical Analysis	88
<b>Chapter 3 Factors affecting <i>P. gingivalis</i> invasion.</b>	<b>89</b>
3.1 INTRODUCTION	90
3.2 METHODS	91

3.3 RESULTS .....	92
3.3.1 The effect of incubation time on the invasion efficiency of <i>P. gingivalis</i> NCTC 11834 in H357 cell monolayers.....	92
3.3.2 Effect of <i>P. gingivalis</i> growth phase on invasion ability.....	94
3.3.3 Effect cell lysis buffer on recovery of <i>P. gingivalis</i> after invasion .....	95
3.3.4 Assessment of <i>P. gingivalis</i> invasion using quantification of DNA. ....	96
3.3.5 Effect of mammalian cell seeding density on the <i>P. gingivalis</i> invasion.....	97
3.3.6 Effect of cell passaging on the <i>P. gingivalis</i> invasion.....	98
3.4 DISCUSSION.....	99
3.5 CONCLUSION .....	107
<b>Chapter 4 Cell response to <i>P. gingivalis</i> stimuli. ....</b>	<b>108</b>
4.1 INTRODUCTION .....	109
4.2 METHODS .....	111
4.3 RESULTS.....	112
4.3.1 Influence of pre-treatment of H357 cells with LPS on the invasion efficiency of <i>P. gingivalis</i> .....	112
4.3.2 Comparison of <i>P. gingivalis</i> invasion of adherent H357 cells to those in suspension.....	113
4.3.3 Genetic characterisation of invaded H357 oral keratinocyte cells by <i>P. gingivalis</i> . ....	114
4.3.3.1 ET-1 mRNA expression in H357 cells following exposure to <i>P. gingivalis</i> NCTC 11834.....	116
4.3.3.2 Urokinase plasminogen activator receptor (uPAR) expression in H357 cells following incubation with <i>P. gingivalis</i> NCTC 11834.....	116
4.3.4 H357 Cell- <i>P. gingivalis</i> association. ....	119
4.4 DISCUSSION.....	121
4.5 CONCLUSION .....	129
<b>Chapter 5 <i>P. gingivalis</i>-cell cycle interaction. ....</b>	<b>130</b>
5.1 INTRODUCTION .....	131
5.2 METHODS .....	133
5.3 RESULTS .....	134
5.3.1 Cell cycle characterization of H357 oral keratinocytes by flow cytometry and immunofluorescence techniques.....	134

5.3.2 Effect of cell cycle synchronization on <i>P. ginigvalis</i> invasion efficiency.....	140
5.3.3 Effect of cell cycle phases on the bacterial association.....	142
5.3.4 Effect of serum synchronization on the H357 oral keratinocyte-protein expression.....	145
5.3.4.1 Effect of serum synchronization on the cellular $\alpha$ 5-integrin and uPAR mRNA expression.....	145
5.3.4.2 Effect of serum synchronization on the cell surface expression of $\alpha$ 5-integrin and uPAR.....	147
5.3.5 Association of <i>P. gingivalis</i> with cells in S-phase using double immunofluorescence.....	151
5.4 DISCUSSION.....	156
5.5 CONCLUSION .....	161
<b>Chapter 6 General discussion and conclusion .....</b>	<b>162</b>
6.1 GENERAL DISCUSSION.....	163
6.2 GENERAL CONCLUSION.....	170
6.3 FUTURE WORK .....	171
<b>Publications .....</b>	<b>171</b>
<b>References.....</b>	<b>172</b>

## List of Figures

### Chapter 1

<b>Figure 1.1</b> Bacterial complexes as described by Socransky and Haffajee (Socransky <i>et al.</i> , 1998). .....	14
<b>Figure 1.2</b> Mechanism of bacterial invasion.....	38
<b>Figure 1.3</b> Mammalian cell cycle .....	53
<b>Figure 1.4</b> Bacterial cyclomodulin influences on the host cell cycle. ....	58

### Chapter 2

<b>Figure 2.1</b> Illustration of how serial dilutions were inoculated in duplicate on two FA agar plates .....	68
--	----

<b>Figure 2.2</b> The amplification plot (A) and the standard curve (B) used to determine the DNA concentration of <i>P. gingivalis</i> .....	72
---	----

### Chapter 3

<b>Figure 3.1</b> Bar chart showing the percentage invasion of H357 cells by <i>P. gingivalis</i> after different incubation times. ....	92
<b>Figure 3.2</b> Example image of viable counting method to determine the number of internalized bacteria. ....	93
<b>Figure 3.3</b> Growth curve of <i>P. gingivalis</i> NCTC 11834 in supplemented BHI broth...94	
<b>Figure 3.4</b> Effect of growth phase of <i>P. gingivalis</i> on invasion of H357 cell monolayer. ....	95
<b>Figure 3.5</b> Recovery of <i>P. gingivalis</i> following invasion of H357 cells using two methods of cell lysis.....	96
<b>Figure 3.6</b> Comparison of methods for estimating invasion of cells by <i>P. gingivalis</i> ..97	
<b>Figure 3.7</b> Effect of H357 cell seeding concentrations on <i>P. gingivalis</i> invasion efficiency.....	98
<b>Figure 3.8</b> Effect of H357 cell passaging on <i>P. gingivalis</i> invasion efficiency.....	99

### Chapter 4

<b>Figure 4.1</b> Effect of <i>P. gingivalis</i> LPS pretreatment on the level of invasion of H357 cell monolayer by <i>P. gingivalis</i> . ....	112
<b>Figure 4.2</b> Comparison of <i>P. gingivalis</i> invasion between cells in suspension and cells on tissue culture plastic using distilled water. ....	113
<b>Figure 4.3</b> Comparison of <i>P. gingivalis</i> invasion between cells in suspension and cells on well plate using 0.1% saponin. ....	114
<b>Figure 4.4</b> ET-1 mRNA expression by H357 cells at different incubation times following infecting with <i>P. gingivalis</i> NCTC 11834.....	117
<b>Figure 4.5</b> uPAR mRNA expression following infecting H357 cells with <i>P. gingivalis</i> NCTC 11834 for different incubation times. ....	118
<b>Figure 4.6</b> Immunocytochemistry images of invaded H357 cells by <i>P. gingivalis</i> NCTC 11834.....	120

## Chapter 5

<b>Figure 5.1</b> Cell cycle analysis of H357 oral keratinocytes .....	135
<b>Figure 5.2</b> DNA content of control (A) and serum synchronized (B) H357 oral keratinocytes .....	136
<b>Figure 5.3</b> Histogram showing the percentage of BrdU-positive cells in synchronized versus unsynchronized samples measured using immunofluorescence.....	138
<b>Figure 5.4</b> Immunofluorescence images of H357 cells stained with DAPI (A) (total cells) and their correspondent BrdU content (B) (S-phase cells). .....	139
<b>Figure 5.5</b> The percentage of <i>P. gingivalis</i> invasion in serum synchronized H357 cells compared to control cells .....	141
<b>Figure 5.6</b> Correlation graph showing the percentage of <i>P. gingivalis</i> invasion in serum synchronized cells versus the percentage of cells in S-phase .....	142
<b>Figure 5.7</b> Effect of cell cycle on <i>P. gingivlias</i> association.....	143
<b>Figure 5.8</b> Flow cytometry analysis of control and <i>P. gingivalis</i> -associated H357 cells. .....	144
<b>Figure 5.9</b> mRNA expression of $\alpha 5$ -integrin and uPAR in serum synchronized and control H357 cells. ....	146
<b>Figure 5.10</b> Correlation of the mRNA expression of $\alpha 5$ -integrin and uPAR with the percentage of cells in S-phase .....	147
<b>Figure 5.11</b> Comparison of surface expression of $\alpha 5$ -integrin and uPAR between serum synchronized and control H357 cells. ....	148
<b>Figure 5.12</b> Correlation of the surface expression of $\alpha 5$ -integrin and uPAR with the percentage of cells in S-phase. ....	149
<b>Figure 5.13</b> Surface expression of uPAR and $\alpha 5$ -integrin 2 hours after re-introduction of serum. ....	150
<b>Figure 5.14</b> Association of <i>P. gingivalis</i> with cells in S-phase. ....	152
<b>Figure 5.15</b> Histogram showing the percentage of BrdU-positive and negative cells with associated <i>P. gingivalis</i> . ....	153
<b>Figure 5.16</b> Example of unequal <i>P. gingivalis</i> association among BrdU-positive cells. .....	154
<b>Figure 5.17</b> Histogram of the number of <i>P. gingivalis</i> cells associated with BrdU- positive and negative cells. ....	155

## List of Tables

### Chapter 1

<b>Table 1.1</b> Subgingival microbiota in healthy periodontal sites, shallow pockets and deep pockets.....	16
<b>Table 1.2</b> The effect of different virulence factors of <i>P. gingivalis</i> on the expression of cytokines by different host cells. ....	49

### Chapter 2

<b>Table 2.1</b> Supplements added to the KGM.....	65
<b>Table 2.2</b> Brain heart infusion (BHI) broth with its related supplements used for the culture of <i>P. gingivalis</i> NCTC 11834 growth phase experiments. ....	66
<b>Table 2.3</b> DNA extraction reagents. ....	70
<b>Table 2.4</b> The reagents used in reverse transcription- Polymerase chain reaction (RT-PCR).....	77
<b>Table 2.5</b> The reagents used for master mix reaction involved in the gene expression analysis by real-time PCR.....	78
<b>Table 2.6</b> The Nucleotide sequence of the forward and reverse primers used for uPAR and ET-1 genes amplification. ....	79
<b>Table 2.7</b> uPAR, $\alpha$ 5-integrin (ITGA5, CD49), and IgG1 Isotype antibodies.....	83
<b>Table 2.8</b> The primary antibodies used in the immunocytochemical analysis of H357 cells infected with <i>P. gingivalis</i> NCTC 11834. ....	86
<b>Table 2.9</b> Counterstaining protocol using the Small Linear Stainer. ....	86
<b>Table 2.10</b> Experimental conditions for double immunofluorescence of H357 cells and invaded <i>P. gingivalis</i> .....	88

### Chapter 4

<b>Table 4.1</b> List of up-regulated and down-regulated genes isolated following <i>P. gingivalis</i> infection with H357 cells for 90 minutes.....	115
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# **Chapter 1**

# **Literature review**

### 1.1 General introduction

**Periodontal diseases are a group of infections** affecting the periodontal apparatus of the teeth including periodontal ligament, gingivae, alveolar bone and cementum, resulting in a range of reversible to irreversible periodontal destruction. Periodontal diseases are highly prevalent and can be present in up to 90% of the global population (Darveau *et al.*, 1997, Pihlstrom *et al.*, 2005) and it have been classified as the most widespread health dilemma among of the worldwide population by the World Health Organization (Newman *et al.*, 2012). The equilibrium between the microbial biofilm and the host immune response plays a significant role in periodontal health. Based on this, the overgrowth of the microbial biofilm including the presence of virulent anaerobic species such as *Porphyromonas gingivalis* (*P. gingivalis*) and/or the increased stimulation of inflammatory markers in response to this microbial load such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor- $\alpha$  can exacerbate periodontal destruction.

Despite the fact that diverse bacterial species are involved with the initiation and progression of periodontal diseases, a large amount of data has been focused on the pathogenic role of *P. gingivalis* in severe manifestation of periodontitis (Socransky and Haffajee, 1992), more particularly in chronic periodontitis. *P. gingivalis* is a Gram negative, anaerobic non motile short rod which can produce a wide variety of virulence factors such as fimbriae (Yoshimura *et al.*, 2009), gingipain proteases (Curtis *et al.*, 2001), lipopolysaccharide (Darveau *et al.*, 2004), and polysaccharide capsule (Laine *et al.*, 1997). Furthermore, several studies have indicated the invasive ability of the organism within a variety of host cells including epithelial cells (Lamont *et al.*, 1995, Madianos *et al.*, 1996). This virulence factor provides considerable value for bacterial survival as it protects *P. gingivalis* from the host defense mechanisms and aids bacterial stability within a site of infection leading to continuous periodontal inflammation. The mechanism of *P. gingivalis* internalization into host epithelial cells is not fully characterized but the interaction of *P. gingivalis* fimbriae with both fibronectin and the fibronectin-binding receptor,  $\alpha 5\beta 1$  integrin, is thought to play a role in the invasion process (Tsuda *et al.*, 2008, Yilmaz, 2008, Yilmaz *et al.*, 2002, Yilmaz *et al.*, 2003). However, there is evidence to support the existence of alternative mechanisms for *P. gingivalis* invasion into eukaryotic cells, such as lipid raft-mediated invasion,



## Chapter 1 Literature Review

clathrin-mediated endocytosis, and intracellular cytoskeletal rearrangement following changes in the intracellular signals activation such as mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), and calcium ion fluxes (Andrian *et al.*, 2006, Boisvert and Duncan, 2008, Tsuda *et al.*, 2008).

Successful establishment of *P. gingivalis* within the site of infection requires an intimate cohabitation of the bacteria with the host cells such as oral epithelial cells. This interaction works in harmony with the bacterial-induced alterations of the cellular gene expression that maintains the epithelial cell integrity (Handfield *et al.*, 2005). For example, *P. gingivalis* has the ability to regulate the host cell cycle for further enhancement of its survival, virulence, and establishment within the host (Inaba *et al.*, 2009, Pischon *et al.*, 2009).

The mammalian cell cycle represents a highly dynamic process that is precisely regulated by a network of signalling pathways (Meloche and Pouyssegur, 2007, Nakayama and Nakayama, 2006), leading to cell division. G1-phase represents a principle regulatory phase which drives the cell cycle machinery for cell growth and development prior to transition to S-phase, in which the main DNA replication takes place (Shaw *et al.*, 2010, Sherr, 1994). The transition between cell cycle phases is controlled through the sequential assembly and interaction of cyclins and cyclin-dependant kinases (CDKs) via post-translational modification (Nakayama and Nakayama, 2006, Pan *et al.*, 2014).

*P. gingivalis* can induce cell cycle progression and apoptosis of the targeted cells, inducing bacterial virulence and survival within the host leading to persistence of infection (Inaba *et al.*, 2009, Pischon *et al.*, 2009). It has been shown that *P. gingivalis* has the ability to enhance the proliferation of immortalized human gingival epithelial cells (Pan *et al.*, 2014), and accelerate the cell cycle of gingival epithelial cells (Kuboniwa *et al.*, 2008). In concert with the acceleration of the cell cycle, *P. gingivalis* has multiple anti-apoptotic pathways that ensure the suitable environment for the intracellular bacterial survival and evading host defense mechanisms (Mao *et al.*, 2007, Moffatt and Lamont, 2011, Yao *et al.*, 2010, Yilmaz *et al.*, 2004, Yilmaz *et al.*, 2008). These studies addressed the effect of bacteria on the cell cycle development/interaction, but did not consider the effect that the cell cycle might have on the bacterial-cell

interaction. We therefore investigated whether cell cycle phases have an influence on the bacterial association with the host cells, providing another possible mechanism of bacterial pathogenicity at the site of infection, information which may have applications to the diagnosis and treatment of periodontal diseases.

### 1.2 Periodontal diseases

Periodontal diseases consist of a set of infections affecting the supporting apparatus of the teeth, including gingival inflammation, periodontal ligament destruction, loss of alveolar bone, and eventually tooth loss. These diseases involve a range of inflammatory changes within the periodontium, from simple reversible inflammation of gingiva (gingivitis), to the most severe form of periodontal tissue destruction and subsequent loss of teeth. It is considered by the world health organization to be one of the most widespread health diseases with tooth loss occurring as a result of the more aggressive forms of periodontitis in 5-15% of the global population (Newman *et al.*, 2012).

#### 1.2.1 Clinical signs of periodontitis

Clinical loss of attachment represents the main characteristic feature which distinguishes periodontitis from gingivitis. This attachment loss may or may not be accompanied by periodontal pocket formation, in which there is pathological deepening of gingival sulcus and apical migration of pocket epithelium (Ide, 2003). Furthermore, changes in the height and density of the related alveolar bone are considered accompanying features (Newman *et al.*, 2012).

The relationship between gingivitis and periodontitis has been debated in the literature. Although many studies have reported that periodontitis should be preceded by gingivitis (Loe and Morrison, 1986, Page and Kornman, 1997, Schatzle *et al.*, 2003), such a relationship has been questioned by others (Goodson, 1986). The clinical manifestations of gingivitis, such as alteration in colour, bleeding on probing, change of consistency, contour and texture of gingiva may not necessarily indicate future development of periodontitis. However, continuous bleeding on probing has been reported to be a good indicator of periodontitis following loss of attachment at inflamed sites (Newman *et al.*, 2012).

Periodontal pocket formation is another clinical sign of periodontitis, considered as a reflection of the severity of attachment loss. The progression of attachment loss has been shown to continue either in a slow continuous manner, or as an intermittent increase of disease activity (Newman *et al.*, 2012).

### 1.2.2 Types of periodontitis

Periodontitis can be distinguished into two main classes, chronic and aggressive. These two distinct types of periodontitis have retained their final characteristic features after several classification attempts over the last 30 years. These attempts have focused on the clinical presentations of periodontitis, followed by classification features concentrated on age of onset, rate of disease development, and the presence of any host defense abnormalities (Addy *et al.*, 1994, Albandar *et al.*, 1999). This classification was agreed at workshops in North America and Europe in 1989 and 1993 respectively and accordingly periodontitis is presented as early onset, adult onset periodontitis, and a necrotizing type. However, inconsistent findings among different countries in the world and lack of strict scientific precision regarding these classifications, including absence of clear involvement of adult periodontitis as well as its different types and the role of the refractory type, lead to a thorough evaluation by the American Academy of Periodontology (AAP) Workshop for the International Classification of Periodontal Diseases in 1999. According to the AAP 1999, the resultant classification differentiated the clinical features of periodontitis into: chronic periodontitis, aggressive periodontitis (localized and generalized), and periodontitis as a presentation of systemic diseases.

### 1.2.3 Features of chronic and aggressive periodontitis

Both chronic and aggressive periodontitis have similar clinical profiles. Offenbacher *et al.* (2008) reported that the existence of dental biofilm and calculus and presence of signs of inflammation, such as gingival redness, increased gingival oedema, bleeding on probing, increase in pocket depth, alveolar bone loss, and loss of clinical periodontal attachment level, were common features between both of the periodontitis entities. However, there are significant differences such as the age of onset, the aggressive type being more common in younger patients than chronic periodontitis which is more specifically related to older patients (Armitage and Cullinan, 2010). Also, chronic

## Chapter 1 Literature Review

periodontitis has been shown to be a slowly developing disease in comparison to aggressive periodontitis which has a rapid rate of destruction. The progression of periodontal attachment loss in aggressive periodontitis is 3-4 times faster than in chronic periodontitis (Baer, 1971).

It has been reported that there was no specific pattern of destruction in chronic periodontitis, in that it could be confined to a few teeth or involve the whole dentition (Armitage and Cullinan, 2010) but has a slight propensity to bilateral presentation (Mombelli and Meier, 2001). In contrast, localized and generalized types of aggressive periodontitis are identified when lower than 30% and higher than 30% of sites intraorally are affected respectively (Armitage, 1999).

It has been shown that the level of gingival inflammation differs between the different types of periodontitis. Low levels of redness and gingival swelling was found among localized aggressive periodontitis patients compared with other types (Baer, 1971). This is in contrast to the findings of Armitage and Cullinan (2010) findings, in which they reported that patients with chronic pattern of periodontitis or generalized aggressive form present with a high level of gingival inflammation.

The amount of plaque accumulation and calculus formation is inconsistently associated with localized, generalized aggressive periodontitis and chronic periodontitis. Thin microbial biofilms with limited or no calculus formation has been found in localized aggressive periodontitis patients (Baer, 1971, Liljenberg and Lindhe, 1980). In contrast, in chronic periodontitis, it has been reported that there tends to be thicker and more complex polymicrobial biofilm (Listgarten, 1976), suggesting the importance of the plaque quantity in the diagnosis of different types of periodontitis.

Recently, there has been more focus on using objective rather than subjective tests to accurately determine the type of periodontitis which is important for early diagnosis and in treatment planning (Offenbacher *et al.*, 2008). Such objectivity may be obtained by utilizing biomarkers in gingival crevicular fluid or within saliva (Chapple, 2009), or example inflammatory mediators, dental plaque microbes, host tissue breakdown-related products and host-related proteolytic enzymes (Khiste *et al.*, 2011). However, the multi-factorial nature of periodontal disease makes the diagnosis of different

categories of periodontal diseases by individual biomarkers, extremely difficult. Furthermore, the diversity of microbial constituents within the plaque biofilm as well as differences in host immune responses to this microbial threat between individuals contributes to the multi-factorial nature of periodontal diseases, which in turn, makes the use of a single biomarker for diagnosis of a specific periodontal disease more complicated (Laine *et al.*, 2012). Further complications are also brought about by a variety of genetic and environmental risk factors.

### 1.2.4 Genetic factors

Several studies have been conducted on the prevalence of different forms of periodontitis within families based on the assumption that there is a genetic basis to the disease (Marazita *et al.*, 1994, Petit *et al.*, 1994). It has been reported that the progeny of subjects with chronic periodontitis have increased prevalence of periodontitis when they were less than 15 years old, which suggests a familial genetic contribution to the disease (Petit *et al.*, 1994).

Studies have been conducted on the association of genetic polymorphisms with the pathogenesis of chronic and aggressive periodontitis which may be useful in diagnosis. Genes related to the immune system in particular have been investigated (Stabholz *et al.*, 2010). Wide variation has been found in the expression of different cytokines, such as IL-1, IL-6, IL-10, and tumor necrosis factor (TNF) (Kinane *et al.*, 1999, Reichert *et al.*, 2008, Sumer *et al.*, 2007), E-selectin, toll-like receptors (TLRs) and CD14 (Stabholz *et al.*, 2010). Most of these studies showed a variable association between such markers and genetic polymorphisms in both types of periodontitis (Loos *et al.*, 2005, Nikolopoulos *et al.*, 2008). There are, however, significant limitations with gene-related studies such as the wide variations between individuals and shared patterns of behaviour within families (e.g. oral hygiene practices).

### 1.2.5 Environmental risk factors

Several environmental factors contribute to the progression of periodontal disease including oral hygiene status/microbial plaque, smoking and stress (Stabholz *et al.*, 2010). Numerous studies have reported a positive relationship between the amount of dental plaque and the level of gingivitis/gingival inflammation (Loe *et al.*, 1965, Marsh, 1994, Silness and Loe, 1964, Theilade *et al.*, 1966). Other studies have showed that

## Chapter 1 Literature Review

there was a significant correlation between plaque measures and chronic periodontitis in subjects in the age range 20-40 years (Craig *et al.*, 2003, Tanner *et al.*, 2005), but not with old subjects (Haffajee *et al.*, 1991, Tanner *et al.*, 1998). There are conflicting reports on the positive relationship of dental plaque with the levels of aggressive periodontitis (Page *et al.*, 1983, Susin and Albandar, 2005), and those suggesting a negative association between these entities (Baer, 1971).

Smoking has a profound influence on the prevalence of different kinds of periodontitis (Gelskey, 1999, Gustafsson *et al.*, 2000, Heikkinen *et al.*, 2008, Laxman and Annaji, 2008, Preber *et al.*, 1980, Rivera-Hidalgo, 2003). Numerous studies confirmed the effect of smoking on microbial ecology (Haffajee and Socransky, 2001, Zambon *et al.*, 1996), the gingival vasculature (Bergstrom and Preber, 1986, Clarke *et al.*, 1981, Tonetti, 1998), the immunological and inflammatory responses (Kamma *et al.*, 2004, Ryder, 2007), and the healing capability of periodontal connective tissue (Palmer *et al.*, 2005). Moreover, it has been shown that smoking even affects the outcome of periodontal therapeutic measures with a poor response to periodontal treatment in smokers compared to non-smokers (Hugoson *et al.*, 2002).

Stress is thought to be another risk factor for the progression of periodontal diseases. Several studies have focused on the effect of different psychological problems, such as stress, anxiety and depression on the prevalence of periodontal diseases (Boyapati and Wang, 2007, Breivik *et al.*, 1996, Freeman and Goss, 1993, Peruzzo *et al.*, 2007). Health-impairing behaviours and pathophysiological factors represent the two main mechanisms in which stress could affect the progression of periodontal diseases as well as wound healing (Stabholz *et al.*, 2010). High rate of tobacco use, poor standard of oral hygiene, and nutrient deficiency represent the health-impairing traits (Stabholz *et al.*, 2010). In contrast, indirect activation of different hormonal, inflammatory and immunological mechanisms by the increased levels of glucocorticoid and catecholamine levels, predispose to higher susceptibility to both forms of periodontitis (Boyapati and Wang, 2007, Wiebe and McCallum, 1986).

It has been demonstrated that a number of systemic diseases have a positive relationship with different types of periodontitis (Cronin *et al.*, 2008, Heitz-Mayfield, 2005, Kuo *et al.*, 2008). Certain haematological and genetic disorders which affect the

host response to bacterial threats, such as neutropenia, chemotactic and phagocytic dysfunction of neutrophils in Chediak-Higashi syndrome (Bailleul-Forestier *et al.*, 2008), histiocytosis syndromes (Deas *et al.*, 2003), immunosuppression following HIV infection (Lamster *et al.*, 1998), and Down's syndrome (Stabholz *et al.*, 2010), lead to an extreme destruction of periodontal tissue. On the other hand, other diseases exhibit a less destructive influence on the host tissue response to bacterial challenge, such as diabetes mellitus (Kinane and Chestnutt, 1997), osteoporosis (Jeffcoat, 1998), and obesity (Nishida *et al.*, 2005, Ritchie, 2007).

### 1.2.6 Pathogenesis of periodontal disease

The accumulation of microbial plaque biofilm on the tooth surface represents the major etiological agent for the inflammatory changes in gingivitis directly (Loe *et al.*, 1965, Theilade *et al.*, 1966), and indirectly (Page, 1986).

The histopathologic findings which accompany the inflammatory changes in gingival tissue are classified into: initial, early, and established lesions, based on vascular, epithelial cell, and connective tissue cell changes (Page, 1986). Although there are certain histopathological similarities between periodontitis and established gingivitis, the etiological entities are different. This has been debated on the basis of whether periodontitis occurs as an inevitable event following gingivitis (Schatzle *et al.*, 2003), or as a separate entity aggravated by certain genetic and environmental conditions independent of plaque formation (Goodson, 1986).

Periodontal disease progression could occur as a result of the disturbance in the dynamic equilibrium between microbial load and host defense response (Darveau, 2010). Such progression may have different patterns of periodontal loss of attachment, ranging from a linear model, with slow progression of periodontal destruction over time, to cyclical pattern, characterized by periods of active bursts of periodontal detachment with a prolonged periods of remission (Socransky *et al.*, 1984). However, it has been suggested recently, that both models of disease progression could reflect the same disease entity, as some periodontal sites show increased periodontal detachment and other sites are characterized by reduced disease activity or even the presence of healthy tissue (Gilthorpe *et al.*, 2003).

### 1.2.7 Role of periodontal pathogens in periodontitis

It has been widely accepted that poor oral hygiene leads to increased accumulation of microbial biofilm on the tooth surfaces as well as intra oral tissue and that the presence of this biofilm is positively correlated with the presence of periodontal disease (Pihlstrom *et al.*, 2005).

The bacterial biofilm represents the preferred growth conditions for most bacterial species involved in the pathogenesis of periodontal diseases. Such preference comes from the significant advantages that bacteria gain by residing within a biofilm structure (Lindhe *et al.*, 2003). These advantages include: protection against competing external microorganisms and host defence factors or toxic substances, such as antibiotics; simplification of nutrient uptake and processing; inter-bacterial feeding and removal of potentially toxic products; and availability of a suitable physico-chemical environment (Lindhe *et al.*, 2003). *Fusobacterium nucleatum* is considered critical for the survival of number of anaerobic microbes, e.g. *Prevotella nigrescens* and *P. gingivalis* (Bradshaw *et al.*, 1998). It provides a protective econiche for anaerobic bacteria by minimizing the oxido-reduction potential (Diaz *et al.*, 2000).

As build-up of the microbial biofilm increases, there is a transition from the early colonizers such as Gram-positive cocci to Gram-negative, anaerobic rods which are found mostly in sub-gingival plaque samples (Marsh, 2005).

To clarify the possible relationship between microbial load/characteristics and periodontal disease development, two hypotheses have historically been proposed: Firstly, the non-specific plaque hypothesis (Theilade, 1986), in which it is claimed that any accumulation of microorganisms predisposes to an inflammatory response and subsequent periodontal tissue destruction. Removal of the bacterial load could thus return the supra and sub-gingival environment to a healthy state. Secondly, the specific plaque hypothesis (Loesche, 1976), proposed that certain microorganisms within the dental plaque are pathogenic, and that their presence is related to disease severity. However, it is well accepted that mere presence of a putative pathogen in a complex community of bacteria is usually insufficient to give rise to disease. Consequently, a variation on both these hypotheses was proposed by Marsh (1994), which is referred to as the 'Ecological plaque hypothesis'. This suggests that when there is a disturbance in



## Chapter 1 Literature Review

the normal homeostasis of the bacterial population either normally dominant species can decline in proportion or pathogenic species, which are present in low numbers, can proliferate or both can occur simultaneously. The resultant population is now referred to being 'dysbiotic' and the types of factors involved include modifications in the host response, dietary influences and intake of antimicrobials. A further consequence of environmental change is often a reduction in diversity of the microbial population and when the microbial balance is disturbed for prolonged periods, disease often follows.

In terms of periodontitis, the continuous accumulation of microbial load (plaque), due for example to inadequate oral hygiene, results in an increase in inflammation providing the local microbial population with new growth factors (e.g. haemin and serum constituents through increased GCF flow). Such changes provide growth advantages to bacteria that require haemin and/or those with a predominantly proteolytic metabolism allowing them to degrade certain host defensive factors (e.g. complement) and contribute to direct tissue damage. These traits are shown by a number of Gram-negative, anaerobic, subgingival species, including those referred to as belonging to the 'red complex'. Thus, disease progression can be prevented by not only targeting the 'disease-causing' bacteria directly but also addressing the environmental factors that are responsible for this breakdown in bacterial homeostasis. For instance, the metabolism and growth of strictly anaerobic bacteria can be prevented by raising the periodontal pocket redox potential (Eh) through using of oxygenating and redox agents.

Previous microbiological findings revealed that the composition of the periodontal microbiota was significantly different in health and disease (Moore *et al.*, 1982, Socransky, 1977). In healthy sites, it has been shown that the Gram-positive bacteria such as *Streptococci* and *Actinomyces* represent the most predominant bacteria, and Gram-negative bacteria comprise about 15% of the total population (Darveau *et al.*, 1997, Tanner *et al.*, 1996). The transition from healthy gingiva to gingivitis is associated with an increase in the proportion of Gram-negative bacteria to up to 50% of the total microbial load (Lai *et al.*, 1987, Moore *et al.*, 1987). The composition of the subgingival microbiota at diseased sites has been investigated for over a century and although research in this field was slowed for several decades by difficulties in identifying some of the taxa present due to inadequate cultural methods, considerable

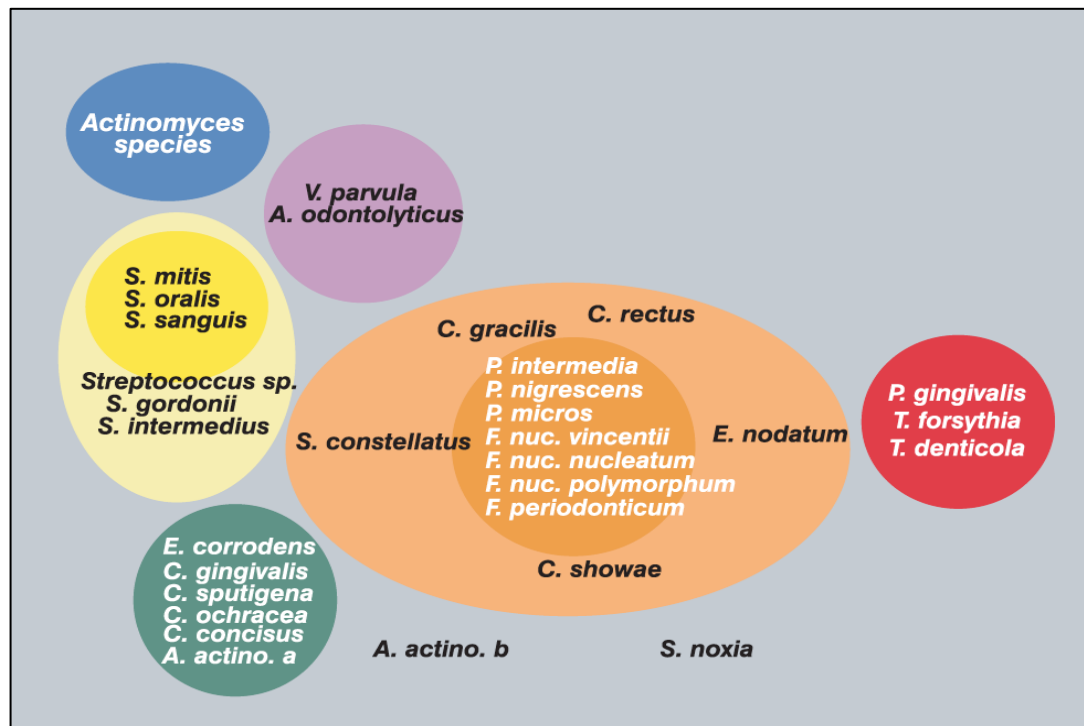
## Chapter 1 Literature Review

advances have been made more recently by the introduction of molecular methods of identification (e.g. PCR-based 16S rRNA analysis, DNA-DNA hybridization, and multi-locus sequence typing). Using a DNA-DNA hybridization method, Socransky *et al.* (1998) divided the subgingival biofilm bacteria into 5 main microbial complexes, with some associated with periodontal health such as the blue, yellow and purple complexes, while others in the orange and red complexes were closely related to disease (figure 1.1). In addition, the involvement of the red complex bacteria and some of the orange complex such as *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Parvimonas micra*, and *Eubacterium nodatum*, with different forms of periodontal diseases has been confirmed using association and elimination studies (Teles *et al.*, 2013). Although DNA-DNA hybridization is a reliable, rapid and semi-quantitative detection of microbial species compared with cultivation studies (Papadimitriou, 2016), it relied on preselected bacterial species which were cultivable in order to obtain appropriate DNA-probes, and did not take account of not-yet cultivable species in the aetiology of periodontal disease (Do *et al.*, 2013). It has been reported that only around half of the oral bacteria are cultivable and now it is thought that there are approximately 1200 taxa in the oral cavity using culture-independent techniques (Dewhirst *et al.*, 2010). Further advances in methodology have now led to full metagenomic sequencing using open-ended PCR analysis, of the 16S rRNA genes directly from sub-gingival plaque samples (Do *et al.*, 2013). This has resulted in the recognition of numerous new uncultivated species associated with periodontitis, such as *Candidatus Saccharibacteria* and *Synergistetes* phyla (Perez-Chaparro *et al.*, 2014). By comparing the subgingival bacterial composition between 29 healthy subjects and 29 with chronic periodontitis using 454 pyrosequencing of 16S rRNA genes, Griffen *et al.* (2012) detected 16 phyla, 106 genera and 596 species. From these phyla, *Spirochaetes synergistetes*, and *Bacteroidetes* were significantly associated with periodontitis, while *Proteobacteria* were significantly associated with health. In addition, from the phylum *Firmicutes*, the class Bacilli was associated with health compared to *Clostridia*, *Negativicutes* and *Erysipelotrichia* that were found within periodontitis subjects. Interestingly, they also found that over half of the periodontitis-associated bacteria and one third of the health-associated bacteria were not yet cultivable, suggesting the importance of these in the pathogenesis of periodontal diseases. Interestingly, within the same study, *Filifactor alocis* was found to be at least as prevalent as *P. gingivalis* and *T. denticola* in periodontitis subjects, suggesting that

this species may be of particular significance in periodontal disease progression. Table 1.1 summarizes the main findings of Griffen *et al.* (2012), in relation to the major phyla of bacteria in the subgingival microbiota, including several newly identified species.

The transition from healthy periodontium to gingivitis followed by periodontitis can be explained by the role of specific periodontal pathogens related to disease initiation and progression as they were initially absent or only present in small numbers and environmental change has allowed their overgrowth. This suggests then that specific virulence factors produced by these pathogens leads to inflammation and tissue destruction. As an alternative explanation, a new paradigm is emerging which is based on the possibility that specific periodontal pathogens colonise and their local virulence contribution, even though only small, produces a marked shift in the rest of the population. This is called dysbiosis and forms the basis of the keystone pathogen hypothesis recently expounded (Hajishengallis *et al.*, 2012). According to this hypothesis, the remodelling of the normal microbiota into a dysbiotic one can be initiated through the action of certain low-abundant pathogenic bacteria. Such remodelling can be induced directly on the commensal bacteria (e.g., changing its transcriptional profile), and/or through indirect impact the local immune surveillance by alterations in host-signalling pathways (Hajishengallis *et al.*, 2012). A considerable amount of data indicates that *P. gingivalis* could act as a successful keystone pathogen with its capability to provoke periodontitis. This was supported by the ability of *P. gingivalis* to evade or breakdown the host defence system constituents such as TLRs and the complement system, rather than acting as a disease-provoking bacteria directly (Darveau, 2009, 2010, Hajishengallis and Lambris, 2011). Manipulation of host defences by *P. gingivalis* can be explained by the ability of these bacteria to exert C5 convertase-like activity through the action of Arg-gingipain protease resulting in enhanced synthesis of C5a and activation of its receptor (C5aR). The resultant receptor signalling leads to a crosstalk with TLR-2 and enhanced periodontal inflammation (Liang *et al.*, 2011, Wang *et al.*, 2010). In addition, *P. gingivalis* serine phosphatase (SerB) can inhibit IL-8 synthesis, leading to a delay in neutrophil recruitment which also facilitates its early colonization within the periodontium (Darveau *et al.*, 1998, Madianos *et al.*, 1997). Possibly of even greater importance though is that these host modulatory strategies may promote the uncontrolled growth of other microbiota within the biofilm (Hajishengallis *et al.*, 2011), which in turn may result in exaggerated

complement-dependent periodontal destruction with accumulation of host tissue breakdown components such as haemin and degraded proteins acting as nutrient sources for these bacteria (Hajishengallis *et al.*, 2012). The direct effect of *P. gingivalis* on other microbes can be followed by introducing *P. gingivalis* into a healthy microbial biofilm. The gene expression profile of the microbial plaque community is altered in such circumstances and there is an up-regulation of growth and division proteins (Frias-Lopez and Duran-Pinedo, 2012).



**Figure 1.1 Bacterial complexes as described by Socransky and Haffajee (Socransky *et al.*, 1998).** The various groups are associated with health or chronic adult periodontitis. Complexes on the left-hand side are thought to be associated with gingival health, whereas the red and orange complexes are mainly associated with periodontitis (Permission obtained to reproduce here).

Exactly which bacteria are responsible for driving the tissue destruction seen in periodontitis as a consequence of over growth in dysbiotic biofilm is unknown. However, *P. gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Streptococcus intermedius*, *Campylobacter rectus*, *Streptococcus sanguinis* and *Streptococcus oralis* represent the main bacterial species that have been found in chronic periodontitis patients (Colombo *et al.*, 2007). Moreover, a wide variety of

## Chapter 1 Literature Review

virulence factors attributed to specific anaerobic microorganisms have been shown to contribute to the pathogenesis of periodontal diseases. For example, virulence factors produced by *T. forsythia* such as Leucine-rich repeat BspA protein (Inagaki *et al.*, 2006) and surface lipoproteins (Hasebe *et al.*, 2004), have been shown to mediate bacterial adherence and internalisation into epithelial cells, and inducing the release of proinflammatory cytokines and apoptosis of the host cells, respectively. In addition, a dentilisin protease (Yamazaki *et al.*, 2006) and a leucine-rich repeat A protein (Ikegami *et al.*, 2004) produced by *T. denticola* have been shown to activate polymorphonuclear (PMN) cells to produce matrix metalloproteinases 9 (MMP-9) and reactive oxygen via the complement pathway, mediating coaggregation with *T. forsythia* and biofilm development, respectively, leading to profound periodontal tissue destruction.

*P. gingivalis* was reported as the most detected subgingival bacterium (approximately 42% among 33 subgingival bacterial species) using modified checkerboard DNA-DNA hybridization taken from chronic periodontitis samples (Colombo *et al.*, 2006). Collagenase enzymes produced by *P. gingivalis* have been shown to degrade the extra cellular matrix (Al-Shibani and Windsor, 2008, Guo *et al.*, 2010). In addition, other factors attributed to these anaerobic microbes such as *T. denticola* including metabolic end products such as H<sub>2</sub>S and dimethyl sulphide (DMS) which are responsible for direct tissue destruction, activation/dysfunction of host immune responses and cytotoxicity to epithelial cells (Dashper *et al.*, 2011, Zhang *et al.*, 2010). Furthermore, suppression of tissue repair and an elevated host inflammatory tissue response can result from increased bleeding at colonized sites which is caused by disruption of the blood coagulation system by *T. denticola* (Bamford *et al.*, 2007).

**Table 1.1 Subgingival microbiota in healthy periodontal sites, shallow pockets and deep pockets.** This table summarizes the subgingival microbiota including several newly identified bacteria arranged from the most likely to the least likely to be detected using 454 pyrosequencing of 16S rRNA genes. Adapted from Griffen *et al.* (2012).

<b>Periodontal health</b>	<b>Shallow pocket periodontitis</b>	<b>Deep pocket periodontitis</b>
<i>Moraxella osloensis</i>	<i>Streptococcus mitis pneumoniae</i>	<i>Streptococcus mitis pneumoniae</i>
<i>Streptococcus intermedius</i>	<i>Acinetobacter junii</i>	<i>Filifactor alocis</i>
<i>Acinetobacter junii</i>	<i>Streptococcus sanguinis</i>	<i>Porphyromonas gingivalis</i>
<i>Granulicatella adiacens</i>	<i>Streptococcus intermedius</i>	<i>Treponema denticola</i>
<i>Acinetobacter sp RUH1139</i>	<i>Moraxella tanneriae</i>	<i>Treponema vincentii medium</i>
<i>Gemella morbillorum</i>	<i>Prevotella tanneriae</i>	<i>Eubacterium brachy</i>
<i>Actinomyces viscosus</i>	<i>Arthrobacter woluwensis</i>	<i>Prevotella tanneriae</i>
<i>Gemella morbillorum</i>	<i>Treponema denticola</i>	<i>Synergistes oral taxon 360 453</i>
<i>Arthrobacter woluwensis</i>	<i>Porphyromonas gingivalis</i>	<i>Leptotrichia oral taxon 210</i>
<i>Actinomyces viscosus naeslundii</i>	<i>Treponema vincentii medium</i>	<i>Anaeroglobus geminatus</i>
<i>Brachybacterium rhamnosum</i>	<i>Filifactor alocis</i>	<i>Prevotella denticola</i>
<i>Lautropia AP009</i>	<i>Prevotella intermedia</i>	<i>Prevotella intermedia</i>
<i>Rothia aeria</i>	<i>Eubacterium brachy</i>	<i>Selenomonas sputigena</i>
<i>Comamonadaceae nbu379c11c1</i>	<i>Eubacterium yurii subsp</i>	<i>Streptococcus sanguinis</i>
<i>Haemophilus parahaemolyticus</i>	<i>Synergistes oral taxon 360 453</i>	<i>Treponema oral taxon 230</i>
<i>Lautropia mirabilis</i>	<i>Bacteroidales oral taxon 274</i>	<i>Tannerella forsythia</i>
<i>Actinomyces massiliensis</i>	<i>Selenomonas sputigena</i>	<i>Treponema socranskii subsp</i>
<i>Actinomyces oral taxon 171</i>	<i>Tannerella forsythia</i>	<i>Desulfobulbus</i>
<i>Haemophilus P3D1 620</i>	<i>Acinetobacter sp RUH1139</i>	<i>R004Bacteroidales oral taxon 274</i>
<i>Streptococcus oral taxon B66</i>	<i>Actinomyces viscosus naeslundii</i>	<i>Synergistes oral taxon 360 453</i>
<i>Comamonadaceae 98 63833</i>	<i>TM7 401H12</i>	<i>Streptococcus intermedius</i>

### 1.2.8 Role of the host response in periodontitis

As a response to bacterial challenge, the host tissue exhibits an inflammatory response to overcome the bacterial attachment, aggregation and subsequent colonization. The disruption of the equilibrium between bacterial challenge and host-related defence mechanisms plays a crucial role in the sustainability of periodontal inflammation and subsequent tissue destruction.

#### **Innate defence**

Although there are beneficial effects of the host response in the suppression of microbial load, inflammatory damage can occur from overstimulation (Preshaw and Taylor, 2011), dysregulation or abnormal functions of this response, such as hyperactive neutrophils (Kantarci *et al.*, 2003), and over stimulation of the host-related proteases (Guo *et al.*, 2010). This damage could add to the destruction which is already produced by the microbial load within the infected sites.

The host immune response can be divided into two main categories: the innate immune response and the adaptive immune response. The innate immune response represents the first line of defence that recognizes the invading pathogens and helps trigger the adaptive immune defences to eliminate these pathogens. In fact, the failure of innate anti-bacterial systems along with increased inflammation could predispose to the continuous activation of adaptive immunity, resulting in chronic infection and microbial persistence (Cekici *et al.*, 2014). Thus, the co-ordination of the innate and adaptive immune systems is a crucial step to return the inflamed tissue to homeostasis. As the first line of defence, the innate defences include components of the structural barrier of the epithelium, such as inter-epithelial cell junctions, epithelial membrane and the continuous turnover of epithelial cells. Also, soluble components in the local environment include antibacterial peptides (Gorr and Abdolhosseini, 2011). Although there are numerous salivary proteins associated with control of the oral microbiota, such as lysozyme, salivary amylase, proline-rich proteins, mucin and peroxidases (Fabian *et al.*, 2008, Fábíán *et al.*, 2007), there is significant doubt as to how much they contribute to defence in the subgingival environment.

## Chapter 1 Literature Review

Periodontal pathogens trigger responses through pathogen-associated molecular patterns (PAMPs) that bind to lipopolysaccharide, bacterial capsule and fimbriin. Pattern recognition receptors, such as TLRs found on the surfaces of host macrophages, dendritic cells, fibroblasts, and epithelial cells (Yoshioka *et al.*, 2008) can initiate the innate immune response against pathogenic microbes by binding to these PAMPs and activating intracellular signalling resulting in release of pro-inflammatory cytokines, chemokines and type I interferons (IFN- $\alpha$  or - $\beta$ ) (Janeway and Medzhitov, 2002, Kawai and Akira, 2005, Takeda *et al.*, 2003). In addition to TLRs, several pattern recognition receptors (PRRs) such as complement receptor 3 (CR3), CD14, nucleotide-binding oligomerization domain, lectins and scavenger receptors are present on the host cell surfaces could bind to PAMPs, triggering the inflammatory response against attacking bacteria (Akira *et al.*, 2001, Arancibia *et al.*, 2007) .

An increasing number of investigations have focused on the role of TLRs on the release of pro-inflammatory cytokines and the resultant inflammatory diseases such as in chronic periodontitis (Hans and Hans, 2011). TLR signalling plays a fundamental role in the innate immune mechanism and periodontal health maintenance. The expression of several TLRs such as TLR-2, TLR-3, TLR-4, and TLR-9 in human gingival tissue has been shown to significantly increased in periodontal disease, suggesting a role for these receptors in the inflammatory response and tissue destruction (Rojo-Botello *et al.*, 2012). Regarding their interaction with PAMPs, Wang *et al.* (2007) reported that *P. gingivalis* has the ability to invade macrophages through an interaction of the microbial fimbrial protein and TLR-2 which leads to activation of the ligand binding capability of complement receptor 3 and eventual *P. gingivalis* uptake. This, in turn results in decreasing T-cell activation by inhibiting IL-12 (Wang *et al.*, 2007). On the other hand, the lipid A component of *P. gingivalis* lipopolysaccharide, has some affinity for TLR-2, which is unlike the majority of Gram-negative bacteria which activates TLR-4. This could be attributed to two different types of lipid A acyl structure, tetra-acylated (TLR-4 antagonist) and penta-acylated (TLR-4 agonist). The switching in lipid A structure is dependent on several environmental conditions such as haem concentration and temperature, which gives rise to a different host immune signalling response (Al-Qutub *et al.*, 2006).



Upon binding with *P. gingivalis* lipopolysaccharides (LPS), it has been reported that there was an increase in MMP-9 expression by human dendritic cells which can result in tissue destruction (Jotwani *et al.*, 2010). In addition, the upregulation of the protease associated receptors (PARs) in neutrophils, fibroblasts, gingival epithelial cells and T-cells can be enhanced through the action of *P. gingivalis* gingipain, contributing to the continuous destruction in chronic periodontitis (Holzhausen *et al.*, 2010).

It has been proposed that the existence of pro-inflammatory cytokines in the gingival crevicular fluid (GCF) of patients experiencing periodontitis (Andrukhov *et al.*, 2011), could be partly explained by an activation of epithelial cell TLRs. In addition, inflammation increases vascular permeability and the presence of secondary inflammatory factors, such as platelet activating factor, histamine and prostaglandins, resulting in vasodilation and increased migration of inflammatory cells to the site of infection. The complement cascade system also participates in the initiation of inflammatory changes through bacterial phagocytosis, opsonisation and lysis (Bascones-Martinez *et al.*, 2009). In addition, the complement system produces biologically active anaphylotoxins such as C3a, C4a and C5a which attract monocytes, lymphocytes and neutrophils, respectively (Cekici *et al.*, 2014).

The PMN leukocytes represent the principal inflammatory cells found within both the gingival sulcus in healthy gingiva and in periodontal pockets. The migration of these cells to the sites of infection is controlled by specific chemotactic factors triggered by the microbial biofilm through the role of chemoattractants such as IL-8 (Lindhe *et al.*, 2003). However, the dysregulation of this chemotaxis was shown to play an important role in the pathogenesis of periodontal disease (Mizuno *et al.*, 2011).

It has been shown that migration of PMNs was higher in inflamed than in healthy gingival sulcus, and that PMNs were more prevalent in sites affected by periodontal disease as opposed to gingivitis (Attstrom and Egelberg, 1970). These PMNs contribute to tissue destruction through serine proteases, such as elastase released from PMN granules. It has been shown that these proteases are present at elevated levels in gingival inflammation (Lindhe *et al.*, 2003). Moreover, such an increase in elastase levels was questioned by others, in studies suggesting that this increase was only in the

## Chapter 1 Literature Review

inactive form of the enzyme (Murray *et al.*, 1995). In addition to elastase enzymes, cathepsin G, MMP 8 and MMP 9 are found to be increased following activation of neutrophils, as well as following their death (Figueredo *et al.*, 2005). However, the importance of PMNs is clear since hyperactivity, hyper-responsiveness and increase in number and/or adherence of PMNs can lead to increased tissue breakdown. Despite these damaging aspects of the neutrophil response, these cells have been shown to be important in limiting periodontal damage. For example, disorders of neutrophil chemotaxis have been shown to be a feature in localized aggressive periodontitis (Daniel *et al.*, 1993). Also, there is histological evidence that neutrophils protect the underlying periodontal tissues by forming a barrier between the junctional epithelium and the dental plaque. This barrier provides both a 'secreting structure' (e.g. reactive oxygen species, antimicrobial peptides) and a phagocytic function (Scott and Krauss, 2011). Their crucial importance is evidenced by the association of periodontal disease with neutrophil deficiency syndromes. Neutrophil hereditary disorders such as Chediak-Higashi syndrome, Chronic granulomatous disease (CGD), Papillon-Lefevre syndrome, Cyclic neutropenia, and Leukocyte adhesion deficiency are widely discussed in the literature with their association with different forms of periodontitis. For example, Chadian-Higashi syndrome is closely associated with early onset periodontitis subjects, and it is characterised by forming large defective granules that undergo delay or incomplete degranulation, and large cytoplasmic inclusions which impair neutrophil migration, leading to inability to metabolize and digest the infecting bacteria (Deas *et al.*, 2003, Huizing *et al.*, 2001). In addition, Papillon-Lefevre syndrome (PLS) is closely associated with aggressive periodontitis and is characterised by various neutrophil defects. These include decreased chemotactic activity/migration, decreased phagocytosis and decreased intracellular killing of bacteria. The latter is associated with the absence of important intracellular proteases within the PMN granules, in particular major serine proteases, including elastase and cathepsin G, leading to the inability to process endogenous cathelicidin hCAP-18 into the antibacterial peptide LL-37, (Sørensen *et al.*, 2014). Moreover, CGD patients are characterised by recurrent fungal and bacterial infection due to its characteristic defect in intracellular microbial killing. This is characterised by inability to produce free oxygen radicals due to defects in the activation of the NADPH oxidase system.

## Chapter 1 Literature Review

Inflammatory cytokines are important inflammatory signals/messengers involved in the regulation and activation of various inflammatory cells within innate and adaptive immune responses. Following bacterial recognition and presentation by the appropriate cells, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are first to be elevated in the pathogenesis of periodontal diseases (Garlet, 2010). IL-1 $\beta$  and IL-6 have been shown to be regulated by TNF- $\alpha$ , and associated with osteoclastogenesis and inflammatory cell migration (Fonseca *et al.*, 2009, Graves *et al.*, 2008). It has been reported that the over-expression of fibroblast-derived cytokines, such as IL-6, plays an important role in the persistence of chronic periodontitis by its direct activation of immune cells. Disease-related periodontal fibroblasts inhibit the natural biological interactions which are necessary to keep the host-bacterial relationship in equilibrium (El-Awady *et al.*, 2010). On the other hand, TNF- $\alpha$  has multiple effects from stimulating inflammatory cell migration to tissue destruction. The inflammatory cell migration is mediated by TNF- $\alpha$  activation of adhesion molecules that promote rolling and adhesion of PMNs to the vessel wall with subsequent extravasation (Cekici *et al.*, 2014). In addition, it stimulates production of several chemokines that are important for cell migration to the infected sites (Kindle *et al.*, 2006, Wajant *et al.*, 2003). Moreover, TNF- $\alpha$  is involved in the extracellular matrix degradation through inducing matrix metalloproteinase secretion and bone resorption through stimulating receptor activator of nuclear factor-kappa B ligand (RANKL) secretion (Garlet *et al.*, 2004, Graves and Cochran, 2003, Graves *et al.*, 2008).

It is not only soft tissue destruction that results from periodontitis, bone resorption is also a key feature. Elevated levels of pro-inflammatory cytokines may increase the rate of bone resorption, suggesting a crucial role for cytokines in the regulation of bone homeostasis (Cochran, 2008). One of the possible mechanisms that the pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , influences normal bone homeostasis is through the regulation of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG), which both control normal bone homeostasis (Mormann *et al.*, 2008). RANKL binds to RANK which is found mainly on the surfaces of osteoclast precursors. This binding leads to osteoclast activation, and bone resorption. In contrast, OPG acts as an antagonist of RANKL inhibiting the interaction between RANK and RANKL, and so preventing bone resorption (Belibasakis *et al.*, 2010). Therefore, the ratio between these two mediators is important for bone health. However, excessive pro-inflammatory cytokines tip the balance towards increased

RANKL expression (Belibasakis and Bostanci, 2012, Mormann *et al.*, 2008, Nagasawa *et al.*, 2007).

A range of cell types can produce prostaglandins (PG), which are arachidonic acid derivatives. These have been suggested to contribute to periodontal destruction by increased vascular dilation and extravasation of inflammatory cells to the infection sites. The concentration of PGE2 in particular has been shown to be increased in gingivitis and its level correlated with increasing periodontal disease severity (Liao *et al.*, 2014, Offenbacher *et al.*, 1993).

### **Adaptive defence**

In addition to innate immunity, the cells of the adaptive immune system have been shown to be important determinants in the destruction of periodontal tissue. A particularly important cell type in this has been suggested to be CD4<sup>+</sup> T helper lymphocytes (Silva *et al.*, 2015). If inflammation persists without resolution, cell-mediated immune response (T-lymphocyte) and the humoral immune response (B-lymphocyte) are activated with subsequent effects such as antigen presentation and neutralisation, activation of other inflammatory cells, and attenuation of inflammation through secretion of various cytokines.

T-lymphocytes are the principle cells of the cell-mediated response and these recognize the alien peptides on the surface of antigen presenting cells (APCs) by specific receptors present in their surfaces. Activation of such receptors on subsets of T-cells requires interaction with the major histocompatibility complex (MHC) on the surface of APCs, leading to killing of infected target cells and activation of various cells such as macrophages, B and T-lymphocytes (Silva *et al.*, 2015). T-lymphocytes have been classified into two main subsets depending on their surface expression of CD8<sup>+</sup> molecules (T-cytotoxic cells) which are activated by antigen presentation with the MHC-I molecule, and CD4<sup>+</sup> molecules (T-helper cells (Th)) which are activated by antigen presentation with the MHC-II molecule. Th cells can be further divided into Th-1 and Th-2 depending on their specific cytokine production (Murphy and Reiner, 2002). Th-1 cells produce IL-2 and IFN- $\gamma$  which enhance cell-mediated responses and stimulate B lymphocyte secretion of IgG2, while Th-2 cells produce IL-4 which suppresses the cell-mediated responses (Modlin and Nutman, 1993), and IL-5, 6, 10 and

13 with the late secretion of IgG2 (Silva *et al.*, 2015). Differences in the responses of these Th subtypes may influence periodontitis activity, as steadily progressing periodontitis correlates with the Th-1 subtype, while rapidly progressing periodontitis is more closely associated with the Th-2 subtype, possibly through an increase in antibody production following B cell activation (Gemmell *et al.*, 2007). In addition, T-cytotoxic cells secrete characteristic cytokines of both Th-1 and Th-2 (Zadeh *et al.*, 1999).

Recently, two additional Th cells have been described as effector cells (Th-17 cells) and suppressor cells (T-regulatory cells (T-reg)) (Appay *et al.*, 2008, Sallusto and Lanzavecchia, 2009, Weaver and Hatton, 2009). Th-17 cells secrete the unique IL-17 and IL-22, which are involved in the stimulation of osteoclastogenesis (Yago *et al.*, 2009), and have been shown to be significantly associated with chronic periodontitis sites (Adibrad *et al.*, 2012, Konermann *et al.*, 2012). In addition, IL-17 has been shown to increase TLR responsiveness, suggesting an active role of this cytokine in influencing innate immune mechanisms (Beklen *et al.*, 2008), and they also stimulate RANKL production, again with possible involvement in osteoclastogenesis (Sbardella *et al.*, 2012). On the other hand, T-reg cells have been shown to have a protective role in periodontal lesions through secretion of various suppressor cytokines, such as TGF- $\beta$  and T-lymphocyte antigen 4, which have been reported to attenuate periodontal inflammation (Cardoso *et al.*, 2008, Nakajima *et al.*, 2005).

B-lymphocytes, through their specific immunoglobulin receptors, can act as APCs and effector cells following antigen recognition and binding, and then further differentiate into plasma cells. Plasma cells release antibodies in response to foreign and self-antigens (Berglundh *et al.*, 2007) and are also a potent source of cytokines, such as IL-6, IL-10, TNF- $\alpha$  and transforming growth factor  $\beta$  (TGF- $\beta$ ). The regulation of extracellular matrix (ECM) turnover is influenced by the action of both TNF- $\alpha$  and TGF- $\beta$ , in that TNF- $\alpha$  can promote the ECM degradation by enhancing expression of MMPs. This action is balanced by TGF- $\beta$  which down regulates the secretion of these MMPs and enhances the production of their inhibitors (tissue inhibitor of matrix metalloproteases (TIMPs)) (Berglundh *et al.*, 2007). This unique ability of B-cells to eradicate pathogens and present them on their surface could further activate T-lymphocytes with subsequent release of different inflammatory cytokines

(Silva *et al.*, 2015). This is importance in severe periodontal lesions as B-lymphocytes represent the major APCs stimulating continuous activation of T-lymphocytes and leading to the persistence of inflammation

This idea of immune dysregulation playing an important role in periodontal disease is confirmed when considering the activity of important MMPs. It has been shown that various MMPs are present at elevated levels within the GCF of periodontitis patients (Hernandez *et al.*, 2006, Kinane *et al.*, 2003), which suggests an increased local proteolytic, tissue remodelling burden. Normal homeostasis is maintained when MMPs are in equilibrium with inhibitors, the TIMPs (Amalinei *et al.*, 2010). However, TIMPs can be degraded by *P. gingivalis* (Grenier and Mayrand, 2001) which could contribute to such dysregulation. Moreover, it has been shown that the production of MMP-9 was disrupted following contact of gingival epithelial cells (GECs) with *P. gingivalis*, resulting in delayed extracellular matrix repair and disorganization in the extracellular matrix compartment (Decarlo *et al.*, 1997, Decarlo *et al.*, 1998, Grayson *et al.*, 2003). In addition, the up-regulation of MMP-3 and MMP-8 genes (Decarlo *et al.*, 1998) and late activation of MMP-1, 3 and 9 (Decarlo *et al.*, 1997), have been demonstrated following the interaction between the purified gingipain proteinases of *P. gingivalis* and rat mucosal epithelial cells. Thus, periodontal destruction is a complex interplay of a number of factors.

### 1.2.9 Treatment of periodontal diseases

Despite the fact that there are differences between chronic and aggressive periodontitis regarding the severity and course of infection, the clinical manifestation of both entities are similar, that is periodontal loss of attachment, the presence of local inflammation, loss of collagen and loss of bone support. Thus, the periodontal treatment for both is fairly similar and directed to achieving an adequate level of plaque control and reduction in pocket probing depth. Periodontal treatment can be performed in five steps: Mechanical therapy, antimicrobial therapy, host modulation therapy, tissue regeneration therapy, and behavioural and economic concerns (Chapple, 2009). The first phase of periodontal treatment involves mechanical debridement of supra and sub-gingival microbial plaque and calculus from teeth and surrounding structures. The removal of sub-gingival plaque from deep periodontal pockets is difficult, however,

## Chapter 1 Literature Review

and so re-colonisation of these sites by pathogenic microbes is tackled by the use of antimicrobial agents and antiseptic substances.

More novel approaches enhancing the immune response might hold promise but are not in general use yet. These include the use of TLR therapy in the suppression of periodontal destruction (Chapple, 2009), vaccination against certain virulent microorganisms, stimulation of T cells into the regulatory subtype (Choi and Seymour, 2010) and the use of certain pro-resolving lipid mediators of inflammation, such as lipotoxin, resolvins and protectines (Serhan, 2008, Van Dyke, 2008).

Another approach that has adoption by some specialities has been to attempt to replacement of the lost soft and hard tissues (Chapple, 2009). Enamel matrix derivatives have been used in tissue regeneration therapy and act by promoting the process of periodontal regeneration (Bosshardt, 2008). Emdogain is one such agent that has been shown, *in vitro*, to stimulate the proliferation of periodontal ligament cells (Gestrelus *et al.*, 1997) and to increase the attachment of periodontal ligament fibroblasts to the diseased root surfaces (Davenport *et al.*, 2003) with enhancement of matrix synthesis (Haase and Bartold, 2001). Therefore, it represents a possible alternative to open surgical debridement (Esposito *et al.*, 2004, Venezia *et al.*, 2004). Furthermore, a favourable clinical outcome can be obtained by using platelet-derived growth factor-B homodimeric peptide in conjunction with beta-tricalcium phosphate (Nevins *et al.*, 2005), in which periodontal osseous defects are filled resulting in increased clinical attachment level and reduced the gingival recession, possibly by its chemotactic and mitogenic effects on periodontal ligament and alveolar bone cells, promoting bone regeneration. More recent studies have concentrated on stimulation and signalling of periodontal stem cells in periodontal sites to complete connective tissue regeneration (Lin *et al.*, 2009).

### 1.3 *Porphyromonas gingivalis*

*Porphyromonas gingivalis* is a Gram-negative anaerobic, asaccharolytic, non-motile cocco-bacillus which has been strongly implicated in the initiation and progression of human periodontal diseases, as well as being a factor in the development of several systemic diseases, such as atherosclerosis (Chun *et al.*, 2005). It was isolated by Oliver and Wherry (1921), in which these rods were shown to produce black-pigmentation when grown on blood agar (Mayrand and Holt, 1988) and was previously classified as *Bacteroides gingivalis* (Coykendall *et al.*, 1980). However, similar anaerobic black pigmenting bacilli were isolated from a range body sites and so were reclassified as *Bacterium melaninogenicum*. Subsequent re-classification saw the *Bacteroides* divided into 3 genera: *Bacteroides*-non-pigmenting, saccharolytic species, *Prevotella*-pigmenting and non-pigmenting species, moderately saccharolytic, and *Porphyromonas*-black-pigmenting species, asaccharolytic type (Shah and Collins, 1989, Shah and Collins, 1990, Shah and Collins, 1988).

*P. gingivalis* is considered to be one of the small number of putative periodontal pathogens whose presence is associated with the progression of periodontitis (Kumar *et al.*, 2005), and is commonly isolated from sub-gingival plaque samples of chronic periodontitis patients along with other periodontal pathogens, such as *T. denticola* and *T. forsythia* (Socransky *et al.*, 1998). Moreover, the detrimental destructive effects of *P. gingivalis* on the tooth supportive structures have been confirmed by several studies (Hojo *et al.*, 2008, Tanner *et al.*, 2007).

The inability of *P. gingivalis* to survive in the exposed environment suggests that the transmission of this microbe is via a preferable vector, such as saliva from an infected person (Greenstein and Lamster, 1997). In order for *P. gingivalis* to develop and express a wide variety of virulence factors required for initiation and progression of periodontal disease, certain pathophysiologic changes are required, such as alterations in the host immune defense mechanisms, changes in the indigenous microbial commensal proportions and/or functions, and the existence of favourable growth factors and nutrients.



### 1.3.1 *P. gingivalis* structure

There is correlation between individual structural components of *P. gingivalis* and its virulence (Yoshimura *et al.*, 2009). As a Gram-negative organism it possesses an outer and a cytoplasmic membrane. The outer membrane contains a number of important proteins including porins, Omp A-like protein (Koebnik *et al.*, 2000); RagA, RagB, and associated Arg-gingipain and Lys-gingipain (Bonass *et al.*, 2000, Kadowaki *et al.*, 2007).

A number of *P. gingivalis* strains possess a capsule which acts to reduce host cell phagocytosis (Singh *et al.*, 2011). *P. gingivalis* colonization, aggregation, and persistence within sites of infection requires adherence to the epithelial surface and other bacterial species. This adherence ability is due to a variety of adhesins which are found within its surface, such as fimbriae and haemagglutinins. However, the sub-gingival colonization process requires expression of certain molecules important for the survival of *P. gingivalis*, such as those involved with oxidative stress resistance (Capestany *et al.*, 2008) and nutrient acquisition (Smalley *et al.*, 2011).

### 1.3.2 Growth demands of *P. gingivalis*

*P. gingivalis* is asaccharolytic and so utilises peptides and amino acids as an energy source (Goulet *et al.*, 2004, Milner *et al.*, 1996, Takahashi *et al.*, 1997, Takahashi and Sato, 2002). The products of their metabolism are fatty acids, amines and ammonia which tend to raise the environmental pH (Takahashi *et al.*, 1997), and its optimum growth pH is between 7.0 and 8.0 (Takahashi and Schachtele, 1990).

Despite *P. gingivalis* being an obligate anaerobe, it is moderately aerotolerant. Consequently it needs to be able to withstand oxidative challenge not only from periods of oxygen exposure but also from the metabolic effects of other commensal species (e.g. H<sub>2</sub>O<sub>2</sub> production by streptococci) and oxide radicals from neutrophils (Barnard and Stinson, 1999). Consequently, *P. gingivalis* possesses antioxidant properties, such as superoxide dismutase (Lynch and Kuramitsu, 1999); rubrerythrin, DNA-binding protein (Dps), and alkyl hydroperoxidaseC (AhpC) which have an antagonistic action against H<sub>2</sub>O<sub>2</sub> (Johnson *et al.*, 2004, Sztukowska *et al.*, 2002, Ueshima *et al.*, 2003). It has been reported that there is a direct relationship between haemin depletion and the level of expression of OxyR protein by *P. gingivalis*, which in turn controls the

## Chapter 1 Literature Review

expression of certain anti-oxidative stress genes, such as *sod*, *trx*, *dps*, and *ahpC* genes (Xie and Zheng, 2012). In addition, haem affects the catalytic degradation of H<sub>2</sub>O<sub>2</sub> (Smalley *et al.*, 2000). Also it has been demonstrated that bacterioferritin co-migratory protein, which is upregulated by *P. gingivalis* under high oxygen tension, plays a vital role in the resistance to elevated levels of oxidative stress (Limauro *et al.*, 2008, Wang *et al.*, 2005).

*P. gingivalis* has an absolute requirement for iron for growth and this is also thought to help protect the organism against oxygen (Smalley *et al.*, 1998). Its importance is illustrated by the expression of more than 50 proteins involved in iron uptake and utilization (Olczak *et al.*, 2008), and the expression of more than 70 proteins and 160 genes are significantly modulated in response to limitation in haemin availability (Dashper *et al.*, 2009). The black pigmentation of *P. gingivalis* is attributed to the accumulation of iron-containing porphyrins on its cell surface (Kuboniwa *et al.*, 1998) this is achieved through the action of its haemolytic and proteolytic activity acquiring haem from haemoglobin, methaemoglobin, myoglobin, lactoferrin as well as from inorganic iron and non-porphyrin compounds (Bramanti and Holt, 1991, Genco *et al.*, 1994).

Temperature is another significant factor affecting the growth of *P. gingivalis*. It has been reported that there is a reduction of approximately 50% in the amount of fimbriae and in the expression of *fimA* mRNA when the temperature is shifted from 37 to 39°C (Amano *et al.*, 1994). Conversely, there was an increase in the activity of *P. gingivalis* *fimA* promoter, adherence to *S. gordonii*, and invasion of primary gingival epithelial cells when the temperature was reduced from 39 to 34 °C (Xie *et al.*, 1997). It has been reported that there is a temperature-dependant change in the lipid A component of the lipopolysaccharide of *P. gingivalis*, towards a higher proportion of monophosphorylated, penta-acylated lipid A. This results in enhanced activation of TLR-4 and increased sensitivity of the bacteria to the antimicrobial peptides,  $\beta$  defensins 2 and 3 (Curtis *et al.*, 2011). Furthermore, the reduction in the gingipain protease activity (Percival *et al.*, 1999) and an increased tolerance towards oxidative stress (Vanterpool *et al.*, 2010) can be observed as a result of increasing temperature.

### 1.3.3 Virulence factors

*P. gingivalis* expresses pathogenic virulence factors that enable this microorganism to colonize and persist at a site. Factors, such as proteases, fimbriae, lipopolysaccharides, extracellular membrane vesicles, hemagglutinin factors, and polysaccharide capsule, represent the main virulence factors that mediate pathogenic alterations within the host.

#### 1.3.3.1 Capsule

The capsular polysaccharide (CPS) represents one of the virulence factors, which has been shown to play a vital role in the pathogenicity of *P. gingivalis*. The evidence for this comes from various studies such as using mouse infection models where non-encapsulated strains of *P. gingivalis* are less virulent than encapsulated strains (Singh *et al.*, 2011). Apart from the anti-phagocytic effect of the capsule (Domenico *et al.*, 1994, Glynn and Howard, 1970, Noel *et al.*, 1992), and resistance to defensins (Igboin *et al.*, 2011), much of its pathogenic properties are associated with its effects on the immune system (Katz *et al.*, 1996, Vernal *et al.*, 2009). In particular, IL-1, IL-6 and IL-8 cytokine production is down-regulated in fibroblast cells after contact with the encapsulated *P. gingivalis* W83 strain (Brunner *et al.*, 2010). Furthermore, there is downregulation of the adaptive immune response to encapsulated strains (Baker *et al.*, 2000, Wilensky *et al.*, 2009).

CPS consists of glucose, glucosamine, galactosamine, and galactosaminuronic acid (Schifferle *et al.*, 1989) and recently it has also been shown to contain mannuronic acid, glucuronic acid, galactose, and 2-acetamido-2-deoxy-D-glucose (Farquharson *et al.*, 2000). Six capsular serotypes have been identified (K1 to K6) (Brunner *et al.*, 2010) and although small differences in virulence have been identified between these serotypes, a significant variation has been reported between the strains within a single capsular serotype (Laine and Van Winkelhoff, 1998). However, the K1 serotype triggers chemokine expression at higher rate compared with other CPS serological types in murine peritoneal macrophages (D'empaire *et al.*, 2006).

A higher rate of adhesion to the epithelial cells has also been associated with non-encapsulated *P. gingivalis* and the level of co-aggregation with different kind of cells has been shown to occur in a CPS dependent manner (Davey and Duncan, 2006, Rosen

and Sela, 2006). All of these characteristics give the CPS its own unique contribution to the virulence of *P. gingivalis*, and explain why it has a significant role in the initiation and progression of periodontal diseases.

### 1.3.3.2 Lipopolysaccharides (LPS)

LPS is a component of the outer membrane of all gram-negative bacteria. It is proinflammatory and is thought to play a damaging role in chronic periodontitis (Park *et al.*, 2010, Trent *et al.*, 2006). The common structure of LPS comprises Lipid A and core and long polysaccharide O-antigen repeats (Jain and Darveau, 2010, Raetz and Whitfield, 2002). It has been found that the virulence of *P. gingivalis* LPS has considerable diversity, which is dependent on multiple factors, such as its structure (Hajjar *et al.*, 2002) and growth conditions (Lee and Baek, 2013) including temperature (Curtis *et al.*, 2011) and on the level of haemin (Al-Qutub *et al.*, 2006, Cutler *et al.*, 1996). High levels of haemin in the environment results in a change in the structure of lipid A toward the tetra-acylated mono-phosphorylated form, which acts as a TLR-4 antagonist and immune suppressor (Reife *et al.*, 2006). Conversely, when low levels of haemin are present a non-phosphorylated structure of lipid A predominates, due to the action of 1- and 4'-phosphatases which does not trigger TLR-4 and results in immune evasion (Coats *et al.*, 2009). Thus, it is possible that high haemin conditions and the associated TLR-4-antagonistic form of Lipid A could contribute to protection of other bacteria in the local biofilm.

There has been some debate over whether some forms of lipid A of *P. gingivalis* can interact with TLR-2 instead of TLR-4 resulting in a different inflammatory signalling pathway (Hajjar *et al.*, 2002). However, according to other studies the stimulation of TLR-2 has been attributed to the phosphorylated dihydroceramide lipids instead of the action of LPS and lipid A (Nichols *et al.*, 2012).

Interestingly, it has been shown that the antigenicity of the *P. gingivalis* LPS as well as the expression of cytokines are exaggerated when grown with *F. nucleatum*, and the lipid A structure, in particular, has shown to be changed as a result of increased expression of *lpxA* and *lpxD* genes, which are related to the LPS biosynthesis (Lee and Baek, 2013).

### 1.3.3.3 Fimbriae

*P. gingivalis* is characterized by the emergence of specific protrusions from its outer surface membrane, called fimbriae. These mediate a range of effects including adherence and invasion of a variety of host cells (Nakagawa *et al.*, 2002a, Yilmaz *et al.*, 2002), colonization within the oral environment (Maeda *et al.*, 2004); auto-aggregation (Lin *et al.*, 2006) and induction of the host immune response (Hajishengallis *et al.*, 2009). There are two types of fimbriae: major fimbriae which are composed of the fimbrillin protein, FimA (a 41-kDa protein encoded by the *fimA* gene (Dickinson *et al.*, 1988)), and minor fimbriae which are composed of the 67-kDa mfa1 protein encoded by the *mfa1* gene (Hamada *et al.*, 1996, Park *et al.*, 2005).

The fimbrillin protein plays an important role, in attachment to a variety of oral structures and subsequent invasion of cells. It has been shown to mediate adherence to human gingival fibroblasts and epithelial cells (Njoroge *et al.*, 1997, Sugano *et al.*, 2004, Weinberg *et al.*, 1997) and to induce periodontal bone loss in rat models (Malek *et al.*, 1994). In addition, fimbrillin modifies the host immune response such as enhancing neutrophil and macrophage production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- $\alpha$  (Ogawa *et al.*, 1994a, Ogawa *et al.*, 1994b). On the other hand, the minor fimbrial protein, mfa1, has been shown to elicit an inflammatory response through expression of IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  in a mouse model (Hiramine *et al.*, 2003) and also to mediate coaggregation with *S. gordonii* (Lamont *et al.*, 2002).

*P. gingivalis* *fimA* genes can be divided into six subsets (I, Ib, II, III, IV, VI) based on their nucleotide sequences (Nakagawa *et al.*, 2002b). Presence of the type II *fimA* gene has been shown to increase the bacterial ability to adhere and invade oral epithelial cells (Kato *et al.*, 2007) and is the most common form isolated from periodontitis patients (Nakagawa *et al.*, 2002a), while type I is the most common form isolated from healthy individuals.

It has been confirmed that *P. gingivalis* fimbriae have the ability to bind with ECM proteins, such as fibronectin and vitronectin. This link between extracellular and intracellular environments is crucial for cell anchorage, migration, survival and intracellular signal transduction pathways, thus the presence of *P. gingivalis* can delay the healing of the diseased periodontal tissue. The competitive binding of the

*P. gingivalis* fimbriae to ECM proteins gives an opportunity for other *P. gingivalis* to bind with  $\alpha_v\beta_3$  (integrin for vitronectin) and  $\alpha_5\beta_1$  (integrin for fibronectin), thus subverting intracellular signal transduction pathways and causing tissue destruction (Amano, 2003). Moreover, disruption of fibronectin and its main receptor ( $\alpha_5\beta_1$  integrin) is attributed to the *P. gingivalis* arginine-specific protease activity (Scragg *et al.*, 1999).

### 1.3.3.4 Gingipains

*P. gingivalis* produces cysteine proteases, named gingipains. There are two classes: arginine specific proteinase (RgpA and RgpB) and lysine specific proteinase (Kgp) (Mezyk-Kopec *et al.*, 2005). Gingipains provide numerous clues for why *P. gingivalis* is one of the most virulent species in the pathogenesis of periodontitis. Gingipains mediate nutrient acquisition through haemagglutination, haemolysis and iron uptake from haem within sites of periodontal infection (Smalley *et al.*, 2008) but they also enhance the attachment of *P. gingivalis* to host tissue cells and other bacteria, stimulate extracellular matrix protein degradation, increase vascular permeability, and induce cleavage of host cellular receptors. A number of other host systems are also potential targets for gingipain such as the blood coagulation pathway (Inomata *et al.*, 2009, Lourbakos *et al.*, 2001), the complement system (O'Brien-Simpson *et al.*, 2003) and cell adhesion molecule cleavage and apoptosis (Sheets *et al.*, 2005). All of these gingipain characteristics contribute to the ability of this anaerobic microorganism to be a successful colonizer and virulent bacterial species in the oral cavity.

It has been reported that immunization of mice with whole *P. gingivalis* cells or with the arginine-specific gingipain, RgpA followed by challenging the animals with virulent *P. gingivalis*, resulted in an overproduction of IgG targeting the hemagglutinin domain of RgpA and a subsequent decrease in the colonization and invasion of *P. gingivalis*. This suggests that the haemagglutinin domain of RgpA plays an important role in the antigenicity of *P. gingivalis* (Genco *et al.*, 1998). In addition, these domains represent the most significant in the adherence of *P. gingivalis* to epithelial cells (Chen and Duncan, 2004).

Kontani *et al.* (1997) have shown that fimbrial binding of *P. gingivalis* to the cultured human fibroblasts and matrix proteins can be facilitated by the exposure of cryptic host ligands by RgpA and RgpB, suggesting that gingipains can work in conjunction with fimbriae to aid colonization. In addition, Nakayama *et al.* (1996) showed that *P. gingivalis* Rgp may be involved in the maturation of its fimbriae and other surface proteins such as 75-kDa protein, suggesting its function as a multifunctional virulence factor involved in periodontopathogenesis.

It has been demonstrated that PARs (1 to 4) can be cleaved by the action of gingipains. The release of pro-inflammatory cytokines, such as IL1 $\beta$ , CXCL8 and TNF- $\alpha$ , can be partly mediated by the action of Rgp gingipains on PAR-1 and activation of PAR-2 on the surface of PMNs (Giacaman *et al.*, 2009). In addition, such activation of pro-inflammatory cytokines may mediate alteration in bone homeostasis and bone resorption by stimulation of RANKL as well as OPG degradation through Kgp related gingipain activity (Yasuhara *et al.*, 2009).

### 1.3.3.5 Other enzymes

Although gingipains are the predominant proteases of *P. gingivalis*, it produces other enzymes that may contribute to periodontitis initiation and progression. These include: endothelin-like converting enzyme which contributes to the disturbance in the blood coagulation pathway (Awano *et al.*, 1999), sialidases and sialoglycoproteases which mediate immune cell evasion through modulating *P. gingivalis* gingipain activity (Aruni *et al.*, 2011), dipeptidyl and tripeptidyl amino peptidases which are considered part of the nutrient acquisition system (Oda *et al.*, 2009); collagenase which is contributes to ECM degradation (Wittstock *et al.*, 2000) and periodontain which has been shown to modulate a variety of host proteases such as neutrophil elastase (Nelson *et al.*, 1999).

### 1.3.3.6 Hemagglutinins

Hemagglutinins can behave as adhesins and have a considerable influence on the virulence of different pathogenic microbial species (Alonso *et al.*, 2002, Chen and Duncan, 2004, Ishibashi *et al.*, 2001, Kuramitsu *et al.*, 2003). It has been found that these cell surface hemagglutinins either exist in concert with fimbriae as fimbrial adhesins or with non-filamentous surface molecules as non-fimbrial adhesins, which

enable the bacteria to bind to host cells (Han *et al.*, 1996). The virulence of hemagglutinins are also involved in the uptake haemin from erythrocytes, which is a vital for *P. gingivalis* growth (Grenier *et al.*, 2003, Lépine *et al.*, 1996).

Five haemagglutinins genes have been identified : *hagA-E* (Han *et al.*, 1996), HagA and HagB of *P. gingivalis* are major hemagglutinins concerned with the adhesion to and invasion of oral epithelial and endothelial cells (Bélanger *et al.*, 2012, Song *et al.*, 2005). Also, HagA plays an important role in co-aggregation of *P. gingivalis* with other microbial species such as *T. denticola* (Ito *et al.*, 2010), so contributing to the ecology of the plaque biofilm.

### 1.3.3.7 Outer membrane proteins

There are number of outer membrane-related proteins that play a significant role in the virulence of *P. gingivalis*. RagA and RagB are important in nutrient acquisition through their transport role (Postle and Kadner, 2003). They are particularly involved in the uptake of glycoproteins (Yoshimura *et al.*, 2009).

*P. gingivalis* membrane stability, adherence to the host cells, invasion capability, formation of plaque biofilm and modulation of immune response has been shown to involve the outer-membrane porin, known as OmpA-like protein (Iwami *et al.*, 2007, Smith *et al.*, 2007). This protein facilitates the passage of various nutrients across the membrane (Sugawara and Nikaido, 1992).

Specific proteins, such as HmuR (Olczak *et al.*, 2008) and haemin-binding protein 35 (Shoji *et al.*, 2011), represent examples of other outer membrane proteins that are part of the haem acquisition system.

### 1.3.3.8 Outer membrane vesicles

*P. gingivalis* is capable of influencing its environment at a distance from the cell surface by the release of outer membrane vesicles. Grenier and Mayrand (1987) showed these vesicles contain proteases that contribute to degradation of collagen, they mediate bacterial adherence to the cells and promote the attachment between the noncoaggregating bacteria such as *Capnocytophaga ochracea* and *Eubacterium saburreum*, suggesting a possible role of these vesicles in the progression of



periodontal disease. Kamaguchi *et al.* (2003) showed that the attachment of the noncoaggregating *Streptococcus* species and *Candida albicans* to *Staphylococcus aureus* was greatly enhanced through the action of *P. gingivalis* vesicles. In addition, the role of these vesicles in mediating the adherence and invasion of other bacteria such as *T. forsythia* has been reported (Inagaki *et al.*, 2006).

### 1.4 Bacterial invasion

#### 1.4.1 Mechanisms of *P. gingivalis* adhesion and invasion

##### 1.4.1.1 Adhesion.

In order to establish permanent colonisation of the oral cavity, *P. gingivalis* must adhere to the available surfaces so as to avoid the various removal forces (Gibbons, 1984). The primary structures on *P. gingivalis* that are involved in adhesion to host cells are fimbriae, LPS, hemagglutinins and proteinases (Njoroge *et al.*, 1997).

Oral epithelial cells are the first type of host cells that *P. gingivalis* is likely to encounter within a periodontal site. The epithelial layer undergoes continuous desquamation which should remove adherent bacteria (Gibbons, 1989, Gibbons and Houte, 1975), however, Rudney *et al.* (2001) proposed that this could help the transmission of *P. gingivalis* to different intra oral locations.

Although the mechanism of *P. gingivalis* adherence to the epithelial cells is not fully understood, it is thought that it involves fimbrial attachment to certain receptors found on the surfaces of epithelial cells, in particular to fibronectin-binding integrin,  $\alpha 5\beta 1$  Nakagawa *et al.* (2002a). Evidence for this comes from the observation that adherence to and subsequent uptake of recombinant *P. gingivalis* type II FimA by the human oral epithelial cell line, HEP-2 was inhibited following addition of antibodies to  $\alpha 5\beta 1$  integrin. Furthermore, it has been shown that  $\beta 1$  integrin antibodies inhibit the attachment of *P. gingivalis* to gingival epithelial cells in a dose-dependent manner, (Yilmaz *et al.*, 2002). Nevertheless, further investigation is needed to elucidate the possible role of other mechanisms which could play an important role in the adherence of *P. gingivalis* to human cells.

Following adhesion of *P. gingivalis* to epithelial cells, cytoskeletal rearrangement occurs along with changes in intracellular  $\text{Ca}^{2+}$  fluxes and triggering of intracellular signalling such as protein phosphorylation and MAPK pathway activation (Andrian *et al.*, 2006). In addition, Andrian *et al.* (2006) proposed that by revealing cryptic receptors on the surface of the host cells, *P. gingivalis* cysteine proteinases (gingipains) may have an important role in the binding of *P. gingivalis* with its targets (Gibbons *et al.*, 1990, Gibbons, 1989).

Agnani *et al.* (2003) demonstrated that N-acetylneuraminic acid and glucuronic acid (complex sugars residues) were involved in the attachment of *P. gingivalis* to the epithelial cell basement membrane. This finding has been studied previously and focused on the role of glycosaminoglycan-binding microbial proteins in the binding of different pathogenic microbes to their targets, for the invasion process and persistent intracellular survival (Wadstrom and Ljungh, 1999).

Sojar *et al.* (2002) have shown that *P. gingivalis* fimbriae can interact effectively with epithelial cell associated cytokeratins which can serve as receptors. In this experiment, a 50-kDa protein shown to be cytokeratin, by its size, amino acid sequence, and immunologic reactivity, can act as a ligand for a purified fimbrial extract of *P. gingivalis*.

Narimatsu *et al.* (2004) showed that glycosyltransferase has a role in *P. gingivalis* attachment to HEp-2 epithelial cells. They discovered that there was a significant reduction in such binding in *P. gingivalis* mutant strains deficient in the *gtfA* gene, the glycosyltransferase encoding gene, suggesting a role for the *gtfA* gene in the fimbrial structure through sugar transfer and for subsequent *P. gingivalis* binding to its targets.

### **1.4.1.2 Invasion.**

The invasion process is considered a common strategy shared between various microbial pathogens and is thought to have evolved in order to access nutrients and shelter from host defences (Finlay and Falkow, 1997). *P. gingivalis* has the ability to bind and subsequently invade a range of eukaryotic cells other than oral epithelial cells (Rautemaa *et al.*, 2004), including fibroblasts (Pathirana *et al.*, 2007), endothelial cells (Dorn *et al.*, 2000), and multiple cell lines (Deshpande *et al.*, 1998, Dorn *et al.*, 1999,

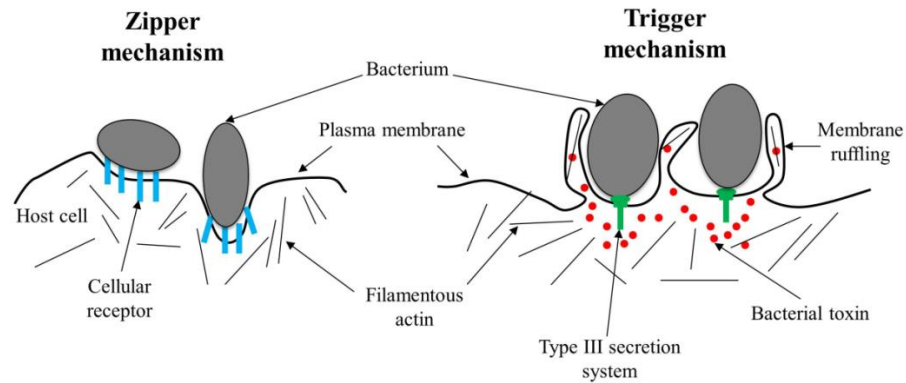
## Chapter 1 Literature Review

Duncan *et al.*, 1993, Lamont *et al.*, 1995, Madianos *et al.*, 1996, Njoroge *et al.*, 1997, Sandros *et al.*, 1994) although it is not clear whether the mechanisms involved are the same for all cell types. As mentioned above, several investigations have demonstrated that *P. gingivalis* fimbriae are important for interaction with the  $\alpha 5\beta 1$  integrin (Njoroge *et al.*, 1997, Weinberg *et al.*, 1997), however, Dorn *et al.* (2000) have shown that the invasive capability of 26 strains of *P. gingivalis* vary but that *fimA* expression is not a requirement for invasion.

Also, the biological relevance of epithelial invasion is not completely understood. Although a higher number of internalized *P. gingivalis* has been found in crevicular and buccal cells associated with increased periodontal destruction sites, a number of those internalized bacteria were found inside crevicular cells at healthy periodontal sites (Colombo *et al.*, 2007).

Bacterial invasion is thought to occur by two major mechanisms; the zipper and trigger mechanisms. The zipper process is mediated by specific binding between certain microbial ligands and host cell surface associated receptors, which lead to clustering of the involved receptors at the membrane surface and subsequent "phagocytic cup" formation. Invasion is completed following activation of intracellular signalling pathways leading to actin remodelling and polymerization followed by microbial engulfment (Cossart and Sansonetti, 2004). Some microbial species are thought to use this mechanism for host cell invasion, such as *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, and *Neisseria gonorrhoeae* (Dramsı and Cossart, 1998) (figure 1.2).

The trigger mechanism uses the type 3 secretion system (T3SS) to deliver certain bacterial-associated virulence factors inside the host cell following contact with the cell surface. This results in the host-cell membrane ruffling due to direct stimulation of intracellular cytoskeletal proteins and consequent microbial entry (Ofek *et al.*, 2003). *Shigella flexneri* and *Salmonella typhimurium* are thought to use this phenomenon to achieve their cellular internalization. However, Lamont and Yilmaz (2002) have proposed that it is unlikely that *P. gingivalis* utilizes the trigger mechanism for host-cellular entry, as it does not have a T3SS (figure 1.2).



**Figure 1.2 Mechanism of bacterial invasion.** The zipper mechanism involves bacteria-cell receptors binding leading to phagocytic cup formation and subsequent bacterial engulfment. Trigger mechanism involves the release of effector molecules through a T3SS leading to the cytoskeletal rearrangement and membrane ruffling, and bacterial internalization (adapted from Eierhoff *et al.* (2012)).

It has been shown that both microtubules and microfilaments in the epithelial cells are important in the intracellular signalling transduction pathways which precede *P. gingivalis* internalization. This was confirmed using inhibitors of actin polymerization, such as cytochalasin D, or using microtubule depolymerization agents, such as nocodazole, which significantly inhibited *P. gingivalis* invasion (Lamont *et al.*, 1995). Belton *et al.* (1999) proposed that after invasion *P. gingivalis* accumulate in the perinuclear region and that they stay in a viable form with a capability of replication 12 minutes after *P. gingivalis* infection. Host GECs do not undergo apoptosis and remain viable for up to 48 hours after invasion of *P. gingivalis* (Belton *et al.*, 1999, Nakhjiri *et al.*, 2001). Phosphorylation of focal adhesion proteins including paxillin following infection of GECs with wild type *P. gingivalis* (Yilmaz *et al.*, 2002), has a role in integrin signalling and activation (Clark and Brugge, 1995, Yamada and Geiger, 1997). The association of integrin-related signaling and activation of cytoskeletal components, such as FAK, paxillin, talin and vinculin is associated with invasion by a range of bacteria, not just *P. gingivalis* (e.g. *S. flexneri*, *Escherichia coli*, *Chlamydia pneumoniae*) (Coombes and Mahony, 2002, Martinez and Hultgren, 2002).

## Chapter 1 Literature Review

Nevertheless, different invasion mechanisms, such as lipid raft mediated (Tsuda *et al.*, 2008) or clathrin-mediated endocytosis (Boisvert and Duncan, 2008) have been suggested as *P. gingivalis* invasion strategies rather than depending on a single mechanism of invasion.

The MAPKs mediate cytoskeletal rearrangement and cytokine responses and act as stress response molecules (Robinson and Cobb, 1997). MAPK consist of 3 serine-threonine kinases: stress-stimulated protein kinases c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERK) and the p38 MAPK (Davis, 1993). As a result of GECs infection with *P. gingivalis*, it has been shown that there was down-regulation of ERK1/2 and marked activation of JNK with no effect on the p38, suggesting that *P. gingivalis* can influence individual MAPK components (Watanabe *et al.*, 2001). Invasion of epithelial cells by *P. gingivalis* is markedly reduced following treatment with staurosporine, a protein kinase inhibitor, and genistein, a specific tyrosine protein kinase inhibitor, suggesting a role for tyrosine phosphorylation in the intracellular signalling transduction pathways linked to invasion. In contrast, Lamont *et al.* (1995) have shown that staurosporine does not block signalling pathways following exposure of epithelial cells to *P. gingivalis*, suggesting that protein phosphorylation has no role in the signalling transduction pathways of invasion.

Tsuda *et al.* (2005) have claimed that the PI3K has a role in *P. gingivalis* internalization. According to their study, phagocytic cup closure is mediated partly by PI3K since specific inhibitors, as wortmannin and LY294002, significantly reduced the number of the internalized fluorescent beads coated with *P. gingivalis* membrane vesicles.

Different studies were conducted on the role of calcium ion fluxes and its effect on the *P. gingivalis* uptake. Andrian *et al.* (2006) have suggested that the cytoskeletal rearrangement can be affected by a transiently elevated concentration of calcium ions which are present after the initial contact between epithelial cells and *P. gingivalis*.

### 1.4.1.2.1 Lipid raft mediated internalization.

Lipid rafts are membrane related sub domains which are rich in cholesterol, glycosylphosphatidylinositol-anchored proteins, glycosphingolipids (GM1, Gb3, and GD1), sphingolipids, and caveolin (Kai and Ikonen, 1997). It has been reported that *P. gingivalis* exploits lipid rafts for its internalization following initial adherence by  $\alpha 5\beta 1$  integrin to the host cell surface (Tsuda *et al.*, 2008). By using cholesterol-blocking agents and lipid raft disruption compounds, such as nystatin, filipin, and methyl- $\beta$ -cyclodextrin, Tsuda *et al.* (2008) reported that there was significant inhibition of internalization of fluorescent beads coated with *P. gingivalis* membrane vesicles. In addition, Caveolae represents another kind of lipid raft, along with GM1 ganglioside clusters, which are thought to play an important role in cell signalling, cholesterol homeostasis and endocytosis through their ability to form cell membrane invaginations (Parton and Richards, 2003). Moreover, the co-localization of caveolin-1 and GM1 clusters with fluorescent beads coated with *P. gingivalis* vesicles provides supporting evidence for the association of lipid rafts with the internalization of *P. gingivalis*. However, the literature demonstrating the importance of lipid rafts is not extensive and so further studies are required.

### 1.4.1.2.2 Clathrin-mediated endocytosis.

Clathrin-mediated endocytosis is a dynamic process by which *P. gingivalis* could be taken into the intracellular environment (Boisvert and Duncan, 2008). According to the study conducted by Boisvert and Duncan (2008), clathrin clusters surround fluorescent beads coated with the adhesin domain of RgpA (HA1). However, these findings were not supported by Tsuda *et al.* (2008), who reported that the uptake of fluorescent beads covered by *P. gingivalis* membrane associated vesicles was not inhibited when clathrin mutant cells were used.

Lamaze *et al.* (2001) has described these vesicles as basket like structures composed mainly of protein, clathrin, and stabilized by adaptins. These structures are surrounded by a ring of the Guanosine 5'-Triphosphate (GTP)-associated protein, Dynamin. This protein has the ability to transfer vesicles via the actin cytoskeleton to the intracellular environment. The recycling of these vesicles in the cytoplasm of the host cells has been described by Henderson *et al.* (1999), in which they claimed that the fate of these vesicles is dependent on its binding to either early or late lysosomes. Vesicular

recycling after ligand dissociation is a characteristic of early lysosome binding, whereas delivery of these vesicles to the active lysosomes for hydrolysis and further recycling is a characteristic of the late lysosome.

Takeuchi *et al.* (2011) has suggested that the close proximity of *P. gingivalis*, when enclosed by endosomal membranes to the cell membrane is brought about by endosomal recycling via the action of early endosomes, and may explain the mechanism by which *P. gingivalis* could exit from the invaded host cells. Moreover, it has been proposed that following invasion for a short period of time, the conjugation of *P. gingivalis* into specific multi-membranous vacuoles called autophagosomes, takes place. These vacuoles play a significant role in organelle recycling (Dorn *et al.*, 2001), and can differentiate into more mature autolysosomes which are characterized by vacuole degradation (Dunn, 1994).

The intravacuole life style of *P. gingivalis* has a number of advantages for the survival including utilization of the elevated levels of free amino acids and the ability to replicate and re-colonize to cause continued infection (Dorn *et al.*, 2001, Sinai and Joiner, 1997).

### **1.4.2 Invasion benefits to the bacteria.**

The establishment of microbe species within an intracellular environment represents a challenging issue for extracellular and intracellular host defence mechanisms. The occupation of such a privileged niche within host cells is considered to be advantageous since the intracellular environment is not exposed to normal host immune defence mechanisms or many therapeutic agents (Falkow, 1991, Finlay, 1990, Sansonetti, 1993, Tilney and Portnoy, 1989). This ability is a feature of a number of virulent organisms. Indeed Meyer *et al.* (1991) showed that *Actinobacillus actinomycetemcomitans* (now *Aggregatibacter actinomycetemcomitans*) can penetrate human epithelial cells by switching its morphological phenotype during the invasion process. Also, Colombo *et al.* (2013) reported that *S. aureus* and *Pseudomonas aeruginosa* exist in a high proportion of epithelial cells from periodontitis samples compared to healthy individuals, and that *E. faecalis* was the most predominant species discovered inside the oral epithelial cells of periodontitis samples.

In addition, Yilmaz *et al.* (2006) noted the long standing viability of *P. gingivalis* within epithelial cells and its capability for inter-cellular spreading. The highly nutrient rich intracellular environment would be expected to favour bacterial survival and consequent reduction in expression of aggressive features of the bacteria should ensure host cell integrity and thus survival for a prolonged period. Indeed there is a marked decrease in cysteine proteinase levels (RgpA, RgpB, Kgp) upon invasion (Xia *et al.*, 2007). By inactivation of caspase-9 and pro-apoptotic Bax, invaded host cells are protected from apoptotic cell death following invasion by *P. gingivalis* (Yao *et al.*, 2010). This protective mechanism is thought to occur by modulation of the JAK/STAT pathways (Mao *et al.*, 2007). However, this has been disputed by several authors who suggest there is stimulation of apoptosis by activation of the pro-apoptotic molecules, such as caspase-9 (O'brien-Simpson *et al.*, 2009, Stathopoulou *et al.*, 2009b).

### **1.4.3 Adhesion and invasion assays.**

#### **1.4.3.1 Adhesion assays.**

A variety of adhesion and invasion experimental models have been used to quantify the number of bacterial cells including *P. gingivalis* which adhere to or invade cells *in vitro*.

Vesterlund *et al.* (2005) described different adhesion assays, such as those using radioactively labelled bacteria; fluorescently labelled bacteria; detection of adherent bacteria by crystal violet; and detection of microbial binding with 4V, 6-diamidino-2-phenylindole (DAPI). In this research, they concluded that the radioactive labelling is the most sensitive and reproducible method used to measure the selective adherence of bacteria. However, now workers strive to find alternatives to the use of radioisotopes.

Hellström *et al.* (2004) used a chromatogram binding assay, to measure bacterial binding to the glycolipid fraction of the epithelial cells, and showed that *P. gingivalis* can bind to both non-acid and acid glycolipid fractions of the cell membrane of epithelial cells, and that the sugar chain of the glycolipid is considered the bacterial receptor for such binding. While this provides some insight into the molecular aspects of adhesion, it is not capable of differentiating adherent bacteria from those that have become internalised within the host cells.



To combine this, Njoroge *et al.* (1997) described an adhesion assay in which they calculated the number of adherent *P. gingivalis* as the difference between the number of recovered bacteria after treatment with an antibiotic that does not penetrate inside the cell (antibiotic protection assay (section 2.4)) compared to the number obtained without antibiotic treatment. An advantage of this approach is that both adherence and invasion data can be obtained from the same experiment (Dorn *et al.*, 2000).

### 1.4.3.2 Invasion assays.

The importance of studying bacterial invasion is mainly dependent on the ability to differentiate between extracellular and intracellular bacteria. The quantification of invaded microbes represents a key characteristic to study and represents a potential risk to host cell integrity and the subsequent disease outcome.

Although high resolution imaging of the invaded bacteria is possible, the difficulty of distinguishing the attached bacteria from the internalized bacteria if serial sectioning is not used. This represents a major drawback of using transmission electron microscopy for studying bacterial uptake by the host cells (Tang *et al.*, 1993). However, the antibiotic protection assay and immunofluorescence microscopy represent the most widely used techniques for studying bacterial invasion.

The addition of antibiotics to kill adherent and non-adherent bacteria that are external to the host cells represent the basic principle of the antibiotic protection assay, hence the name of this assay (Kihlström and Andåker, 1985, Mandell, 1973). Host cells are seeded and then cultured with bacteria to allow bacterial internalization to take place. Antibiotic is added to kill the external bacteria, and the cell monolayer washed to remove the antibiotic followed by cell lysis to release invaded bacteria and viable counting then conducted (Tang *et al.*, 1993).

It has been proposed that the invasion assay parameters, such as incubation time, type of antibiotic used, and a variety of culture conditions such as temperature and oxygen, should be optimized as these can affect the level of invasion independently (Tang *et al.*, 1993). For instance, Finlay *et al.* (1991) have shown that 20-30 minutes was optimal for *S. typhimurium* uptake by HeLa cells, whereas it took 2-3 hours for the invasion of HeLa cells by *E. coli* (EPEC) (Donnenberg *et al.*, 1989). In addition, the use of

kanamycin antibiotic is considered efficient for killing the extracellular gentamicin resistant bacteria (Une, 1976). Sodium deoxycholate and Ethylene glycol tetraacetic acid are examples of widely used detergents for lysing cultured cells and releasing the intracellular invaded bacteria (Lissner *et al.*, 1983, Shaw and Falkow, 1988).

Immunofluorescence microscopy can be used as an alternative to the simple antibiotic protection assay. Differentiation between internalized and the external adherent bacteria is based on a double immunofluorescence labelling technique. Briefly, a labelled antibody is added to the cells which bind to the external bacteria, then the cells are permeabilised to allow entry of another antibody with a different label (Heesemann and Laufs, 1985, Sveum *et al.*, 1986).

#### **1.4.4 Evidence of bacterial coaggregation/interactions and their subsequent effects on adhesion and invasion of host-related cells.**

The wide diversity of microbial species at a site of infection creates a favourable environment for inter-bacterial adhesion and co-operation. For example, strains which do not have the capability to invade host cells in an independent manner can gain entry through their association with invasive bacteria (Edwards *et al.*, 2006, Grenier, 2013, Inagaki *et al.*, 2006, Saito *et al.*, 2008).

It has been shown that *F. nucleatum* ATCC 10953 is protected against antimicrobial agents found in saliva when coaggregated with *Streptococcus cristatus*, suggesting an enhanced viability of *F. nucleatum* could be attributed to this bacterial interaction (Rudney and Strait, 2000).

Edwards *et al.* (2006) demonstrated that the ability of *S. cristatus* to invade TERT-2 epithelial cells can be greatly enhanced when they are co-incubated with an adherent/invaded *F. nucleatum*. Such invasion was mediated by the induction of membranous blebbing in host cells as seen under scanning electron microscopy. The adherence and invasion of *S. cristatus* was abolished following inhibition of *F. nucleatum* invasion by galactose pretreatment.

Using fluorescence *in situ* hybridization (FISH) and confocal microscopy, Rudney *et al.* (2005) reported that an intracellular consortium of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythensis* (now *T. forsythia*) in buccal epithelial cells was seen in 38 healthy subjects, suggesting that polymicrobial interactions may be important in the invasion process, although all of these bacterial species are considered powerful invaders individually.

Furthermore, Saito *et al.* (2008) have documented that the invasion of the gingival epithelial cell line Ca9-22 and human aortic endothelial cells by *P. gingivalis* ATCC 33277 was significantly increased (2-20 fold) when coinfecting with a clinical isolate of *F. nucleatum* TDC100, providing additional evidence for the synergistic polymicrobial activity between *P. gingivalis* and *F. nucleatum*. Lipid rafts are thought to play an important role in mediating this effect (Saito *et al.*, 2012).

It has been shown that the outer membrane vesicles of *P. gingivalis* can mediate the coaggregation of *Lachnoanaerobaculum saburreum*, which is a non-motile microbe, to *T. denticola*, which has the ability to migrate to the deeper periodontal tissue, the coaggregation of *L. saburreum* with *T. denticola* is characterized by "piggyback appearance" (Grenier, 2013). Furthermore, the beneficial effect of the *P. gingivalis* outer membrane vesicles has been documented in relation to the increased adherence and invasive capabilities of *T. forsythia* with epithelial cells in the presence of these vesicles (Inagaki *et al.*, 2006).

### 1.5 Invaded host cells

#### 1.5.1 Types of cells used in invasion assays and range of invasion.

As described above, *P. gingivalis* has the ability to adhere and invade a variety of host cells in different *in vivo* and *in vitro* experimental models. Lamont *et al.* (1995) showed that differences in the invasion percentages of primary gingival epithelial cells by *P. gingivalis* 33277 were attributed to the inoculum concentrations (12.8% at multiplicity of infection (MOI) of 1:100 in comparison to 1% at MOI of 1:10000), incubation time (nearly 26% after 300 minutes of infection in comparison to 1% after 30 minutes), and bacterial growth phase (15% in mid log phase in comparison to 2.2% of the lag phase).

Amornchat *et al.* (2003) used transmission electron microscopy to show that the percentage invasion of the human gingival fibroblasts by *P. gingivalis* 381 was 0.17%. Examination of such invaded fibroblasts, after 90 minutes incubation, showed *P. gingivalis* moving freely in the cytoplasm and not surrounded by vacuoles.

Rudney *et al.* (2001) used laser scanning confocal microscopy and FISH, to show that *P. gingivalis* 33277 can invade human buccal epithelial cells in a 3D reconstruction mucosal model. These results were supported by Colombo *et al.* (2007), in which they showed that *P. gingivalis* can invade crevicular and buccal epithelial cells. A higher number of invaded bacteria were noted among periodontitis subjects. Using a 3D oral mucosal model (OMM) to study *P. gingivalis* invasion, Pinnock (2012) showed that the total invasion of the OMM by *P. gingivalis* was similar to the monolayer cultures although longer incubation was required (4 hours) for OMM to be invaded by the bacteria compared to 1.5 hour for the monolayer cultures. While both systems are reliable for studying *P. gingivalis* invasion then, OMM are considered more representative of the oral mucosa.

It has been confirmed that *P. gingivalis* has the ability to invade different kinds of endothelial cells in a fimbriae dependent manner. The invasion percentages of bovine aortic endothelial cells, human umbilical vein endothelial cells, and fatal bovine heart endothelial cells by *P. gingivalis*, were 0.1%, 0.2% and 0.3% respectively (Deshpande *et al.*, 1998). In addition, Dorn *et al.* (2001) proposed that the invasive ability of *P. gingivalis* 381 to human coronary artery endothelial cells favoured the late autophagosome instead of the early endocytic pathway, providing a potential mechanism to explain the close association between cardiovascular disease and periodontal disease.

### **1.5.2 Host cell response to *P. gingivalis* challenge.**

A number of reports have outlined the key responses of different kinds of host cell to *P. gingivalis* infection. Chemokine expression, which has a vital role in leukocyte activation and attraction to the site of infection, as well as changes in the expression of the surface adhesion molecules has been reported following bacterial-epithelial cells interaction (Huang *et al.*, 2001). However, it has been documented that *P. gingivalis* reduced the expression of IL-8 and intercellular adhesion molecule-1 (ICAM-1),

resulting in more tissue destruction by delaying the action of immune defence mechanisms (Darveau *et al.*, 1998, Huang *et al.*, 1998a, Madianos *et al.*, 1997). Although such attenuation has been attributed to the action of *P. gingivalis*-gingipain proteases, the precise mechanisms for the down-regulation of IL-8 and ICAM-1 are still unclear (Darveau *et al.*, 1998, Mikolajczyk-Pawlinska *et al.*, 1998, Zhang *et al.*, 1999). Decreased expression of IL-8 can exaggerate the host tissue destruction by decreasing the host defense mechanisms through limiting circulation of neutrophils at the site of infection (Huang *et al.*, 2001). This has been supported by the finding that recruitment and/or stimulation of PMNs was inhibited by the action of Rgp of *P. gingivalis* (Nakayama *et al.*, 1995), suggesting a direct effect on PMN cell activity. Table 1.2 highlights several studies investigating the effect of different virulence factors of *P. gingivalis* on the expression of cytokines in a variety of cell types. Watanabe *et al.* (2001) proposed that the reduced expression of IL-8 could be attributed to the inability of *P. gingivalis* to activate nuclear factor-Kappa-B (NF- $\kappa$ B) as a result of ERK1/2 down-regulation. Furthermore, Hasegawa *et al.* (2008) described a serine phosphatase protein (SerB) which is excreted by *P. gingivalis* upon contact with gingival epithelial cells and aids in the invasion process, which can reduce the IL-8 expression.

In contrast, up-regulation of IL-8 and ICAM-1 has been shown following infection of gingival epithelial cells with *F. nucleatum* or *A. actinomycetemcomitans* (Han *et al.*, 2000, Huang *et al.*, 1998a). However, Huang *et al.* (2001) suggested that, following exposure of gingival epithelial cells to *P. gingivalis*, down-regulation of IL-8 and ICAM-1 was correlated with the translational and/or post-translational regulatory pathways, finding that there was an increase in the transcription of ICAM-1 and IL-8 genes in the presence of all 3 species.

Other cytokine responses to gingipain- mediated *P. gingivalis* interaction include soluble TNF- $\alpha$  which has been reported to be degraded by the action of HRgpA, RgpB, and Kgp cysteine proteinases (Calkins *et al.*, 1998). In addition, Mezyk-Kopec *et al.* (2005) showed that arginine gingipain and, to a lesser degree, lysine-gingipain have the ability to degrade the soluble and membranous forms of TNF- $\alpha$ , resulting in evasion of the host immune defences through decreased synthesis of IL-8, cell adhesion receptors, IL-6, and major histocompatibility complex I (Mezyk-Kopec *et al.*, 2005). Furthermore, the decrease in TNF- $\alpha$  secretion has been reported following degradation

## Chapter 1 Literature Review

of the major LPS receptor, CD14 by the selective binding of gingipains and outer membrane vesicles, resulting in diminished stimulation of LPS and subsequent down-regulation of TNF- $\alpha$  (Duncan *et al.*, 2004, Sugawara *et al.*, 2000).

It has been confirmed that the pro-inflammatory cytokine release from gingival epithelial cells was stimulated through the *P. gingivalis* LPS and fimbriae (Asai *et al.*, 2001). Such cytokine release is mediated through activating protein-1 (AP-1) and activation of NF- $\kappa$ B and the interaction between *P. gingivalis* LPS and its receptor, CD14, on the outer surface of the human gingival fibroblasts (Wang and Ohura, 2002). The expression of pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  correlates with the invasion of oral epithelial cells by *P. gingivalis*, suggesting that the could have real importance in the pathogenesis of periodontal diseases (Sandros *et al.*, 2000).

**Table 1.2 The effect of different virulence factors of *P. gingivalis* on the expression of cytokines by different host cells.**

<b>Cytokine(s) of interest</b>	<b>Cell type</b>	<b>Source of stimulation</b>	<b>Increase/Decrease</b>	<b>Reference</b>
IL-8, ICAM-1	Human oral keratinocytes	<i>P. gingivalis</i>	Decreased expression of IL-8, ICAM-1	Huang <i>et al.</i> (2001)
IL-8	Human gingival epithelial cells	Whole <i>P. gingivalis</i>	Decreased expression of IL-8 Via ERK1/2 downregulation	Watanabe <i>et al.</i> (2001)
IL-8	Human gingival epithelial cells	Serine phosphatase protein (SerB) of <i>P. gingivalis</i>	Decreased expression of IL-8	Hasegawa <i>et al.</i> (2008)
IL-8	Human neutrophils	<i>P. gingivalis</i> gingipain	Decreased expression of IL-8	Mikolajczyk-Pawlinska <i>et al.</i> (1998)
IL-8	Oral epithelial cells (TERT-2) cell line	<i>P. gingivalis</i> Rgp gingipain	Decreased expression of IL-8	Giacaman <i>et al.</i> (2009)
IL-8	Human gingival epithelial cells	<i>P. gingivalis</i> fimbriae	Increase expression of IL-8 by NF- $\kappa$ B activation	Asai <i>et al.</i> (2001)

IL-1 $\beta$ , IL-6, IL-8	Human gingival epithelial cells	<i>P. gingivalis</i> Kgp gingipain	Decreased expression of IL-6, IL-8 Increase expression of IL-1 $\beta$ .	(Stathopoulou <i>et al.</i> , 2009a)
TNF- $\alpha$	The mouse L929 connective tissue cell line	<i>P. gingivalis</i> HRgpA, RgpB, and Kgp gingipains	Increase degradation of TNF- $\alpha$	Calkins <i>et al.</i> (1998)
TNF- $\alpha$	<ul style="list-style-type: none"> <li>• ADAM17/ Ras-Myc-immortalized murine fibroblasts.</li> <li>• Human histiocytic lymphoma cell line.</li> <li>• Acute promyelocytic leukemia</li> </ul>	<i>P. gingivalis</i> HRgpA, RgpB, and Kgp gingipains	Increase degradation of soluble and membranous TNF- $\alpha$	Mezyk-Kopec <i>et al.</i> (2005)
TNF- $\alpha$	GCF	<ul style="list-style-type: none"> <li>• Purified Arg-and Lys- gingipain of <i>P. gingivalis</i></li> <li>• <i>P. gingivalis</i> outer membrane vesicles</li> </ul>	Decrease TNF- $\alpha$ secretion by degradation of CD14 (LPS receptor)	Duncan <i>et al.</i> (2004)
IL-6	Human gingival fibroblasts	<i>P. gingivalis</i> LPS	IL-6 upregulation via TLR-4 activation	Wang and Ohura (2002)
IL-6, IL-8	Human gingival fibroblasts	<i>P. gingivalis</i> LPS via lipid A action	Upregulation of IL-6, IL-8	Herath <i>et al.</i> (2013)



### 1.6 The mammalian cell cycle

#### 1.6.1 General overview

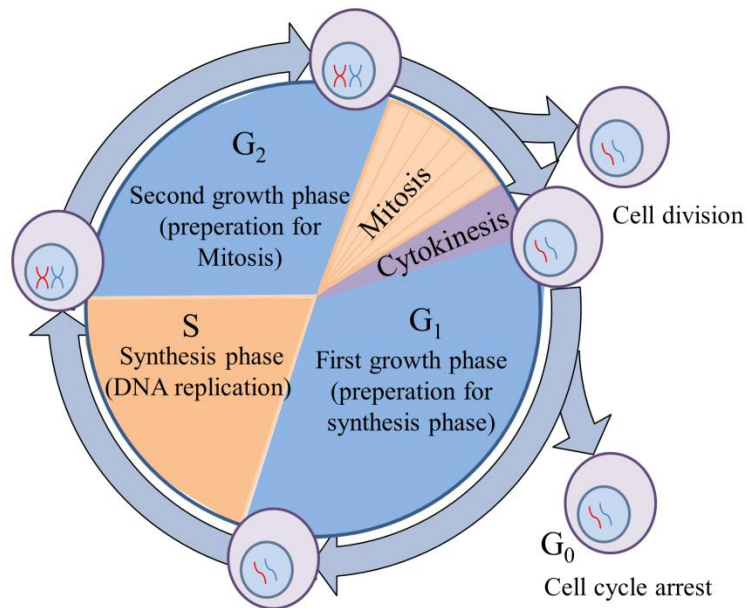
The ability of bacteria to become internalised into host cells, gain nutrition and stimulate cytokine release could be influenced by the growth phase of the host cells. It is possible that bacteria themselves influence the host cell growth cycle or in contrast, that the physiological state of the cell influences the events of cell invasion and intracellular lifestyle of the bacteria. For this reason it is important to review what is known about the cell cycle and its interaction with/influence on bacteria.

The mammalian cell cycle is a highly dynamic conserved process which is tightly orchestrated by series of regulatory pathways and molecular events that enable a single cell to be duplicated. The main function of the cell cycle is to transfer the genomic and cellular contents of a cell into two identical daughter cells. This process is achieved following completion of the two basic cycles which form the main skeleton of the entire cell cycle. These cycles are the chromosome cycle and growth cycle (Alberts *et al.*, 2002, Tyson *et al.*, 2002). The chromosome cycle consists of DNA replication followed by the segregation of the copied genome to the daughter cell nuclei, while the growth cycle represents the replication of the all cytoskeletal components, proteins, membranes, and organelles with the subsequent separation to daughter cells (Tyson *et al.*, 2002). It is essential for both processes to act in harmony to ensure that the cells can replicate their DNA and divide each time they grow under internal and external stimuli. Dysregulation of these cycles would result in cells with multiple cellular and genetic errors which would have potentially fatal consequences for the organism.

S-phase represents the DNA synthesis phase, in which the main replication of the double stranded DNA takes place to form pairs of “sister chromatids” joined together by specific proteins called cohesins (Nasmyth *et al.*, 2000). Following S-phase, the DNA content is condensed into two compartments to form two identical sets of chromosomes, followed by their separation to each of the newly formed daughter cell through a phase called M-phase (mitosis). The main nuclear and cytoplasmic divisions of the cells occur during the mitotic phase. The nuclear division consists of five sub-phases named: prophase, prometaphase, metaphase, anaphase, and telophase (Pollard *et al.*, 2008). The cells are then “nipped” and separated by cytoplasmic division through a

process called cytokinesis, in which the main cell division is accomplished (Alberts *et al.*, 2002).

In order to replicate the DNA and cytoplasmic components for the next generation of cells in a proper sequence and time, the cells require time for growth to increase their cellular mass, proteins, enzymes required for DNA synthesis and cell mitosis. Cell growth mainly exists through two gaps in the cell cycle called G1 and G2-phases. G1-phase represents the key regulatory growth phase of the cell cycle which is characterized by synthesis of the essential proteins with metabolic changes required for DNA synthesis in S-phase (Shaw *et al.*, 2010, Sherr, 1994). It starts following completion of cell mitosis and ends at the entry of the DNA synthesis phase. The length of time required for G1-phase is variable dependent on the nature of the extra-cellular stimuli such as nutrient depletion and loss of cell-cell contact. In addition, Cells may temporarily or permanently leave the cell cycle during G1 and enter an arrested state known as G<sub>0</sub> dependent on environmental and developmental signals (Morgan, 2006). However, cells may re-enter the cell cycle if the extracellular conditions are favourable enabling cells to progress through the point at the end of G1-phase known as the restriction point. Passing this point, cells commence DNA replication and continue through the cell cycle (Alberts *et al.*, 2002). Following DNA replication, cells continue their growth, synthesis of the necessary enzymes and proteins required for mitosis through the second growth gap called G2-phase. G2-phase is shorter than G1-phase, and is considered a crucial step to complete the cell mitosis through M-phase. Thus, the generic cell cycle sequence follows: G1-S-G2-M. G1, S and G2-phases are collectively known as interphase (Morgan, 2006) (figure 1.3).



**Figure 1.3 Mammalian cell cycle.** Cells start growing through the first growth phase (G<sub>1</sub>) to increase the cellular mass, proteins and enzymes necessary before transition into S-phase in which the main DNA replication events take place. Cells then pass through the second growth phase (G<sub>2</sub>) in which they synthesise the required proteins and enzymes for mitosis. The DNA condenses into chromosomes which are equally divided during nuclear division in the mitosis phase prior to the cytoplasmic division which is known as cytokinesis. Two daughter cells are generated which may then enter the cell cycle. If unfavourable signals are present, cells might transiently or permanently exit the cycle and remain in the arrested state (G<sub>0</sub>). (Adapted from © Clinical Tools, Inc- permission obtained to reproduce here).

### 1.6.2 Cell mitosis

As cells continue growing in the G<sub>2</sub>-phase, the cells begin to organize their genomic and cellular compartments prior to nuclear and cellular segregation into two identical sets of daughter cells.

The mitosis phase (M) is characterised by important cellular rearrangements with subsequent division of the cell nucleus and cytoplasm. As mentioned above it consists of five phases depending on the physical status of the chromosomes (Satzinger, 2008). Many biochemical studies of the mitotic phase has revealed that this process is highly

regulated, involving hundreds, if not thousands, of cellular proteins (Mitchison and Salmon, 2001).

The main nuclear rearrangements that exist during mitosis phase include: chromosomes condensation to form sister chromatids, disassembly of the nuclear envelope, alignment of each chromatid at the cellular poles with its related microtubules, separation of each chromatid through the action of anaphase-promoting complex (APC), and re-formation of the nuclear membrane around the separated sister chromatid with de-condensation of the chromosomes into their original state at interphase (Pollard *et al.*, 2008).

### 1.6.3 Cell cytokinesis

The physical division of the cells into two identical daughter cells is achieved through a process termed cytokinesis. It consists of dramatic biological changes within the cellular compartment. These include: signalling changes related to the division plane assembly, organization of the contractile apparatus, changes within the plasma membrane, and the final segregation of the two daughter cells termed as abscission (Pollard *et al.*, 2008). Fededa and Gerlich (2012) have reported that the chromosomal segregation and cytokinesis are tightly co-ordinated processes, which are activated following activation of the APC.

### 1.6.4 Cell cycle regulation

The progression of the cell cycle requires an ordered set of events that control the transition from the genomic duplication phase (S-phase) to the complete chromosomal segregation into two resultant identical daughter cells (M-phase) of the cell cycle. These phase transition inductions cannot continue without regulators that control the progression of the growth phases. In addition, cells at G1 are dependent on favourable or unfavourable extracellular signals, which may lead to transient or permanent exit from the cell cycle and a quiescent or arrested phase at G0 (Van Den Heuvel, 2005). The interaction between the extrinsic and intrinsic cell signals exists to determine whether cells commence cell division. For such a significant commitment, it is not surprising that extrinsic factors influence the entrance to the cell cycle only until cells go through G1 at a point known as a “Restriction point” in mammals and “START” in yeast (Van Den Heuvel, 2005). From that point, the entire cell cycle is controlled internally through the basic machinery which is conserved in all eukaryotes. Thus

research conducted in yeast genetics, biochemistry studies in frog eggs, and mammalian tissue culture models have provided a molecular foundation and understanding of cell cycle regulation. Thus, this regulation is considered one of the most complicated paradigms in the cell biological interactions as it involves a complex between nuclear genetics, cytoplasmic organelles, and the cell surface components with its external environmental surroundings (James Jr, 2012).

### 1.6.4.1 Cyclins and cyclin-dependant kinases (CDKs)

During the last two decades, several studies have reported that the progression of the cell cycle is brought about by through the activity of the key regulatory proteins termed CDKs and their active subunits, cyclins. CDKs are small serine/threonine protein kinases which become activated when conjugated with their cyclin substrates. The CDKs-cyclin complex activation controls the vast majority of the signalling pathways involved in the regulation of the cell cycle. These represent the consecutive expression/destruction of cyclins, phosphorylation/dephosphorylation of the CDKs, and the activation/deactivation of the CDK inhibitory proteins (Van Den Heuvel, 2005).

At least five of nine different CDKs are active during the mammalian cell cycle. The CDK4/6 are known to be active during G1-phase, CDK2 is the active kinase during S and G2-phases, while CDK1 is the active form present during G2-phase and mitosis. Two steps are essential for the CDKs to be fully activated: CDKs need to be associated with their subunits, cyclins, and the CDKs threonine residue in the activation loop needs to be phosphorylated by the combination of the CDK7 with cyclin H, which acts as a CDK activating kinase (Schachter *et al.*, 2013). While the level of the majority of cyclins remains fluctuant throughout the cell cycle, the stable level of the CDKs remains one of the characteristic features of the cell cycle development (Morgan, 2006).

Sherr *et al.* (1994) have shown that the cell entry to G1-phase is mediated by binding of cyclins D1/2/3 with CDK4/6, while the transition from G1 to S-phase is regulated through association of cyclin E and CDK2 (Ohtsubo *et al.*, 1995). As cells progress through S-phase, the association of cyclin A with CDK2 become more prominent (Girard *et al.*, 1991). In addition, at late G2-phase, cyclin A-CDK2 complex is formed

to permit cell's entry into mitosis, which is then regulated by an association between cyclin B with CDK1 (Nurse, 1990).

CDKs and cyclin activity can be inhibited through the action of the cyclin-dependent kinase inhibitors (CKIs), which can associate with CDKs or with CDK-cyclin complexes. The regulation of these inhibitory proteins is controlled by different extracellular and intracellular signals. For example, TGF $\beta$  has the ability to stimulate p27 expression, inducing cell cycle arrest. On the other hand, a tumor suppressor gene, p53, can control the transcription of p21, affecting cell growth (El-Deiry *et al.*, 1993, Harper *et al.*, 1993, Polyak *et al.*, 1994, Toyoshima and Hunter, 1994).

### 1.6.4.2 Cell cycle checkpoints

The cell cycle checkpoints act as quality control points which allow the cell to continue its growth through the cell cycle. Pardee (1974) described a restriction point "R" within the G1 gap, which determines the cell commitment to enter the cell cycle, as "the point of no return". Serum starvation or removing of the critical amino acids required for cell growth before R stimulated cells to stay at G0, while serum and amino acids starvation following the "R" point have no effect on the cell cycle progression (Pardee, 1974). In addition, other checkpoints have been reported in the cell cycle to ensure that each cell cycle phase is completed without errors before the next phase begins.

Ko and Prives (1996) have reported the significance of G1/S transition checkpoint which prevents duplication of damaged DNA before cell transition into S-phase. The level of p53, a regulator of G1/S transition, is available at low level under normal circumstances but which rises significantly if DNA damage occurs, leading to the cyclin-dependent kinase (CDK) inhibition and cell cycle arrest following *p21* gene activation (Ko and Prives, 1996). However, in severe cases of DNA damage, the level of p53 is extremely high and is involved in inducing cellular death by activation of apoptotic genes (Gottlieb and Oren, 1998). In addition, the importance of G2/M checkpoint has been investigated to determine whether the DNA damage existed during G2, inducing cellular arrest at this stage (Peng *et al.*, 1997).

When the chromosomes are present in the unattached form to the spindle, another control gate termed the “spindle assembly checkpoint” is activated, preventing the cell transition from metaphase to anaphase (Lara-Gonzalez *et al.*, 2012).

Recently, an additional checkpoint has been reported to exist within cytokinesis. Studies demonstrated that the unseparated chromosomes within the division plane can be sensed through the activity of the so called ‘Aurora B kinases’, which inhibit abscission when activated until the complete clearance of the division plane. This process is termed the “NoCut pathway” (Norden *et al.*, 2006, Steigemann *et al.*, 2009).

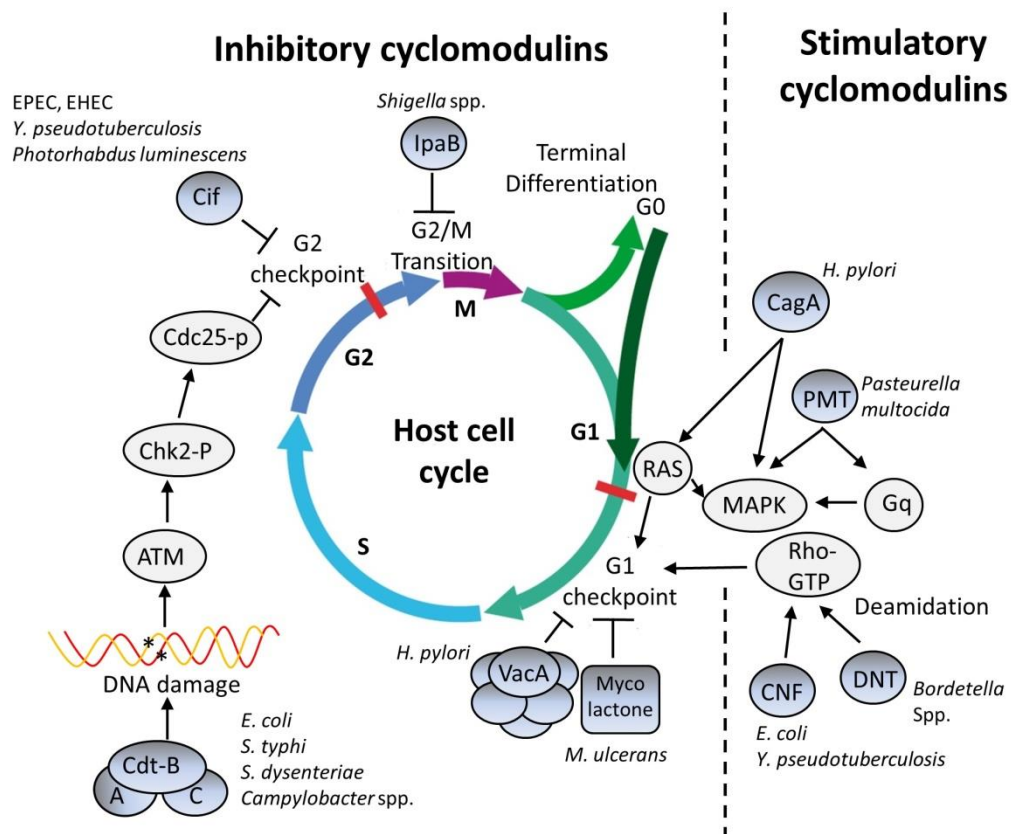
### 1.6.5 Bacterial interaction with cell cycle.

The ability of pathogenic bacteria to manipulate host-cell functions to favour their survival, colonization, and spread of infection represents a key strategy for the bacteria to be successful pathogens. One of these interactions is the modulation of the host cell cycle by a group of bacterial toxins termed “cyclomodulins”. these can have both stimulatory and inhibitory influences on the progression of the cell cycle (Nougayrède *et al.*, 2005).

The inhibitory cyclomodulins such as cytolethal distending toxins (CDTs) from *Salmonella typhi*, *E. coli*, *Shigella dysenteriae*, *Campylobacter* spp. (Guerra *et al.*, 2011, Pérès *et al.*, 1997), mycolactone from *Mycobacterium ulcerans* (Pahlevan *et al.*, 1999), Cycle inhibiting factor from enteropathogenic and enterohaemorrhagic *E. coli* (Cui *et al.*, 2010, Jubelin *et al.*, 2010, Morikawa *et al.*, 2010, Samba-Louaka *et al.*, 2009), and IpaB effector protein secreted by *Shigella* species (Iwai *et al.*, 2007) have an inhibitory effect on the cell cycle progression by inducing G1/G2 arrest. Such factors can contribute to avoiding immune surveillance. In addition, they might impair the integrity of the epithelial barrier, facilitating pathogen intrusion into the host tissue or preventing of epithelial shedding, which encourages bacterial persistence.

In contrast, stimulatory cyclomodulins can enhance cellular proliferation, and interfere with normal cell differentiation pathways, affecting cell development. Bacterial effectors such as Cytotoxin-associated antigen A protein from *Helicobacter pylori* induces a rapid gastric cell progression from G1 into G2/M by activation of the MAPK

pathway (Oswald *et al.*, 2005). In addition, cytotoxic necrotizing factors (CNFs) secreted by *E. coli* and *Y. pseudotuberculosis*, and dermonecrotic factors from *Bordetella* species stimulate Rho GTPases activity, affecting the levels of cyclin D1 and CKIs and contributing to the cell cycle progression (Horiguchi, 2001). Figure 1.4 highlights the effect of various stimulatory and inhibitory bacterial effectors on the cell cycle progression. The stimulatory and inhibitory effects of various cyclomodulins on the cell cycle could be applied by different periodontal pathogens as described in section 1.6.6.



**Figure 1.4 Bacterial cyclomodulin influences on the host cell cycle.** Four successive phases constitute the host cell cycle: G<sub>1</sub> (1<sup>st</sup> growth phase), S (DNA replication), G<sub>2</sub> (2<sup>nd</sup> growth phase), and M (mitosis phase). Cells in G<sub>1</sub> can enter a resting state at G<sub>0</sub> (resting state). Inhibitory cyclomodulins block the cell cycle progression whereas stimulatory cyclomodulins promote cell proliferation (Permission obtained to reproduce here).



The influence of these cyclomodulins in the understanding of bacterial virulence and host tissue destruction needs further study and may have applications for novel therapeutic strategies.

### 1.6.6 Periodontal pathogens-cell cycle interactions

The association of the periodontal pathogens with the host cell cycle has been investigated within the last few years. For example, it has been shown that leukotoxic strains of the early onset periodontal pathogen *A. actinomycetemcomitans*, have the ability to induce membrane lysis and apoptosis of neutrophils, monocytes, and T cells (Henderson *et al.*, 2010, Henderson *et al.*, 2002, Kachlany, 2010). This cell-induced apoptosis might be attributed to G2-phase blockage induced through the action of an additional major toxin secreted by *A. actinomycetemcomitans*, an *A. actinomycetemcomitans*-related CTD (Saiki *et al.*, 2004, Sugai *et al.*, 1998). This CTD toxin has the ability to induce expression of p21 (CIP1/WAF1), a cyclin-dependent kinase inhibitor, with subsequent G2 cell cycle arrest through p53-independent pathways (Sato *et al.*, 2002).

Various cell cycle effects have been reported in host cells exposed to *F. nucleatum*. Uitto *et al.* (2005) showed that the levels of collagenase 3 and 12 protein kinases involved in the cell proliferation, migration and survival signalling were increased following infection of human epithelial cells with *F. nucleatum*, suggesting a role for this microorganism in pocket formation and periodontal diseases. From these 12 kinases, protein kinase C epsilon (Comalada *et al.*, 2003) and death-associated protein kinase 1 (Shohat *et al.*, 2002) induced cell apoptosis, suggesting that in addition to the stimulatory effect on cell proliferation, *F. nucleatum*-infected epithelial cells might also undergo apoptosis (Uitto *et al.*, 2005).

Demuth *et al.* (1996) showed that the *F. nucleatum* immunosuppressive protein (FIP) has the ability to arrest the cell cycle at mid G1-phase and inhibit T cell activation. This apoptotic effect has also been reported in peripheral blood mononuclear cells and PMNs following infection with *F. nucleatum* which leads to an increase in DNA fragmentation and subdiploid DNA content (Jewett *et al.*, 2000). However, *F. nucleatum*, can activate the NF- $\kappa$ B transcription factor which increases the

expression of DNA-activated protein kinase which is the principle kinase involved in the repair of the damaged DNA preventing cell apoptosis (Durocher and Jackson, 2001). Recently, a significant association between *F. nucleatum* and colorectal cancer was observed through increased cell proliferation of the colon cancer cells stimulated by activation of the  $\beta$ -catenin signalling. This activation was triggered by binding of fusobacterial adhesin FadA to E-cadherin on colon cancer cells (Rubinstein *et al.*, 2013).

*T. denticola*, one of the red complex periodontal pathogens, has been reported to activate MAPK signalling pathway and apoptosis in a murine calvarial bone and soft tissue infection model (Bakthavatchalu *et al.*, 2010). Lee *et al.* (2004) have shown that Sip (*T. denticola*-immunosuppressive protein) has the ability to impair the proliferation of human lymphocytes, and that the pre-treatment with this protein resulted in irreversible G1-phase arrest and activation of the apoptotic cascade. This was accompanied by a significant reduction in the number of cells in S and G2/M-phases, leading to the immunodeterioration and enhanced survival of the spirochetes at the site of infection.

The role of *T. forsythia* in host cell cycle modulation has been investigated in recent years. Nakajima *et al.* (2006) showed that following infection of the human epidermal carcinoma cells with *T. forsythia*, a significant arrest at G2-phase occurs and there is accumulation of a sub-G1 cell population. Elevated cyclin B1 and p53 were observed due to the effect of G2AIF, a cytopathic protein factor. In addition, *T. forsythia*, has been shown to exert an apoptosis-stimulating effect with severe membrane ruffling, DNA ladder formation and caspase 3 activation upon infection of the human leukaemia cell line (HL-60) (Arakawa *et al.*, 2000). *T. forsythia* surface-lipoproteins stimulate cellular apoptosis and proinflammatory cytokine release through activation of caspase-8, which initiates the caspase activation cascade involved in apoptosis (Hasebe *et al.*, 2004).

A growth inhibitory effect of *P. intermedia* endotoxin on proliferation of the periodontal ligament cells (PDLs) was observed. A significant increase in the proportion of cells at G1-phase and a decrease in cell number in S-phase was observed following 6 hours and 12 hours incubation with *P. intermedia* endotoxin (Ling *et al.*, 2010). However, others have shown a dose-dependent inhibition of the PDLs proliferation following 24 hours exposure to this endotoxin accompanied by ultrastructural changes such as Golgi body swelling (Yuan *et al.*, 2002).

The effect of other periodontal pathogens such as *P. nigrescens*, *C. rectus*, *Eubacterium nodatum*, *S. intermedius*, *Micromonas micros*, *Eikenella corrodens*, *C. ochracea*, *Veillonella parvula*, *Actinomyces naeslundii* on the host cell cycle has not been previously documented. Regarding *P. gingivalis* interaction with the cell cycle, several studies showed that the bacteria have both inhibitory and stimulatory effects on the cell cycle progression and apoptosis. This will be discussed in details in chapter 5.

### 1.7 Hypothesis, aims and objectives

The influence of the phases of the cell cycle on host cell interaction with *P. gingivalis* has not been previously documented. Others have noted that not all cells in a culture become equally invaded by *P. gingivalis* or other bacteria. Consequently the question arises as to whether subpopulations of oral keratinocytes display differences in their interaction with *P. gingivalis*, which reflect differences in the phase of the cell cycle that they are in. Therefore, the primary aim of this study was to investigate whether different phases of the epithelial cell cycle are more susceptible to invasion by *P. gingivalis*.

To test this hypothesis, the following aims were carried out:

- 1- Establish a reproducible *P. gingivalis* assay using H357 oral keratinocytes and investigate the effect of various host and bacterial factors on the level of *P. gingivalis* invasion.
  - Investigate the effect of different incubation times on *P. gingivalis* invasion.
  - Characterise the bacterial growth phases and determine their effect on *P. gingivalis* invasion.

## Chapter 1 Literature Review

- Compare the *P. gingivalis* invasion efficiency using two models: antibiotic protection assay and DNA extraction method.
  - Explore the optimum conditions for release of *P. gingivalis* from cells using different cell lysis buffers.
  - Characterise the influence of the epithelial cell density and passage number on the invasion of *P. gingivalis*.
- 2- Investigate the epithelial cell response to *P. gingivalis* invasion
- Determine the influence of pre-treating H357 cells with the *P. gingivalis* LPS on the level of invasion.
  - Investigate the effect of different incubation times of *P. gingivalis* on the gene expression of the invaded H357 cells such as Endothelin-1 (ET-1) and Urokinase Plasminogen Activator Receptor (uPAR).
  - Address whether the physical state of the cells (adherent or in suspension) influences *P. gingivalis* invasion.
  - Determine whether *P. gingivalis* localisation is apparent within H357 cells using immunocytochemistry.
- 3- Investigate the invasion of *P. gingivalis* in synchronised cultures to identify whether invasion varies according to the cell cycle phase.
- Investigate the cell cycle of H357 cells using flow cytometry versus immunofluorescence techniques.
  - Determine *P. gingivalis* invasion with various times of cell cycle synchronization.
  - Optimise the bacterial association with cell cycle phases.
  - Investigate the effect of serum synchronization on the mRNA and cell surface protein expression of uPAR and  $\alpha 5$ -integrins.
  - Determine the association of cells in S-phase with the invaded *P. gingivalis* using double immunofluorescence technique.

# **Chapter 2**

# **Materials and Methods**

### 2.1 Materials

All materials were purchased from Sigma, UK unless otherwise specified.

### 2.2 Epithelial cell culture conditions

All experiments were conducted with the oral keratinocyte cell line H357, a squamous cell carcinoma (SCC)-derived cell line isolated from the tongue (kindly provided by Professor S.Prime, University of Bristol). H357 cells were cultured in keratinocyte growth medium (KGM) at 37°C, 5% CO<sub>2</sub>. Table 2.1 shows the supplements for KGM added to a 3:1 ratio of Dulbecco's modified eagles medium (DMEM) and Nutrient Mixture F-12 (Ham's F12). Routine subculturing of H357 cells was performed twice weekly to maintain the viability of the cells. Subculturing was performed by washing the flask containing the H357 cell monolayer with phosphate buffered saline (PBS) without Mg<sup>2+</sup> or Ca<sup>2+</sup>, followed by addition of 0.05% (v/v) porcine trypsin / 0.02% (v/v) ethylene diamine tetra-acetic acid (EDTA) at 37°C for 5-10 minutes until the cells became detached. To neutralize the enzymatic activity of trypsin, KGM was added to the flask and the suspension centrifuged at 179 g for 5 minutes. The supernatant was removed and cell pellet was resuspended with KGM and adjusted to 0.5-1x10<sup>6</sup> cells/75 cm<sup>3</sup> flask. All cell samples were subjected to periodic testing for mycoplasma infection to confirm that the experiments were carried out on uncontaminated samples.

For the standard invasion experiments, the seeding of H357 cells was optimized at 5x10<sup>4</sup>/well into 24 well plates (NUNC, UK, 1 ml per well) two days prior to starting the experiments, while for the purpose of testing the invasion efficiency of different growth phases of *P. gingivalis* and for the purpose of screening H357 gene expression in response to *P. gingivalis* invasion, H357 cells were seeded at 1x10<sup>5</sup>/well and 2x10<sup>5</sup>/well in 24 and 6 well plates respectively, 24 hours prior to commencing the assay.

**Table 2.1 Supplements added to the KGM.** All supplements were added to a 3:1 ratio of DMEM and Ham's F-12, to culture the H357 oral keratinocyte cell line.

Supplement	Final concentration	Company
Fetal bovine serum	10% (v/v)	Sigma, UK
Streptomycin	100 IU/ml	Invitrogen, UK
Penicillin	100 IU/ml	Invitrogen, UK
Amphotericin B	2.5 µg/ml	Invitrogen, UK
L-Glutamine	2mM	Sigma, UK
Adenine	0.18 mM	Sigma, UK
Hydrocortisone	5 µg/ml	Sigma, UK
Cholera toxin	1 nM	Sigma, UK
Insulin	5 µg/ml	Sigma, UK
Epidermal growth factor	10 ng/ml	Sigma, UK

### 2.3 General bacterial growth and culture conditions

*P. gingivalis* NCTC 11834 (Coykendall *et al.*, 1980) lab strain was obtained from the National Collection of Type Cultures (Public Health England, Porton Down, UK). *P. gingivalis* was grown and maintained on blood agar plates containing fastidious anaerobe agar medium (FA; LabM, UK), supplemented with 5% (v/v) oxalated horse blood (Oxoid, UK). Bacterial subculturing and incubation were performed under anaerobic conditions (10% H<sub>2</sub>, 10% CO<sub>2</sub>, and 80% N<sub>2</sub>) at 37° C in an anaerobic cabinet two days prior to starting the experiments. For the purpose of testing *P. gingivalis* NCTC 11834 growth phases, the bacteria were grown and maintained in brain heart infusion (BHI) broth supplemented with yeast extract, haemin, vitamin K, and cysteine. Table 2.2 shows the concentrations of BHI broth with related supplements used for *P. gingivalis* NCTC 11834 growth phase experiments. Periodic microscopic examination of Gram stained *P. gingivalis* NCTC 11834 was performed to confirm the purity of the bacterial culture prior to starting the experiments.

**Table 2.2 Brain heart infusion (BHI) broth with its related supplements used for the culture of *P. gingivalis* NCTC 11834 growth phase experiments.** Brain heart infusion broth and yeast extract were autoclaved, and the filter sterilized supplements added prior to bacterial culture.

Supplement	Concentration	Company
Brain heart infusion broth	37 g/L	Oxoid, Hampshire, UK
Yeast extract	5 mg/ml	Oxoid, Hampshire, UK
Haemin	5 µg/ml	Oxoid, Hampshire, UK
Vitamin K	1 µg/ml	Sigma, UK
Cysteine	0.5 mg/ml	ICN Biomedical Inc., UK

#### **2.4 *P. gingivalis* invasion of H357 cell monolayer (Antibiotic protection assay).**

An antibiotic protection assay was used to determine the level of invasion of H357 cells by *P. gingivalis* (Duncan *et al.*, 1993, Lamont *et al.*, 1995, Sandros *et al.*, 1994, Tang *et al.*, 1993). Briefly, two day old plate-cultured bacteria were harvested using a sterile swab and transferred into 1 ml of PBS in an Eppendorf tube, followed by 3 washes in PBS with centrifuging at 13000 g for 2 minutes. Bacterial counting was performed using a Helber chamber (Hawksley, Sussex, UK). The bacteria were resuspended in DMEM and adjusted to a multiplicity of infection 1:100, i.e. every epithelial cell was exposed to 100 bacterial cells. Following 48 hours incubation of the H357 cells in 24 well plates at 37°C, 5% CO<sub>2</sub>, the confluent monolayers were washed 3 times with 1 ml of PBS and incubated with 2% (w/v) bovine serum albumin (BSA) in DMEM to block the non-specific binding of bacteria for 1 hour at 37°C. To count the exact number of H357 cells in each well prior to commencing the invasion assay, at least 3 wells were trypsinized (0.05% trypsin-0.02% EDTA), followed by counting using a haemocytometer (Hawksley, UK). The incubation of bacterial cells with the epithelial cells was conducted for 90 minutes at 37°C. Infected H357 cells were then washed with 1 ml PBS 3 times in order to remove the non-adherent bacteria. To kill the extracellular adherent bacteria, 1 ml/well of 200 µg ml<sup>-1</sup> metronidazole in DMEM was added for 1 hour 37°C. Cells were then washed 3 times with PBS and 1 ml/well distilled water was added to lyse the cells

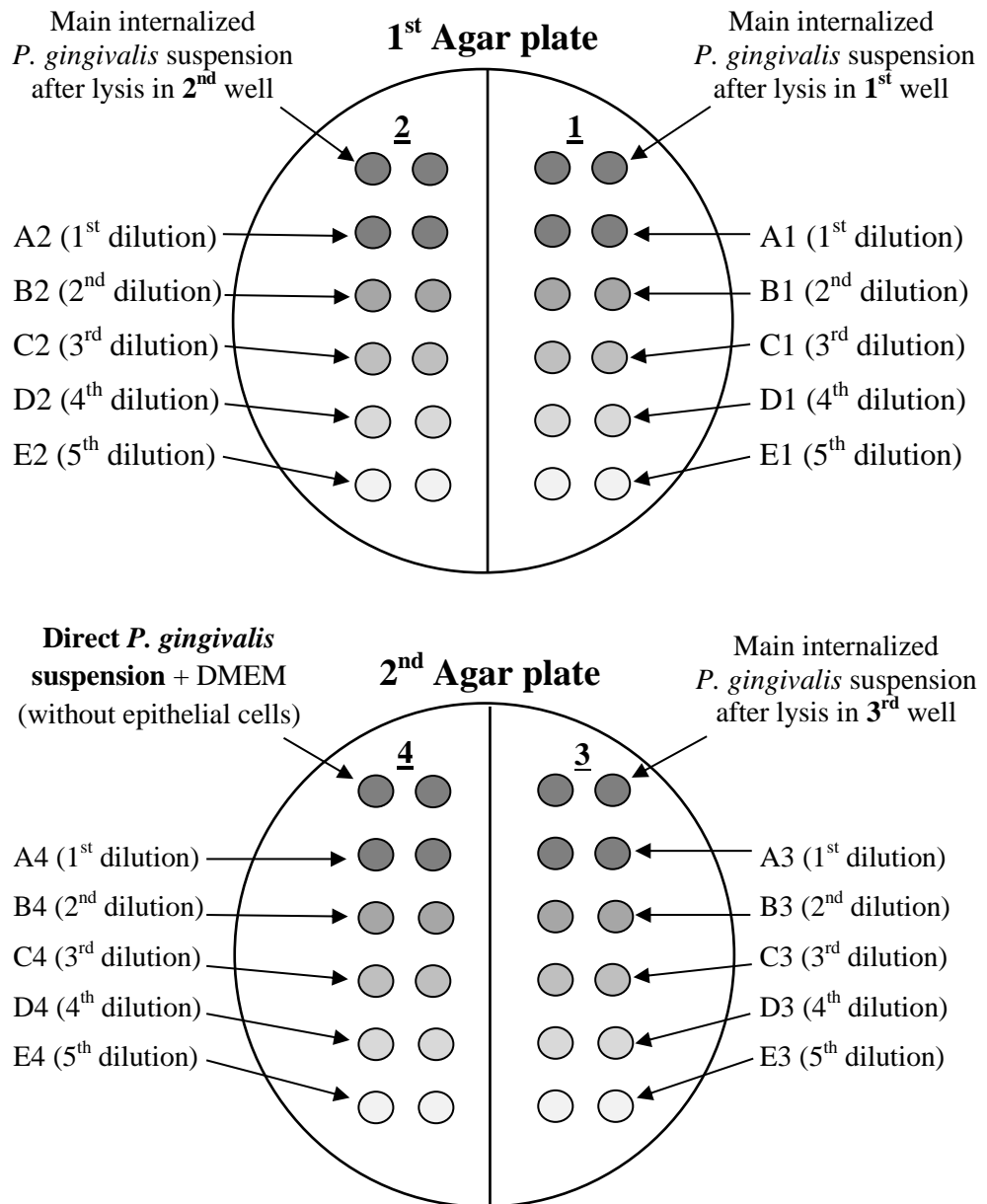


## Chapter 2 Materials and Methods

for 20-30 minutes followed by 1 minute of scraping and agitation using a sterile pipette tip to ensure release of the internalized bacteria. The resultant bacterial suspension then was serially diluted to five dilutions as shown in figure 2.1, plated in duplicate on FA agar supplemented with 5% horse blood and incubated for 5 days under anaerobic conditions at 37°C. Viable counting was conducted and the percentage of invasion was expressed as the proportion of the number of viable bacteria recovered after 5 days incubation, divided by the visible count of the main bacterial inoculum, multiplied by 100.

$$\% \text{ Invasion} = \frac{\text{Number of viable recovered bacterial count}}{\text{Number of initial bacterial inoculum}} \times 100$$

All the invasion assays were performed at least 3 times in triplicate and the final results were presented as mean±SD.



**Figure 2.1 Illustration of how serial dilutions were inoculated in duplicate on two FA agar plates.** Two FA agar plates were divided into two halves as shown above. **1, 2 and 3** represent lysates from triplicate wells of H357 cells that had been invaded by *P. gingivalis*. **4**, represents the original bacterial suspension that was used in the invasion experiments. Each *P. gingivalis* suspension (lysates and initial inoculum of H357 cells) were then serially diluted through five dilutions (A-E) as illustrated above. Plates were incubated under anaerobic conditions prior to viable counting.

### 2.5 Analysis of *P. gingivalis* invasion using DNA extraction.

To characterize the *P. gingivalis* invasion efficiency using a method not dependent on viable counting, the *P. gingivalis* invasion percentage was calculated using a DNA extraction method, in which the bacterial DNA was purified and the number of the invaded bacteria was calculated using the DNA concentration. H357 cells were seeded and infected with *P. gingivalis* NCTC 11834 as described in section 2.4. Following the distilled water lysis step, the lysate was transferred to an Eppendorf tube and centrifuged at 13000 g for 2 minutes. To count the number of invaded *P. gingivalis* using DNA extraction method, the experiment was conducted in two complementary steps:

1. *P. gingivalis* DNA extraction.
2. Calculating the number of invaded *P. gingivalis* according to its DNA concentration.

#### 2.5.1 *P. gingivalis* DNA extraction.

The *P. gingivalis* DNA was extracted and purified using QIAamp DNA mini kit (Qiagen) according to the manufacturer instructions. Briefly, following centrifugation, the pellet was re-suspended with 300µl PBS. For bacterial lysis, lysozyme, mutanolysin, and lysostaphin were prepared as shown in table 2.3, added to the samples, and incubated at 37°C for 1 hour. To eliminate the effect of protein contamination and nucleases which degrade the bacterial nucleic acids, 24 µl proteinase K 20 mg/ml was added to the samples in addition to 4.8 µl RNase A (100mg/ml) to degrade the RNA content, and 300µl lysis buffer AL were added and incubated for 10 minutes at 56°C. For DNA precipitation, 400µl of 100% ethanol was added, and samples were briefly vortexed. Next, 700 µl of the lysate was transferred to the silica membrane spin columns for DNA binding and isolation, and centrifuged at 6000 g for 1 minute. The supernatant was removed from the column, and the process was repeated with the remaining lysate. Serial washings with 500 µl buffer AW1 (centrifuged at 6000 g for 1 minute), and 500 µl buffer AW2 (centrifuged at maximum speed for 1 minute) were performed to remove any impurities and leave the *P. gingivalis* DNA bound to the membrane. The membrane was then dried by centrifugation at maximum speed for 3 minutes. Finally, the bacterial DNA was eluted from the membrane by

adding 100 µl of distilled nuclease free water in a new collection tube and centrifuging at 6000 g for 1 minute.

**Table 2.3 DNA extraction reagents.** The concentration, volume added, and the type of buffers used for *P. gingivalis* lysis prior to DNA extraction.

Reagent	Concentration	Volume added	Type of buffer
Lysozyme	10 mg/ml	90µl	Tris-EDTA buffer
Mutanolysin	25,000 U/ml	3.6µl	Tris-EDTA buffer
Lysostaphin	4000 U/ml	1.8µl	Nuclease free water

### 2.5.2 Calculating the number of invaded *P. gingivalis* according to its DNA concentration.

Prior to calculating the *P. gingivalis* DNA concentration, a standard curve of *P. gingivalis* DNA concentrations was created using *P. gingivalis*-universal primers (In collaboration with Dr. Sarhang Gul, former PhD student in Oral and Maxillofacial Pathology Department/University of Sheffield). *P. gingivalis* gene amplification as shown in figure 2.2.A was performed by qPCR as described in section 2.15. The nucleotide sequence of the forward *P. gingivalis* primer used was: GCG AGA GCC TGA ACC AGC CA, while for the reverse primer was: ACT CGT ATC GCC CGT TAT TCC CGT A (Ammann *et al.*, 2013). The primer amplification was conducted in triplicate using the 7900HT Fast Real-Time PCR Detection System (Applied Biosystems) in a 96-well plate format (Semi Skirted, FAST). The qPCR cycle reactions were as follows:- initial denaturation for 5 minutes at 95°C, followed by 40 cycles of amplification comprising dissociation of DNA at 95°C, annealing of primers, and extension at 72°C for 1 minute each, and final extension for 7 minutes at 72°C. The reaction mixture was prepared in a total volume of 20µl containing 10µl SYBR® Green Master Mix (Life Technologies), 7µl nuclease free water, 1µl each of forward and reverse primers (5nM) and 1µl DNA template.

## Chapter 2 Materials and Methods

The DNA concentration was measured using a NanoDrop 1000 spectrophotometer (ThermoScientific) at 260/280 nm, and adjusted to 1 ng/μl with nuclease free water. A standard curve (from 1-10<sup>-5</sup> ng) was created following addition of tenfold serial dilutions, by plotting the known DNA concentrations against the corresponding point at which the *P. gingivalis* primers showed high linearity (R>0.99). To determine the efficiency of *P. gingivalis* primers used for the genomic quantification, the standard curve slope value was used which was -3.542, indicating an efficiency of 90-100%. The final DNA concentration of *P. gingivalis* was determined by locating the value on the standard curve of *P. gingivalis* against its corresponding Ct. value as shown in figure 2.2.B.

This concentration was also logarithmically calculated using excel software (Microsoft office 2010) by the following formula:

$$Y=M.LOG(X) +B$$

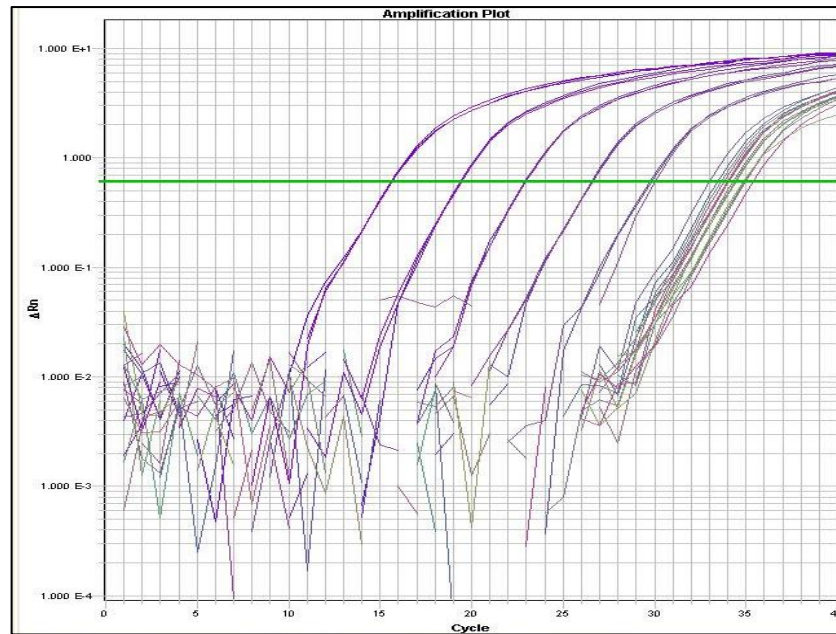
In which; Y =Ct. value, M= slop (-3.5421), X= Concentration, B=Y-intercept (15.74542). Therefore, **X = INV. LOG [(Y - B)/M]**.

Finally, the number and percentage of invaded *P. gingivalis* was calculating using following formula:

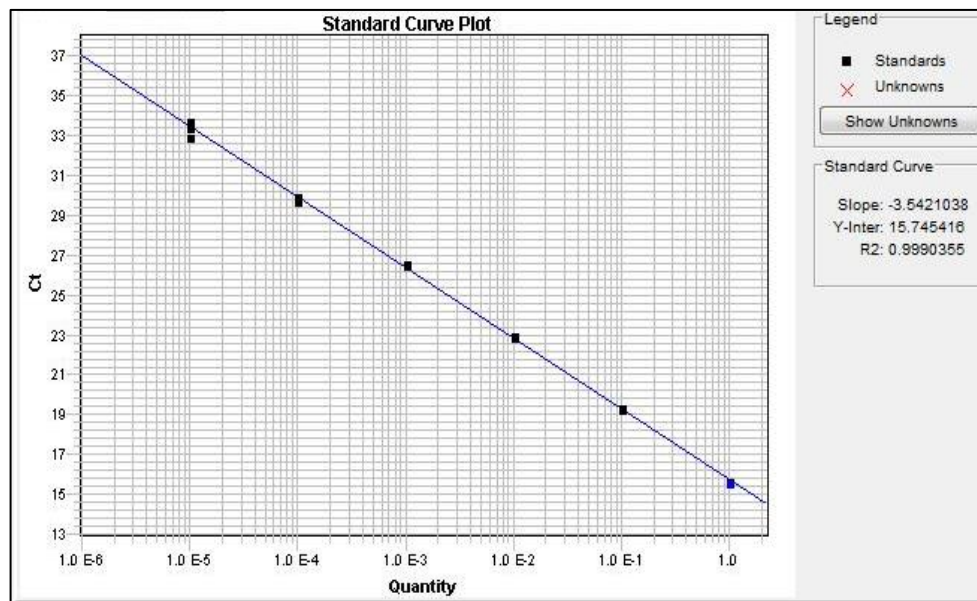
$$\text{Number of invaded } P. \text{ gingivalis} = \frac{\text{DNA conc. (ng/}\mu\text{l)} \times 100 \mu\text{l} \times 1000000}{2.58 \text{ femtogram}}$$

In which; 100 μl = *P. gingivalis* DNA volume, 1000000= to convert from nanogram to femtogram, 2.58 femtogram= genomic weight of *P. gingivalis* (Ammann *et al.*, 2013). For comparison purposes, a standard antibiotic protection assay was conducted in parallel, and the number of invaded bacteria was calculated using the viable counting method as described in section 2.4.

A



B



**Figure 2.2** The amplification plot (A) and the standard curve (B) used to determine the DNA concentration of *P. gingivalis*. The amplification plot was obtained using *P.gingivalis* specific primers and qPCR, and the standard curve was generated by plotting the known DNA concentrations against its correspondent point in which the *P. gingivalis* primers showed high linearity ( $R>0.99$ ). The DNA concentration of *P. gingivalis* was determined by locating the value on the standard curve against the corresponding Ct. value.

### **2.6 Incubation time and the invasion efficiency of *P. gingivalis*.**

To determine the effect of the infection time on the invasion efficiency of H357 cells by *P. gingivalis*, an antibiotic protection assay was conducted as described in section 2.4 with 30, 90, and 240 minutes incubation time. The invasion assays were conducted at least 3 times in triplicate, and the results were analyzed and compared between these incubation times.

### **2.7 *P. gingivalis* growth phases.**

#### **2.7.1 Analysis of *P. gingivalis* growth curve.**

For the purpose of studying the growth curve of *P. gingivalis* NCTC 11834, two day old cultures were inoculated in 5 ml of supplemented BHI broth (section 2.3), which was maintained under anaerobic conditions at 37°C overnight. The next day, 1 ml of the overnight culture was transferred in to 20 ml of supplemented BHI broth, and the first optical density (time zero) at 600 nm was recorded using a UV/visible spectrophotometer (Bio-Rad, USA). The OD measurements were repeated every 2 hours until 30 hours of incubation. At each time point, Gram staining and inoculation onto FA agar was performed separately to confirm the purity of the *P. gingivalis*.

#### **2.7.2 Effect of growth phases on *P. gingivalis* invasion efficiency.**

Invasion assays were performed with midlog and stationary phases of *P. gingivalis* NCTC 11834. H357 monolayers were prepared as previously described in section 2.2, and exposed to 12 hours (midlog) and 30 hours (stationary) BHI broth grown cultures of *P. gingivalis* for 90 minutes according to the antibiotic protection assay as described in section 2.4.

### **2.8 Effect of different lysis buffers on *P. gingivalis* invasion efficiency with H357 monolayer cell culture.**

An antibiotic protection assay as described in section 2.4 was conducted by using 0.1% Saponin (1 mg/ml) (w/v) dissolved in PBS as a lysis buffer, and the results were compared to experiments using distilled water. Cells were seeded at conc.  $5 \times 10^4$ /well in 24 well plate to be nearly confluent after 2 days of incubation, and infected with *P. gingivalis* NCTC 11834 for 90 minutes. After killing the adherent bacteria, cells were washed twice with PBS, and incubated with 0.1% (w/v) Saponin dissolved in PBS

for 5-10 minutes, as it forms pores in eukaryotic membranes enhancing permeabilization to release the internalized bacteria. Finally, cells were inoculated on FA agar and the viable counting was performed after 5 days to estimate the percentage of *P. gingivalis* invasion as described in section 2.4. Control samples (H357 cells) infected with *P. gingivalis* were lysed using distilled water, and the results were compared.

### **2.9 Effect of cell seeding density on *P. gingivalis* invasion.**

To determine whether *P. gingivalis* invasion is affected by the confluency of the cells, H357 cells were seeded at  $2.5 \times 10^4$ ,  $5 \times 10^4$ ,  $7.5 \times 10^4$  cells/well in 24 well plate at 37°C, 5% CO<sub>2</sub> overnight. *P. gingivalis* was grown two days prior to the starting of invasion assay. H357 cells were infected with *P. gingivalis* NCTC 11834 for 90 minutes at 37°C, 5% CO<sub>2</sub> as described in section 2.4. Direct comparisons of the *P. gingivalis* invasion efficiency were made between these different seeding concentrations.

### **2.10 Effect of cell passage on *P. gingivalis* invasion**

To determine whether the number of culture passages of H357 cells has an effect on their sensitivity to *P. gingivalis* invasion, comparison was made between cultures of cells with low passage number and cultures of cells that had been passaged many time. 3 biological repeats of the antibiotic protection assay, as described in section 2.4, were conducted with the limited (early) passage cells (p=56, p=57, and p=58) and compared to cells passaged many times (late) (p=218, p=219, and p=220). H357 cells were seeded at  $5 \times 10^4$ , cells/well in 24 well plate at 37°C, 5% CO<sub>2</sub> for 2 days. *P. gingivalis* was grown two days prior to starting of invasion assay. H357 cells were infected with *P. gingivalis* NCTC 11834 for 90 minutes at 37°C, 5% CO<sub>2</sub> as described in section 2.4. Direct comparisons of the *P. gingivalis* invasion efficiency were made between both passage groups.

### **2.11 *P. gingivalis* invasion of H357 cells pretreated with *P. gingivalis* Lipopolysaccharide (LPS).**

To investigate the effect of the pretreating H357 cell monolayers with *P. gingivalis* LPS on the invasion efficiency of *P. gingivalis*, H357 cells were seeded in 24 well plates at  $1 \times 10^5$ /well for 24 hours at 37°C, 5% CO<sub>2</sub>, followed by medium replacement with KGM



containing *P. gingivalis* LPS (1 µg/ml, LPS-PG Ultrapure, InvivoGen, USA) for 24 hours prior to the commencement of the antibiotic protection assay with 90 minute incubation time as described in section 2.4. Control cells without LPS underwent medium replacement at the same time as treated cells.

### **2.12 Comparison of *P. gingivalis* invasion of adherent H357 cells to those in suspension.**

To determine whether *P. gingivalis* internalization into H357 oral keratinocytes is affected by the physical state of the cells, a modified antibiotic protection assay was conducted with cells in suspension and compared to a standard antibiotic protection assay with cells in monolayer culture. For invasion of cells in suspension, H357 cells were cultured in T75 flask size for 2 days to be a nearly confluent. Meanwhile, *P. gingivalis* NCTC 11834 was cultured on FA agar for 2 days too. Following 2 days of incubation, cells were washed with PBS, and detached with 1 ml of trypsin/EDTA. The detached cells were transferred to a 15 ml centrifuge tube, washed with 5 ml DMEM, and centrifuged at 179 g for 5 minutes. The cell pellet was re-suspended with 1ml DMEM, counted and adjusted to  $1 \times 10^6$ /ml in triplicate. Cells were centrifuged at 664 g for 5 minutes, re-suspended with 1 ml of 2% (w/v) BSA in DMEM, and incubated for 1 hour at 37°C, 5% CO<sub>2</sub> on an end-over-end mixer. After BSA incubation, cells were counted again to monitor the final cell number prior to centrifugation at 664 g for 5 minutes, and re-suspension with bacteria at multiplicity of infection (MOI) 1:100 for each Eppendorf tube. Next, cells were infected with bacteria on an end-over-end mixer for 90 minutes at 37°C, 5% CO<sub>2</sub>, centrifuged at 664 g for 5 minutes, re-suspended with 1ml DMEM containing Metronidazole to kill the adherent bacteria, and incubated on an end-over-end mixer for 1 hour at 37°C, 5% CO<sub>2</sub>. Cells were washed twice with PBS, and centrifuged prior to cell lysis with either distilled water or 0.1% (w/v) saponin on an end-over-end mixer for 30 minutes at room temperature. Finally, cells were vortexed at maximum speed for another 20 minutes as an extra step to disrupt the cells and release the bacteria. The lysates then serially diluted, inoculated on FA blood agar, and the viable counting was performed as described in section 2.4. In parallel, a standard antibiotic protection assay was performed with monolayer cell culture as described in section 2.4, using 0.1% (w/v) Saponin or distilled water to compare the *P. gingivalis* invasion between cells in suspension and monolayer cell cultures.

### **2.13 Gene expression of H357 cells exposed to *P. gingivalis*.**

Confluent H357 cell monolayers in 6 well plates (2 ml/well) were seeded at  $2 \times 10^5$ /well 2 days prior to the invasion assay, and washed twice with 2 ml of DMEM then exposed to a 2 days old *P. gingivalis* suspension in DMEM at a MOI 1:100 for 30 minutes, 90 minutes, 150 minutes, and 240 minutes. Control wells were left with DMEM alone.

After each specified incubation time, all wells were washed once with 2 ml DMEM and the cells detached with 0.5 ml of 0.05% trypsin-0.02% EDTA (v/v). After trypsinization, 0.5 ml KGM was added to each well and mixed well by pipetting up and down several times with scraping and agitation. The samples were centrifuged at 3000 rpm for 5 minutes, the supernatant removed and the resultant pellet of cells was frozen at -80 °C.

### **2.14 RNA extraction of H357 cell monolayers following exposure to *P. gingivalis* NCTC 11834.**

RNA was purified from isolated H357 cell pellets following *P. gingivalis* infection using the Rneasy mini kit (Qiagen) according to the manufacturer's instructions. Briefly, the cell pellets were lysed in a buffer containing a chaotropic salt used to avoid RNA degradation by deactivation the RNase enzymes. The lysates were then transferred to a spin column and centrifuged at 11000 g for 1 minute. 350 µl of 70% (v/v) ethanol was added and mixed well by pipetting up and down several times, and the solution transferred to another spin column containing a silica membrane for binding RNA, and centrifuged at 11000 g for 30 seconds. 350 µl of membrane desalting buffer then was added and centrifuged at 11000 g for 1 minute to dry the membrane. DNA digestion was performed using a solution of DNase 27 K unit/sample which was added to the centre of the silica membrane for 15 minutes at room temperature. The membrane was washed 3 times and the RNA eluted in 60 µl RNase free water by centrifuging at 10000g for 1 minute. All RNA samples were stored at -80 prior to cDNA synthesis.

### 2.15 Reverse transcriptase-Polymerase chain reaction (RT-PCR).

cDNA was synthesized from the total RNA isolate using a high capacity cDNA reverse transcription kit (Applied biosystem, Life Technologies Ltd., UK) according to the manufacturer instructions. Briefly, the RNA concentration of individual samples was measured using a NanoDrop 1000 spectrophotometer (ThermoScientific, USA) at 260/280 nm. The kit reagents used to prepare the reaction master mix are illustrated in table 2.4. The volume of RNA was adjusted to obtain 500 ng RNA /10  $\mu$ l, and this was mixed with 10  $\mu$ l master mix. An extra tube containing master mix and nuclease free water only was prepared as a control without any RNA content. All tubes were then placed into a thermal cycler (BIO-RAD, USA), to run the reverse transcription process. The initial temperature was 25°C for 10 minutes, followed by 37 °C for 120 minutes, then 85°C for 5 minutes, after which all synthesized cDNA samples were stored at -20°C.

**Table 2.4 The reagents used in reverse transcription- Polymerase chain reaction (RT-PCR).**

Component	Volume/reaction ( $\mu$ l)	
	Kit with RNase inhibitor	Kit without RNase inhibitor
10 X RT Buffer	2.0	2.0
25 X dNTP Mix (100mM)	0.8	0.8
10 X RT Random Primers	2.0	2.0
MultiScribe Reverse Transcriptase	1.0	1.0
RNase Inhibitor	0.5	
Nuclease-free H <sub>2</sub> O	3.7	4.2
<b>Total per Reaction</b>	<b>10</b>	<b>10</b>

### 2.16 Real time- Polymerase chain reaction (qPCR).

Quantification of the target genes expression, such as Urokinase plasminogen activator receptor (uPAR) and Endothelin-1 (ET-1) genes, was performed by using SYBR green (Applied biosystem, life technologies, UK) master mix. The reaction mixture components are shown in table 2.5. The sequence of forward and reverse primers of uPAR and ET-1 genes are shown in table 2.6 (Primer designed by Dr. Dan Lambert, University of Sheffield). The endogenous control (House Keeping gene) for the

## Chapter 2 Materials and Methods

reaction was U6 gene. The total reaction volume was 20  $\mu$ l which included the SYBR green master mix, forward and reverse primers, Nuclease free water, and cDNA, which was loaded into a 96 well plate in triplicate. Additional wells containing reaction mixture and nuclease free water were used as control samples. The 96 well plate containing reaction mixture was centrifuged for 1 minute prior to loading into a real time PCR machine (7900HT fast real time PCR system), with a thermal cycle composed of two stages of 50°C for two minutes, followed by 95°C for 10 minutes for initial warming, then 40 cycles of denaturation and annealing at 95°C for 15 seconds and 60°C for 1 minute, respectively. The number of cycles in which the fluorescent signal exceeding the pre-selected threshold, called the Threshold cycle ( $C_T$ ) value for every reaction, were analyzed by using the RQ manager software program (Applied biosystem). The target  $C_T$  value ( $\Delta C_T$ ) was calculated by subtracting endogenous control  $C_T$  value, which reflects the amount of cDNA available in every sample, from the total sample  $C_T$  value. This was followed by subtracting the value of untreated control (H357 cells without *P.gingivalis*) from the  $\Delta C_T$  for each sample. Lastly, the fold change of the target genes expression of each individual sample was calculated in relation to the untreated control values.

**Table 2.5 The reagents used for master mix reaction involved in the gene expression analysis by real-time PCR.**

Reagent	Volume ( $\mu$ l)
SYBR Green Master mix	10
Nuclease - free water	7
Forward Primer	1
Reverse Primer	1
cDNA	1
<b>Total</b>	<b>20</b>

**Table 2.6 The Nucleotide sequence of the forward and reverse primers used for uPAR and ET-1 genes amplification.**

<b>uPAR</b>	Forward	5' GCCCAATCCTGGAGCTTGA 3'
	Reverse	5' TCCCCTTGCAGCTGTAACACT 3'
<b><math>\alpha</math>5-integrin</b>	Forward	5' ACAAAGCCCTGAAGATGCCC 3'
	Reverse	5' ATCCACAGTGGGACGCCATA 3'
<b>ET-1</b>	Forward	5' CTGCCACCTGGACATCATTG 3'
	Reverse	5' TCTCACGGTCTGTTGCCTTTG 3'
<b>U6</b>	Forward	5' CTCGCTTCGGCAGCACA 3'
	Reverse	5' AACG TTCACGAATTTGCGT 3'

### 2.17 Cell cycle analysis.

The general principle of the cell cycle analysis conducted in our work is based on serum starving overnight to arrest the growth of all cells and synchronize them to the starting point of the cell cycle at G<sub>0</sub>-phase. Serum represents the main cell growth promoting factor which drives cell division and when it is re-introduced cells enter the cell cycle at G<sub>1</sub>.

### **2.18 Cell cycle characterisation of H357 oral keratinocytes.**

H357 cells were cultured in keratinocyte growth medium at conc.  $4 \times 10^5$ /well in a 6 well plate for 24 hours. The cells were then serum starved by replacement with serum free medium (SFM), which consisted of 3:1 DMEM and Ham's-F12 medium with Streptomycin (100 IU/ml), Penicillin (100 IU/ml), and Amphotericin B (2.5  $\mu$ g/ml), for 24 hours. to arrest the growth of cells and synchronize them to the same point of the cell cycle at G<sub>0</sub>-phase. The following day, the SFM was replaced with KGM for stimulating cells to divide and developed through the cell cycle phase's characterization. The serum was re-introduced for 2, 9, and 16 hours. The cells were washed with PBS, trypsinised, centrifuged and re-suspended with 500  $\mu$ l 70% (v/v) ethanol (ThermoFisher, UK) added dropwise and incubated on ice for 30 minutes for fixation. The cells were then washed with PBS and incubated with a mixture of propidium iodide (PI) (50  $\mu$ g ml<sup>-1</sup>) to stain the DNA content, and RNase (100  $\mu$ g/ml) in PBS to degrade RNA ensuring that only DNA is stained by PI. The incubation was performed in the dark for 30 minutes at room temperature. Cells were analysed for the cell cycle distribution by flow cytometry using LSRII flow cytometer (BD Bioscience) at the Flow Cytometry Core Facility, Medical School, University of Sheffield. The analysis was performed using 10,000 cells per sample with excitation at 488 nm using BD FACS Diva software (Version 7.0, BD Bioscience). All experiments were conducted in triplicate and repeated at least 3 times. Control wells were investigated in parallel with the same steps as described above except that the KGM in these wells was not replaced with SFM.

### **2.19 Effect of cell cycle phases on the bacterial association.**

To explore whether the cell cycle phases have an influence on the cell-bacterial association, plate cultured *P. gingivalis* NCTC 11834 were harvested and labelled with fluorescein 5(6)-isothiocyanate (FITC) 1mg/ml of 0.1 M Sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>, pH=9) in the dark for 1 hour at 4°C on rotating roll shaker. The bacterial cells were washed with PBS four times by centrifuging at 13793 g for 2 minutes, counted and the number adjusted prior to performing the antibiotic protection assay. Meanwhile, the overnight seeded H357 cells (1x10<sup>6</sup>/25 cm<sup>2</sup> flask) were washed and infected with FITC-labelled *P. gingivalis* for 30, 90, and 240 minutes according to the antibiotic protection assay as described in section 2.4. This protocol was performed without

metronidazole treatment to reflect the total number of bacteria associated with the cells. Cells were then washed twice with PBS, trypsinized, and transferred to an Eppendorf tube for cell cycle analysis which was performed as described in section 2.18.

The fluorescence expression index in each cell cycle phase was obtained by dividing the median FITC fluorescence by the percentage (%) of cells in each cell cycle phase.

$$\text{Fluorescence expression index} = \frac{\text{Median fluorescence from } P. \text{ gingivalis}}{\text{Percentage (\%) of cells in each cell cycle phase}}$$

### **2.20 Effect of cell cycle synchronization on the *P. gingivalis* invasion efficiency.**

H357 cells were cultured at  $5 \times 10^4$ /well in a 24 well plate and subjected to serum synchronization for 0, 2, 9, and 16 hours as described in section 2.18. After each time point of re-introducing serum, cells were washed twice with PBS and exposed to *P. gingivalis* NCTC 11834 for 90 minutes at 37°C as described in section 2.4. For comparison purposes, control cells remained in KGM throughout the experiment.

### **2.21 Effect of serum synchronization on cell surface proteins.**

To determine the effect of serum synchronization on the cell surface proteins which may be possible mediators of the host-bacterial interactions, gene and cell surface protein expression levels  $\alpha 5$ -integrin (ITGA5, CD49) and uPAR were analyzed following serum synchronization treatment.  $\alpha 5$ -integrin (ITGA5) mediates *P. gingivalis* adherence to the cell membrane and subsequent internalization following activation of cell signaling transduction pathways (Nakagawa *et al.*, 2002a, Tsuda *et al.*, 2008, Yilmaz *et al.*, 2002). On the other hand, uPAR expression has been reported following treatment of fibroblasts with *P. gingivalis* LPS (Ogura *et al.*, 1999), co-localizes with  $\alpha 5\beta 1$  integrin and is thought to modify its activity (Wei *et al.*, 2005).

#### **2.21.1 Effect of serum synchronization on cellular gene expression.**

The mRNA expression of both  $\alpha 5$ -integrin and uPAR has been investigated following re-introducing serum to H357 cells for a period of 2, 9, and 16 hours. H357 cells were seeded at  $2 \times 10^5$ /well in a 6 well plate. After each time of serum synchronization, cells

were washed, trypsinized, centrifuged, and stored at -80 °C prior to RNA extraction. RNA was extracted as described in section 2.14, and reverse transcribed to synthesis the cDNA of serum synchronized cells as described in section 2.15. Finally, both genes quantification was performed using real time PCR as described in section 2.16. The nucleotide sequences of the specific primers used to amplify the  $\alpha 5$ -integrin and uPAR genes are shown in table 2.6

### **2.21.2 Effect of serum synchronization on cell surface protein expression.**

To determine whether cell-surface protein expression of possible mediators of *P. gingivalis*-cell association are altered following serum synchronization, the surface expression of  $\alpha 5$ -integrin and uPAR was investigated. H357 cell monolayers were cultured to confluence in a 75 cm<sup>2</sup> flask, followed by replacement of KGM with SFM for 24 hours and subsequent re-introducing serum for a period of 2, 9, 16 hours as described in section 2.18. Following serum synchronization, cells were washed twice with PBS, trypsinized, and neutralized with KGM. The cell suspension was transferred to an Eppendorf tube and centrifuged at 1000 rpm for 5 minutes. The cell pellet was re-suspended with PBS containing 10% foetal bovine serum (v/v), and adjusted to 1x10<sup>6</sup> cells/ml. 100  $\mu$ l (1x10<sup>5</sup> cells) was added to each sample, and the primary antibodies against  $\alpha 5$ -integrin, uPAR, and IgG1 control isotype as shown in table 2.7 were added to triplicate samples, and incubated with ice for 1 hour. Cells were washed with 1 ml of PBS containing 10% FCS (v/v) and centrifuged at 179 g for 5 minutes. The pellet was re-suspended with 100  $\mu$ l of PBS containing 10% FCS (v/v) and incubated with 4  $\mu$ g/ml (1:500) Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate (ThermoFisher,UK) on ice for 30 minutes. Cells were centrifuged at 73 g for 5 minutes, and re-suspended with 400  $\mu$ l of PBS containing 10% FCS (v/v). Finally, the cell surface expression of uPAR,  $\alpha 5$ -integrin, and IgG1 was determined by FACs Calibur (BD Biosciences, Oxford, UK). Prior to FACs determination, 5  $\mu$ l TO-PRO®-3 (Invitrogen) (approximately 4 $\mu$ M) was added to each sample to stain live/dead cells as it has the ability to bind to the DNA of the dead cells whilst it cannot penetrate the outer membrane of the live cells. The visualization of Alexa fluor 488-conjugated secondary antibody was through FL-1 channel while for TO-PRO®-3 it was through the FL-4 channel. Gating was performed around the live cells only to avoid false positive results, and the comparison was made between the



serum synchronized cells and unsynchronized control cells. The median fluorescence expression of each marker was analyzed between synchronized and unsynchronized control cells using CellQuestPro Software (BD Biosciences).

**Table 2.7 uPAR,  $\alpha$ 5-integrin (ITGA5, CD49), and IgG1 Isotype antibodies.** The table shows the primary antibodies used for the surface expression analysis of uPAR and  $\alpha$ 5-integrin (ITGA5, CD49) by FACs.

Antibody	Clone	Conc./(Dilution)	Species	Origin
uPAR	62022	5 $\mu$ g/ml (1:100)	Mouse monoclonal	R&D systems (Biotechne, USA)
$\alpha$ 5-integrin (ITGA5, CD49)	IIA1	5 $\mu$ g/ml (1:100)	Mouse monoclonal	BD Pharmingen™, UK
IgG1 (Isotype control)	DAK- GO1	10 $\mu$ g/ml	Mouse monoclonal	Dako, Copenhagen, Denmark

## 2.22 Immunofluorescence of serum synchronized cells using BrdU staining

To identify cells in S phase, BrdU stain (5-bromo-2-deoxyuridine, ThermoFisher, USA) was used according to manufacturer's instructions. Briefly, H357 cells were seeded at  $5 \times 10^4$ /well on a cover slip in a 24 well plate for 24 hours at 37°C, 5% CO<sub>2</sub>. The cells were then serum starved and synchronized the following day for 2, 9 and 16 hours as described in section 2.18. The BrdU labeling solution was prepared by diluting the 10 mM BrdU- dimethyl sulfoxide stock solution in KGM to give a final concentration of 10 $\mu$ M BrdU. Following the specified time of serum reintroduction, the KGM was replaced with the BrdU labeling solution and incubated for 2 hours at 37°C, 5% CO<sub>2</sub>. The wells were then washed for 2 minutes with PBS 3 times. The cells were fixed with 4% formaldehyde (v/v) for 15 minutes at room temperature and washed for 2 minutes with PBS 3 times. To permeabilize the cell membrane, the wells were incubated with a permeabilization buffer of 0.1% Triton® X-100 (v/v) dissolved in PBS for 20 minutes at room temperature. The buffer was replaced with 1M HCl

(Fisher,UK), for 10 minutes on ice, followed by 2M HCL for 10 minutes at room temperature to separate the DNA strands to allow the primary antibody to bind to the BrdU. The HCl was replaced with phosphate/citric acid buffer (pH 5) and incubated for 10 minutes at room temperature, and then washed with 0.1% Triton® x-100 (v/v) for 2 minutes 3 times. Buffer was replaced with 0.4 µg/ml (1:500) of anti-primary BrdU antibody (mouse monoclonal antibody, MoBU-1, ThermoFisher, USA) in 0.1% Triton (v/v) and 5% normal goat serum (v/v) in PBS (Antibody staining solution), and incubated overnight at room temperature. The next day, the wells were washed with 0.1% Triton® X-100 buffer (v/v) for 2 minutes 3 times, and antibody staining buffer containing 4 µg/ml (1:500) goat anti-mouse IgG polyclonal secondary antibody (Alexa Fluor® 488 conjugate, ThermoFisher, USA) added for 1 hour at room temperature. The wells were washed with PBS, and cover slips were mounted using ProLong® diamond antifade mountant with 4', 6-diamidino-2-phenylindole (DAPI) (ThermoFisher, USA).

All stained coverslips were visualized using the Zeiss Axioplan 2 fluorescent microscope and the image-ProPlus 7.0.1 imaging software (Zeiss, Ltd). All these staining steps were made in parallel with the control wells which underwent KGM replacement without serum starvation and re-introduction. The percentage of cells in S-phase was calculated by dividing the number of cells stained with BrdU by the total number of cells stained by DAPI.

$$\text{Percentage of cells in S-phase} = \frac{\text{Number of cells stained by BrdU}}{\text{Total number of cells stained by DAPI}} \times 100$$

### 2.23 Cell-bacterial co-localization:

#### 2.23.1 Immunocytochemical staining of *P. gingivalis* invaded H357 cells.

H357 cells were cultured on sterile coverslips and infected with *P. gingivalis* NCTC 11834 as described in section 2.1.3. Following the metronidazole treatment step, cells were fixed and permeabilized with cold 100% methanol (Fisher Scientific, UK) for 15 minutes and 3% (v/v) of hydrogen peroxide in methanol (Fisher Scientific, UK) was added for 15 minutes to quench endogenous peroxidase activity. Cells were then blocked with 100% goat serum for 1 hour to avoid non-specific binding of the *P. gingivalis* primary antibody. Cells were washed twice with PBS and incubated with two types of primary antibodies (table 2.8) in separate samples to determine if there is

## Chapter 2 Materials and Methods

any difference in the pattern of association between *P. gingivalis* and H357 cells in the antibody staining buffer (50% Goat serum and 50% PBS) overnight at 4°C. The next day, cells were washed 3 times with PBS and incubated with freshly prepared rabbit/mouse biotinylated secondary antibodies as appropriate for 30 minutes according to the manufacturer's instructions (VECTASTAIN® Elite Avidin Biotinylated enzyme Complex (ABC)-Peroxidase Kits, Vector Laboratories, Peterborough, UK). The secondary antibodies used correspondent to the species in which the whole *P. gingivalis* and Mab 1B5 primary antibodies were raised. Following washing cells 3 times with PBS, freshly prepared Avidin Biotinylated enzyme Complex reagent was added for 30 minutes to bind to the biotinylated secondary antibody. Cells were then washed 3 times with PBS, and incubated with 3, 3'-diaminobenzidine (Vector Laboratories) substrate at room temperature until a dark brown precipitate was formed. Finally, the coverslips were washed 3 times with distilled water and counterstained with haematoxylin as shown in table 2.9, using a Small Linear Stainer (Leica ST4020 Microsystems, Milton Keynes, UK). The coverslips were then mounted onto microscope slides using DPX aqueous mounting medium (Thermo Fisher Scientific, UK). All slides were visualized using the BX51 upright microscope (Olympus, Essex, UK) and cell<sup>^</sup>D imaging software (Olympus UK Ltd). Control wells without *P. gingivalis* or with *P. gingivalis* without primary antibody were subjected to the same protocol described above to confirm the specificity of the primary *P. gingivalis* antibody.

**Table 2.8 The primary antibodies used in the immunocytochemical analysis of H357 cells infected with *P. gingivalis* NCTC 11834.** The table shows the commercial supplier of each antibody, the clone of the antibody, the expected locality of staining, species isolated, and the optimised concentration at which the antibody was used.

Antibody	Clone	Suppliers	Species	Expected locality of staining	Concentration
<i>P. gingivalis</i>	Mab 1B5	M. Curtis, Barts and the London, School of Medicine	Mouse monoclonal	<i>P. gingivalis</i> RgpAcat, mtRgpAcat, mt-RgpB, APS (Curtis <i>et al.</i> , 1999)	1:50
<i>P. gingivalis</i>		Graham Stafford, University of Sheffield	Rabbit polyclonal	Whole <i>P. gingivalis</i>	1:1000

**Table 2.9 Counterstaining protocol using the Small Linear Stainer.** The table shows the order in which the coverslips were processed and stained, each step was for duration of 1 minute.

Step	Process
1	Harris's haematoxylin (x2)
2	Running tap water
3	1% (v/v) acid alcohol (1% HCl in 70% isopropanol)
5	Running tap water
4	Scott's tap water substitute (3.5g/L sodium bicarbonate & 20g/L magnesium sulphate)
5	Running tap water
6	95% ethanol (x2)
7	100% ethanol (x2)
8	Xylene (x3)

### 2.23.2 Double immunofluorescence of oral keratinocytes and invasive

#### *P. gingivalis*.

To determine whether there was co-localization of invaded *P. gingivalis* with cells in S-phase, double immunofluorescence staining of BrdU stained cells and internalized *P. gingivalis* was conducted. Optimization was performed using four different experimental conditions regarding the temperature of overnight incubation of primary antibody and the type of fixation used. These experimental conditions are shown in table 2.10. After visualization by immunofluorescence microscope, the best quality image of co-localization relationship was obtained with condition 1. Thus all subsequent experiments were conducted using condition 1. H357 cells were seeded on cover slips in triplicate at  $5 \times 10^4$  cells/well in a 24 well plate, and underwent the antibiotic protection assay as described in section 2.4. After antibiotic treatment, cells were washed twice with PBS and BrdU staining performed as described in section 2.22. At the step of primary antibody incubation, both primary anti-BrdU mouse monoclonal antibody (section 2.21), and primary anti- whole *P. gingivalis* antibody (section 2.23) were added and incubated at room temperature overnight. Cells were then washed with PBS, and incubated with 1ml antibody staining solution containing 4  $\mu\text{g/ml}$  (1:500) of goat anti-mouse IgG polyclonal secondary antibody (Alexa Fluor® 488 conjugate, ThermoFisher, USA) directed against BrdU stained cells (FITC stain), and 4  $\mu\text{g/ml}$  (1:500) of goat anti-rabbit IgG secondary antibody [Alexa Fluor® 594 conjugate (Texas Red), ThermoFisher, USA] directed against whole *P. gingivalis* bacteria for 1 hour at room temperature. Cells were washed with PBS, and the cover slips were mounted on microscope slides and visualized using fluorescence microscopy as described in section 2.22. Each stain was analyzed separately, captured, and extracted using the representative color channel within the image-ProPlus 7.0.1 imaging software (Zeiss, Ltd). For the dual staining, images were merged, and analyzed through multi-statistical parameters including counting, comparisons, and correlations.

**Table 2.10 Experimental conditions for double immunofluorescence of H357 cells and invaded *P. gingivalis*.** The table shows the four conditions investigated for the co-localization between BrdU stained cells and invaded *P. gingivalis*.

Condition	Description
1	Fixation cells with 4% paraformaldehyde + incubation primary antibodies overnight at room temperature
2	Fixation cells with 4% paraformaldehyde + incubation primary antibodies overnight at 4°C
3	Fixation cells with 100% cold methanol + incubation primary antibodies overnight at room temperature
4	Fixation cells with 100% cold methanol + incubation primary antibodies overnight at 4°C

### 2.24 Statistical Analysis

All experiments were carried out in triplicate and repeated at least 3 times. The data were presented as mean  $\pm$  standard deviation, and comparisons were performed using a student's unpaired, two-tailed t-test. The correlation analysis was performed using Pearson's correlation coefficient (r). P-values < 0.05 were considered statistically significant. The data were analysed using GraphPad Prism, version 7.

**Chapter 3**  
**Factors affecting**  
***P. gingivalis* invasion.**

### 3.1 INTRODUCTION

As described earlier, *P. gingivalis* is a key periodontal pathogen which possesses a wide range of virulence factors. Amongst these is the ability to invade a number of different mammalian cell types and it is thought that the intra-cellular lifestyle facilitates its persistence at a site, and so possibly contributes to continued progression of the disease (Kumar *et al.*, 2005, Madianos *et al.*, 1996).

H357 oral keratinocytes have been used to study *P. gingivalis* NCTC 11834 invasion in monolayer cultures through an antibiotic protection assay (Stafford *et al.*, 2013, Suwannakul *et al.*, 2010, Wayakanon *et al.*, 2013). Therefore, our study was conducted using the same experimental conditions to enable standardization and comparison.

Several factors have been shown to influence the uptake of *P. gingivalis* by host cells. These include a variety of *P. gingivalis* virulence factors such as fimbriae (Nakagawa *et al.*, 2002a, Yilmaz *et al.*, 2002), gingipain proteases (Stafford *et al.*, 2013), and hemagglutinins (Belanger *et al.*, 2012). In addition, the host cell receptors which are thought to mediate *P. gingivalis* invasion such as  $\alpha 5\beta 1$  integrin (Yilmaz *et al.*, 2002), toll-like receptors (TLRs) (Wang *et al.*, 2007), and cytokeratins (Sojar *et al.*, 2002) appear to play a role. Moreover, several environmental factors have been shown to modulate *P. gingivalis* virulence and these can affect its interaction with the host cells. For example, temperature affects on fimbrial expression (Amano *et al.*, 1994, Xie *et al.*, 1997), haemin concentration affects on lipid A structure (Al-Qutub *et al.*, 2006) and hemagglutination (Smalley *et al.*, 1991) and pH affects on the proteolytic activity of *P. gingivalis* (Takahashi and Schachtele, 1990).

However, the influence of other environmental factors on the *P. gingivalis* invasion have not been well characterised such as incubation time, bacterial growth phase, cell density and passaging, lysis buffer used, and the use of other methods to quantify invasion such as the method of DNA extraction.

Therefore, all of these factors were taken into consideration and carefully investigated in this chapter to determine which influence *P. gingivalis* invasion of host cells. This, in addition to the previously documented factors throughout the literature, could establish



a strong foundation explaining the underlying mechanisms involved in *P. gingivalis*-cell interactions.

### **The aims of this part of the study were:**

- 1- To investigate the effect of infection time on the invasion of H357 cells by *P. gingivalis*.
- 2- To investigate the influence of the bacterial growth phase on bacterial invasion efficiency.
- 3- To determine whether the cell lysis buffers employed has an influence on the release of internalised bacteria.
- 4- To compare two methods of quantifying the bacteria that have invaded; the standard antibiotic protection assay and total DNA assay
- 5- To assess whether density and passage number of the mammalian cells have any effect on the uptake of *P. gingivalis*.

## **3.2 METHODS**

The following methods were used in this chapter:

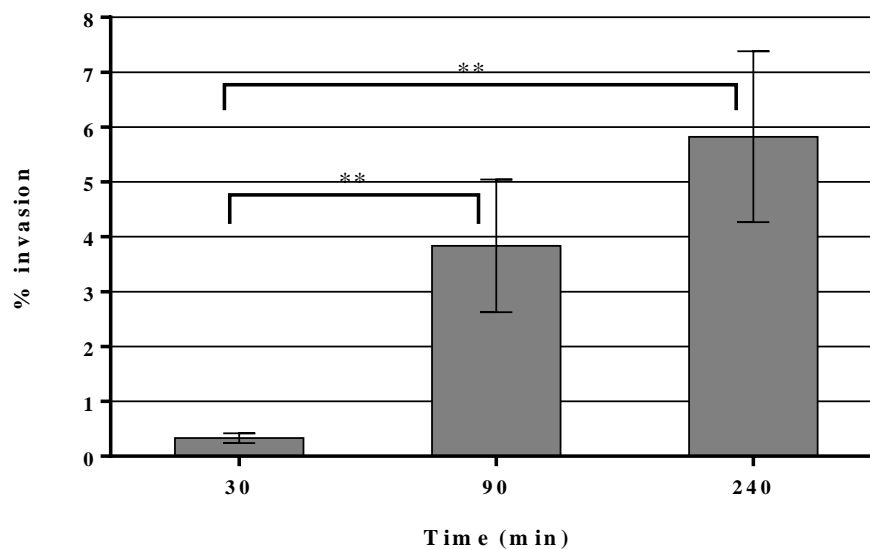
- Epithelial cell culture (section 2.2)
- *P. gingivalis* culture (section 2.3)
- Antibiotic protection assay (section 2.4)
- Using DNA extraction to analyse *P. gingivalis* invasion (section 2.5)
- Incubation time and the invasion efficiency of *P. gingivalis* (section 2.6)
- Effect of growth phases on *P. gingivalis* invasion efficiency (section 2.7.2).
- Effect of different lysis buffers on *P. gingivalis* invasion (section 2.8)
- Effect of cell seeding density on *P. gingivalis* invasion (section 2.9).
- Effect of cell passage number on *P. gingivalis* invasion (section 2.10).

### 3.3 RESULTS

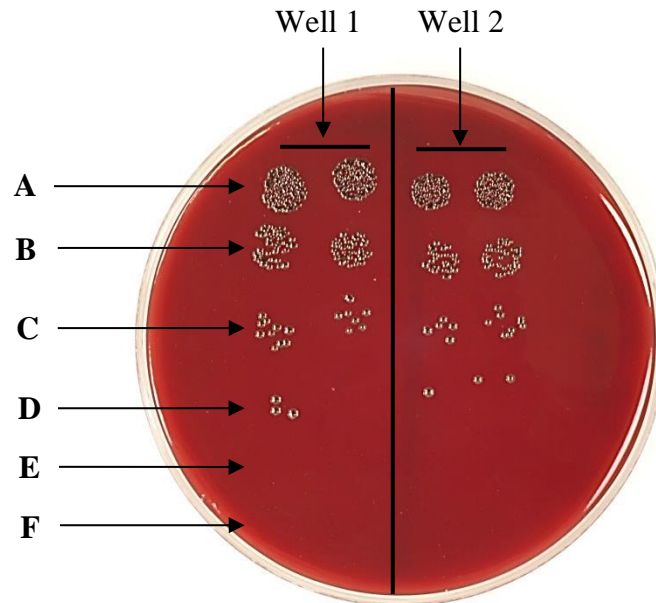
#### 3.3.1 The effect of incubation time on the invasion efficiency of *P. gingivalis*

##### NCTC 11834 in H357 cell monolayers.

Using the antibiotic protection assay, incubation of *P. gingivalis* NCTC 11834 with H357 cells for 30, 90, and 240 minutes were investigated. The results showed that the invasion of *P. gingivalis* was significantly increased with increasing time of infection from  $0.33\% \pm 0.08$  with 30 minutes to  $3.83\% \pm 1.2$  with 90 minutes, and  $5.82\% \pm 1.55$  with 240 minutes (Figure 3.1). The number of internalized bacteria after osmotic cell lysis was determined by serial dilution and viable counting is expressed as the percentage of the number of recovered bacteria by the main bacterial inoculum (Figure.3.2).



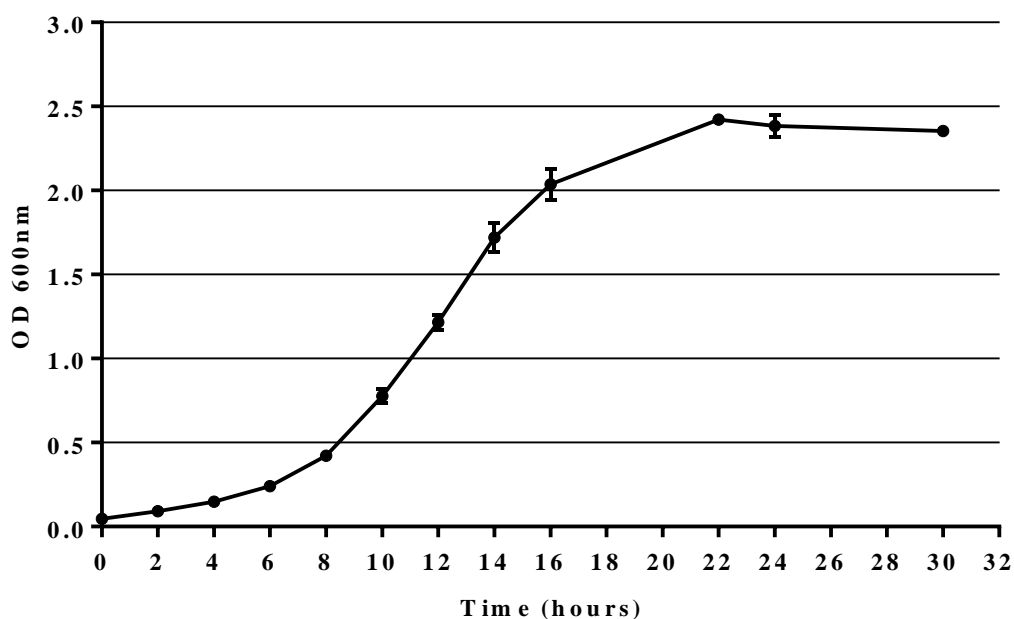
**Figure 3.1** Bar chart showing the percentage invasion of H357 cells by *P. gingivalis* after different incubation times. The number of recovered bacteria expressed as a percentage of the bacterial inoculum. Data are mean percentage  $\pm$  SD of the 3 independent experiments,  $**P \leq 0.01$ .



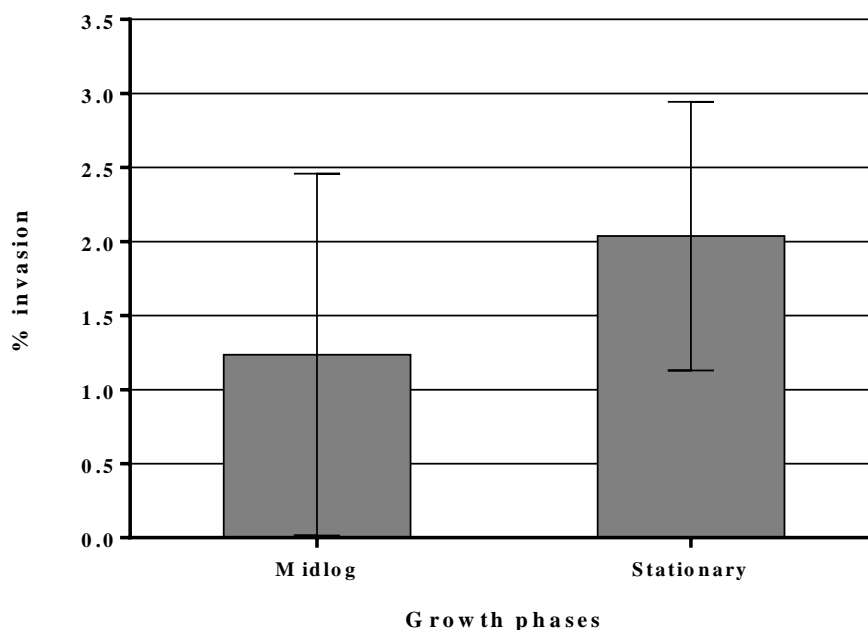
**Figure 3.2 Example image of viable counting method to determine the number of internalized bacteria.** After osmotic lysis, samples were serially diluted and 10 $\mu$ l spots were placed in duplicate on FA plates. The viable count was expressed as the proportion of the number of bacteria recovered, divided by the viable count of the bacterial inoculum placed on the H357 cells, multiplied by 100. **A**, Internalized *P. gingivalis* suspension after lysis/well; **B**, internalized *P. gingivalis* after first dilution; **C**, internalized *P. gingivalis* after second dilution; **D**, internalized *P. gingivalis* after third dilution; **E&F**, clear zones as there are no recovered bacteria in the fourth and fifth dilutions.

### 3.3.2 Effect of *P. gingivalis* growth phase on invasion ability.

In order to select bacteria in different growth phases, growth curves of *P. gingivalis* were performed as described in section 2.7.1 (Figure 3.3). *P. gingivalis* was found to reach mid log phase after 12 hours anaerobic incubation at 37 °C in supplemented BHI broth. Late stationary phase was reached after 30 hours. Consequently, *P. gingivalis* NCTC 11834 grown for the above periods were harvested by centrifugation and adjusted to give an MOI of 100 relative to the H357 cell monolayer for 90 minutes. The results showed that invasion by bacteria in late stationary phase was slightly higher ( $2\% \pm 0.9$ ) than by cells in midlog phase ( $1.2\% \pm 1.2$ ), although this difference was not statistically significant (Figure 3.4).



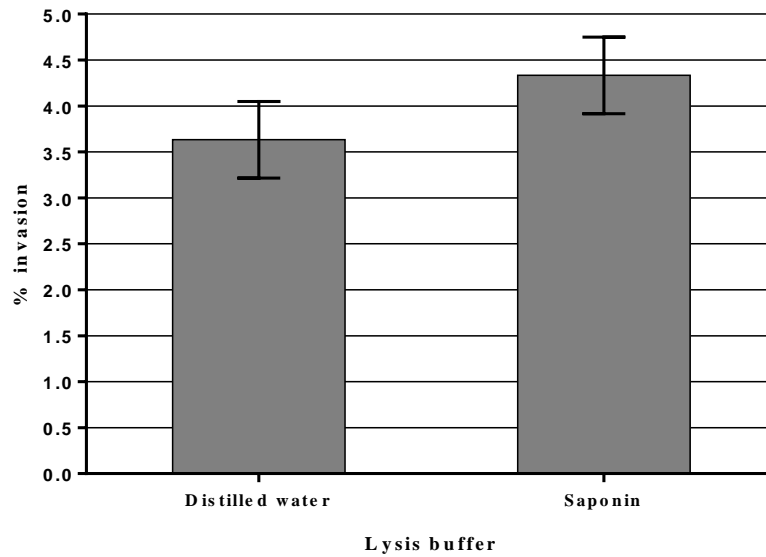
**Figure 3.3** Growth curve of *P. gingivalis* NCTC 11834 in supplemented BHI broth. Cell density was determined by UV/visible spectrophotometry at 600 nm. In parallel with each measurement, Gram staining and culturing into FA agar plates were performed to confirm the purity of the culture. Graph shows mean percentage  $\pm$ SD.



**Figure 3.4 Effect of growth phase of *P. gingivalis* on invasion of H357 cell monolayer.** *P. gingivalis* was harvested following 12 hours (midlog phase) and 30 hours (stationary phase) growth in BHI broth, and adjusted to a MOI 100:1. Antibiotic protection assays were performed in triplicate on 3 separate occasions. Graphs show mean percentage $\pm$ SD.

### 3.3.3 Effect cell lysis buffer on recovery of *P. gingivalis* after invasion

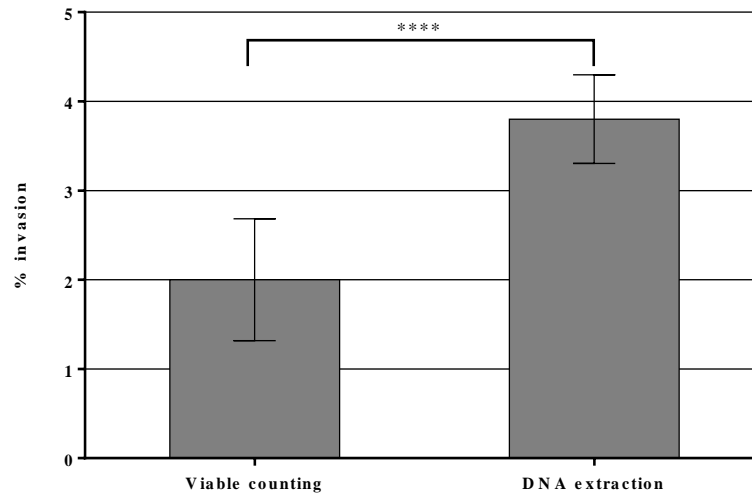
Two methods of cell lysis were compared; detergent lysis with 0.1% saponin (Sigma, UK) and osmotic lysis (section 2.8) with sterile distilled water. The resultant recovery of bacteria is shown in figure 3.5. A slightly higher recovery of *P. gingivalis* was obtained following cell lysis with 0.1% saponin ( $4.33\% \pm 0.4$ ) compared with that obtained using distilled water ( $3.63\% \pm 0.4$ ). However, the difference was not statistically significant ( $p=0.1$ ). Therefore, for convenience, all subsequent antibiotic protection assays were conducted using osmotic lysis.



**Figure 3.5 Recovery of *P. gingivalis* following invasion of H357 cells using two methods of cell lysis.** H357 cells infected with *P. gingivalis* for 90 minutes aerobically were lysed with either 0.1% saponin or distilled water. Data shown are the mean percentage $\pm$ SD of viable bacteria recovered expressed as a proportion of the bacterial inoculum. (N=3)

### 3.3.4 Assessment of *P. gingivalis* invasion using quantification of DNA.

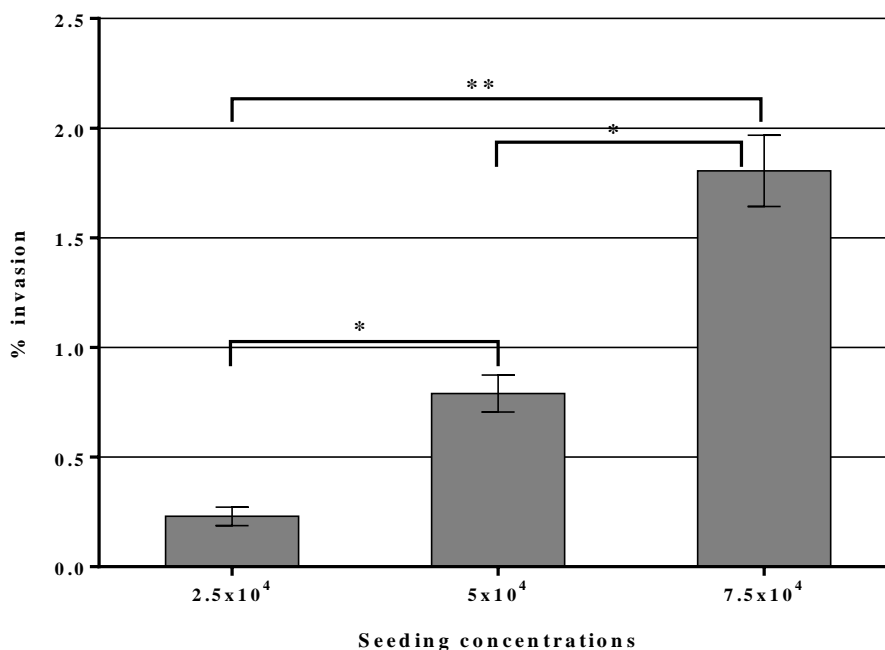
*P. gingivalis* invasion was assessed by two methods, viable counting following the standard antibiotic protection assay compared with estimates of bacterial DNA by qPCR from infected cells. The number of invading bacteria was determined from a standard curve as described in section 2.5.2. The results showed that a significantly higher number of bacteria were found to have invaded the H357 cells using the qPCR method (3.8% $\pm$ 0.16) compared to the standard viable counting method (2% $\pm$ 0.22) (figure 3.6). This difference is likely to reflect measurement of both living and dead bacteria by the qPCR method.



**Figure 3.6 Comparison of methods for estimating invasion of cells by *P. gingivalis*.** H357 cells were infected *P. gingivalis* NCTC 11834 for 90 minutes according to the antibiotic protection assay. Following cell lysis, bacteria were quantified by qPCR of *P. gingivalis* DNA relative to a standard curve, or by viable counting. Bar chart shows mean percentage $\pm$ SD of 3 independent experiments performed in triplicate, \*\*\*\*  $p < 0.0001$ .

### 3.3.5 Effect of mammalian cell seeding density on the *P. gingivalis* invasion.

The influence of seeding density of H357 cells on *P. gingivalis* invasion was determined using 3 cell densities,  $2.5 \times 10^4$ ,  $5 \times 10^4$ ,  $7.5 \times 10^4$  cells/well. An antibiotic protection assay was performed following overnight seeding of H357 cells at these concentrations instead of two days incubation to avoid full confluency of the monolayer cell cultures. The results showed that the internalization rate of *P. gingivalis* by H357 cells was significantly increased with increasing number of growing cells giving  $0.23\% \pm 0.03$  invasion at  $2.5 \times 10^4$  cell/well,  $0.79\% \pm 0.06$  at  $5 \times 10^4$  cell/well, and the highest invasion percentage ( $1.8\% \pm 0.11$ ) was obtained at  $7.5 \times 10^4$  cell/well (figure 3.7).

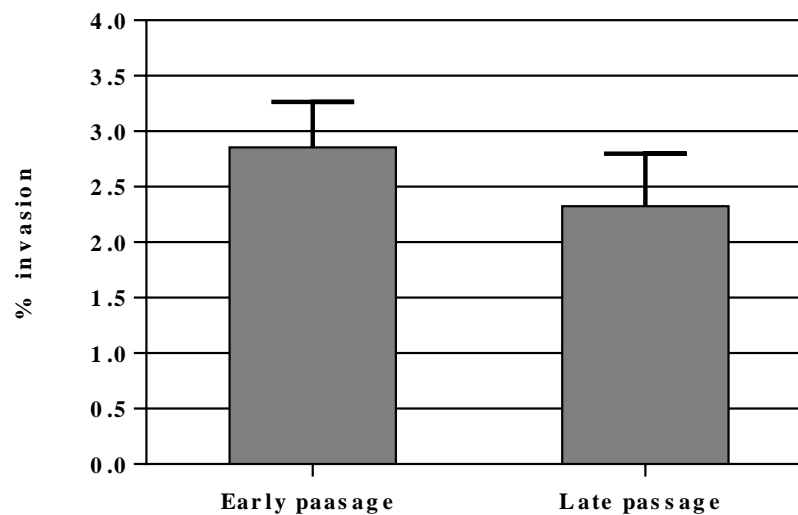


**Figure 3.7 Effect of H357 cell seeding concentrations on *P. gingivalis* invasion efficiency.** H357 cells were seeded at  $2.5 \times 10^4$ ,  $5 \times 10^4$ , and  $7.5 \times 10^4$  cells/well overnight, and infected with *P. gingivalis* according to the antibiotic protection assay. Viable counting of the invaded bacteria was conducted, and the comparison was made between these concentrations. Graph indicates means $\pm$ SD of 3 independent experiments repeated in triplicate. \*  $P \leq 0.05$ , \*\* $P \leq 0.01$ .

### 3.3.6 Effect of cell passaging on the *P. gingivalis* invasion.

It is possible that the age of cell stocks used for invasion assays could affect the internalization of *P. gingivalis*. Consequently two groups for cells of different passage number were compared for *P. gingivalis* invasion. The results showed that the average *P. gingivalis* invasion was slightly higher ( $2.8\% \pm 0.2$ ) in early passage cells (P=56, P=57, and P=58) than the late passage cells (P=218, P=219, and P=220) ( $2.3\% \pm 0.2$ ) (figure 3.8) but this was not statistically significant.





**Figure 3.8 Effect of H357 cell passaging on *P. gingivalis* invasion efficiency.** H357 cells were seeded at  $5 \times 10^4$  cells/well for 2 days, and infected with *P. gingivalis* using an antibiotic protection assay. The bar chart shows mean invasion percentage  $\pm$ SD of 3 independent experiments repeated in triplicate for the two groups of passaged cells.

### 3.4 DISCUSSION

The internalization of pathogenic bacteria into a variety of eukaryotic epithelial cells is one of the strategies that the bacteria are thought to follow to ensure the re-colonization and persistence of infection at the target sites. This is achieved by allowing bacteria to be hidden from immune surveillance, providing a rich source of nutrients in the intracellular compartment, and allowing cell to cell movement or destruction of the target host cells (Malek *et al.*, 1994, Sansonetti, 1993). *P. gingivalis* has the capacity to invade a variety of cell lines (Dorn *et al.*, 1999) mediated by several mechanisms such as those involving lipid rafts (Tsuda *et al.*, 2008), clathrin-mediated endocytosis (Boisvert and Duncan, 2008), and accompanying intracellular signalling changes (e.g. mitogen-activated protein kinase, PI3K, and calcium ion fluxes). The result is cytoskeletal rearrangement and subsequent *P. gingivalis* engulfment (Andrian *et al.*, 2006, Cossart and Sansonetti, 2004, Watanabe *et al.*, 2001). However, laboratory assessment of bacterial invasion could be affected by a number of parameters and the work described in this Chapter aimed to evaluate the effect of several such factors.

### **Incubation time and the invasion efficiency of *P. gingivalis*.**

The invasive efficiency of *P. gingivalis* NCTC 11834 with H357 cells was tested with different incubation times using a standard antibiotic protection assay as described previously (section 2.4). Our results showed that the invasion percentage was increased 17 fold (0.33% to 5.82%) with an 8 fold increase in incubation time (30 minutes to 240 minutes). Increased invasion with longer exposure time has been reported by others. Lamont *et al.* (1995) found an approximate 3 fold increase of *P. gingivalis* invasion with a 3 fold increase in incubation time. In addition, a time-dependent increase in invasion of HT-29 epithelial cells by *Mycobacterium avium* has been reported with invasion approximately doubling over an increased incubation time of 2.66 fold (90 minutes to 240 minutes) (Bermudez and Young, 1994). Consequently the proportional increase in invasion that we found was greater than the previous workers although the reason for this is not clear. It may however, reflect differences in the mammalian cell types used.

It has been shown using scanning electron microscopy, that *P. gingivalis* attached to the cell membrane of the human gingival epithelial cells (HGEC) relatively quickly and became completely surrounded by numerous microvilli followed by complete encapsulation after 2 to 4 hours (Njoroge *et al.*, 1997, Sandros *et al.*, 1994). This results in bacterial engulfment by cellular microvilli and represents the first step of cytoplasmic vacuole synthesis (Njoroge *et al.*, 1997). In addition, Yilmaz *et al.* (2003) have shown that after 30 minutes incubation time, which represents the shortest infection time point in our experiments, numerous long, stable microfilaments and organized microtubules were prominent. However, clear depolymerization of microfilaments and degradation in the microtubule network occurred after 24 hours of incubation. According to these results, the effect of increased incubation time on uptake can be explained partly through the more stable organization, stimulation and maturation of the microfilament and microtubule networks. This could be attributed to the influence of the *P. gingivalis* fimbriae on the formation of integrin-associated molecules such as FAK and paxillin (Yilmaz *et al.*, 2003), with subsequent remodelling of the intracellular cytoskeletal components including the actin and tubulin network, which in turn, affect the *P. gingivalis* uptake. It is possible that with increasing incubation time of H357 cells with *P. gingivalis*, there is simply more opportunity for

### Chapter 3 Factors affecting *P. gingivalis* invasion

host-bacterial contact and for the subsequent signalling by the intracellular transduction pathways prior to *P. gingivalis* uptake which will in turn increase invasion efficiency.

Intracellular replication of *P. gingivalis* has also been shown during incubation times of up to 4 hours (Deshpande *et al.*, 1998, Lamont *et al.*, 1995). This may explain our results in that with increasing incubation time up to 240 minutes, high invasion efficiency was obtained which could reflect intracellular growth. However, comparison between the invasion percentage found in others studies with those found here are difficult to make because of the use of different strains and different host cell lines. It is interesting that Madianos *et al.* (1996) failed to find viable bacteria after 8 hours of incubation under cell culture condition without epithelial cells, suggesting the importance of the intracellular lifestyle for *P. gingivalis* replication. Further studies are required to explain the reasons of this increased invasion efficiency in respect to prolonged incubation time.

#### **Effect of growth phase on *P. gingivalis* invasion efficiency.**

We found that *P. gingivalis* grown to mid log phase or to late stationary phase in BHI broth appeared to differ little in their ability to invade epithelial cells. This is in disagreement with several previous studies which showed that the invasion rate of *P. gingivalis* was higher for exponential phase cells (early, mid, and late) than stationary phase cells (Lamont *et al.*, 1995). A similar finding has been reported for other species too (Bermudez and Young, 1994, Ernst *et al.*, 1990, Lee and Falkow, 1990). The high rate of metabolic activity of the bacteria and *de novo* synthesis of proteins required for the invasion process in the exponential phase of the bacteria could explain this variability in the invasion efficiency. For example, the production of certain bacterial surface proteins, such as fibronectin binding protein, is influenced by the microbial growth phase (Lammers *et al.*, 1999). This has been shown for *S. aureus* in which fibronectin binding proteins are more highly expressed during exponential phase than stationary phase, and so suggests a role for fibronectin binding proteins in bacterial adhesion and invasion of host cells. Indeed, *P. gingivalis* fimbriae are known to bind to fibronectin via interaction with fimbriae. The latter are influenced by the action of *P. gingivalis* proteases (Kontani *et al.*, 1997), suggesting a role for both fimA and proteases in *P. gingivalis* adhesion and invasion. Xie *et al.* (1997) showed that *P. gingivalis* growth phase has a negligible effect on *fimA* gene expression and so

### Chapter 3 Factors affecting *P. gingivalis* invasion

fimbrial synthesis. Similarly, RgpA expression which contributes to fimbrial maturity (Nakayama *et al.*, 1996), was not influenced by growth phase (Muthiah *et al.*, 2013, O'Brien-Simpson *et al.*, 2001, Tokuda *et al.*, 1998, Vanterpool *et al.*, 2005). In contrast, but still supportive of our findings, previous studies (Murakami *et al.*, 2004, Rangarajan *et al.*, 1997) showed higher gingipain activity and higher expression of fimA protein in stationary versus log phase (Nishikawa *et al.*, 2004), suggesting that adhesion and invasion was less likely to be reduced in stationary phase. There are numerous similar findings reported for other bacterial species showing that adhesion and cell invasion is at least as high in stationary and log phase and this is explained by differences in expression of various surface factors (Badger and Kim, 1998, Ha and Jin, 2001, Kim *et al.*, 2005, Lapenta *et al.*, 1994, Schiemann and Shope, 1991).

Taken together then, our failure to find a clear effect of growth phase on *P. gingivalis* uptake by epithelial cells may reflect the fact that the important molecules that mediate the process are present in both growth phases, whereas in other species (e.g. *S. aureus*) that is not the case. Furthermore, the variation in the invasion efficiency between exponential and stationary growth phases in our study compared to the previous investigation could also be attributed to the use of different host cell lines and strains of *P. gingivalis*.

#### **Effect of different lysis buffers on the assessment of *P. gingivalis* invasion.**

The estimation of the invasion of cells by bacteria is, in most experiments, dependent on the method used to release the bacteria from the cells. In this study osmotic lysis with distilled water was compared with membrane disruption by the detergent Saponin. Distilled water has been used in many studies as the lytic medium (Aruni *et al.*, 2011, Deshpande *et al.*, 1998, Guilak *et al.*, 2002, Irshad *et al.*, 2012, Lamont *et al.*, 1995), Crowston *et al.* (2004) showed that exposing epithelial cells to distilled water for 60 and 120 seconds is adequate to induce marked swelling and plasma membrane disruption, respectively. However, incubating cells with distilled water for 20-30 minutes in our experiments was standardized, and it is consistent with previous studies (Lamont *et al.*, 1995) where cell lysis was performed for 20 minutes, a period during which bacterial viability was not reported to be adversely affected. This gentle method, together with the cell agitation and scraping described in section 2.8, leaves the cell membrane completely disrupted, releasing the invaded bacteria.

### Chapter 3 Factors affecting *P. gingivalis* invasion

Saponin is well known glycoside detergent which exerts a wide variety of biological effects such as cytotoxicity, haemolysis, and anti-bacterial, anti-viral, and anti-fungal effects (Bachran *et al.*, 2006, Leung *et al.*, 1997, Sen *et al.*, 1998). It has the ability to form pores in the cell membrane resulting in long-lasting permeability allowing passage of large molecules such as ferritin (Seeman, 1974). The cellular effects observed upon treatment with saponin are concentration dependant. These include permeabilization at 0.01-0.05% (Hedman, 1980, Jalal *et al.*, 1992), and complete dissociation at 0.09% concentration (Dourmashkin *et al.*, 1962). For cellular lysis as part of the antibiotic protection assay, saponin has been used at concentrations of 0.01%, 0.1%, and 1% for *H. pylori* (Chu *et al.*, 2010), *P. gingivalis* (Wayakanon *et al.*, 2013) and *Lactococcus lactis* (Asmat *et al.*, 2012), respectively. Its primary effect is through its ability to complex with cholesterol (Baumann *et al.*, 2000, Gögelein and Hüby, 1984), resulting in pore formation (Gögelein and Hüby, 1984). While saponin concentrations from 25 to 100 µg/ml (0.01%) are adequate to cause lysis of erythrocytes (Arabski *et al.*, 2012), it was not sufficient to cause lysis of H357 oral keratinocytes in our study (data not shown). Therefore, we induced cell membrane lysis with a concentration of 1 mg/ml (0.1%). This is in agreement with Wayakanon *et al.* (2013), who use 0.1% saponin to lyse the keratinocyte cell membrane and in order to ensure complete cell lysis. We also used gentle agitation in our experiments to release the internalized *P. gingivalis*. However, although it is possible that saponin may influence the viability of released bacteria, as has been reported for Gram-positive bacteria (Oyekunle *et al.*, 2006), our data would not support that since there was no significant difference between osmotic and detergent lysis. Having said that, it is documented that saponin can form complexes with the lipid A content of the Gram-negative LPS, increasing the permeability of the bacterial outer membrane (Arabski *et al.*, 2012) and, therefore possibly affecting the viability of *P. gingivalis*.

As distilled water has been widely used in the literature as a lytic agent in invasion assays and saponin did not significantly influence the level of recovered bacteria, we used distilled water in all monolayer culture experiments.

#### **Estimating *P. gingivalis* invasion by measurement of bacterial intracellular DNA.**

Since quantification of cell invasion commonly depends upon release of viable bacteria, it is possible that this leads to an underestimate of invasion if some die or enter an uncultivable state. Consequently we sought to compare cell invasion by *P. gingivalis* using conventional viable counting with quantitative DNA measurement using real time PCR. Real time PCR-dependent quantification of various subgingival plaque bacteria has been compared with culture-based methods in a range of studies (Asai *et al.*, 2002, Boutaga *et al.*, 2003, 2005, Jervøe-Storm *et al.*, 2005, Lau *et al.*, 2004, Lyons *et al.*, 2000, Nonnenmacher *et al.*, 2004). The real time PCR technique has the advantage of being rapid, sensitive, and reliable for identification of very small populations of bacteria (Boutaga *et al.*, 2007). Viable counting has been the gold standard for much of this type of work over the years but suffers from the requirement for anaerobic culture (for *P. gingivalis*), which is time consuming and the sensitivity can be low (Boutaga *et al.*, 2003, 2005).

We observed a significant difference between methods employed to detect bacterial invasion. There was a 2 fold higher *P. gingivalis* level of invasion detected using real time PCR ( $3.8\% \pm 0.16$ ) compared to our standard culture method ( $2\% \pm 0.22$ ) (section 3.3.4). This is in agreement with previous studies (Boutaga *et al.*, 2007, Boutaga *et al.*, 2003) which showed increased detection of *P. gingivalis* using PCR compared to culture methods although these studies were conducted on subgingival plaque samples rather than lysates of mammalian cell following invasion. Nonetheless, it is unsurprising to find such a difference since the higher level detected by PCR could be attributed to DNA of both non-viable *P. gingivalis* and viable cells. It is likely there would be more dead bacteria when exposed to the aerobic laboratory environment, as it has been shown that approximately 7 fold more *Bacteroides gingivalis* were killed when incubated aerobically for 6 hours compared to 1 hour aerobic incubation (Thompson and Wilton, 1991).

Despite its appeal and some advantages, there are two major limitations of the real time PCR-dependant method for use in invasion studies. First, qPCR cannot differentiate between viable and dead bacteria, so there is no knowledge gained on the viable state of the bacteria that have become cell-associated. Secondly, the method cannot differentiate between cell adhesive but not internalised bacteria from those that have

### Chapter 3 Factors affecting *P. gingivalis* invasion

been internalised. Consequently, there is always likely to be an overestimate of the level of invasion and so the term “measurement of cell-associated bacteria” should be used in place of invaded or internalised bacteria. Perhaps a combination of qPCR with the fluorescence microscopy after live/dead staining (Ammann *et al.*, 2013), would be a useful approach for future studies.

For the reasons described above, and despite its time consuming nature, a culture-dependent method for enumeration of the invaded bacteria was carried throughout our entire studies on cell invasion by *P. gingivalis*.

#### **Effect of cell seeding density on the *P. gingivalis* invasion.**

The density of the cell layer used to assess bacterial invasion could influence the apparent efficiency of the process. This is because cellular receptors involved in bacterial adhesion and internalisation for example can have different cell polarisation patterns according to their degree of confluence (Hu and Kopecko, 1999, Pinto *et al.*, 1983, Velge *et al.*, 1997). To determine whether there was likely to be any effect, we seeded cells at different densities, allowed them to adhere to the tissue culture plastic and then challenged them with *P. gingivalis*. The highest *P. gingivalis* invasion was obtained with the highest seeding density ( $7.5 \times 10^4$  cell/well giving  $1.8\% \pm 0.11$  invasion). This could be explained simply by the higher availability of receptors involved in *P. gingivalis* entry in to the host cells, such as  $\alpha 5\beta 1$  integrin (Yilmaz *et al.*, 2002), TLR (Wang *et al.*, 2007), and cytokeratins (Sojar *et al.*, 2002) because more cells provide a higher surface area for attachment.

Fortunately, our H357 cells did not reach full confluence, in which it has been shown that the invasion of other bacteria decreased dramatically compared to semi-confluent cells (Gaillard and Finlay, 1996, Hu and Kopecko, 2008, Mooney *et al.*, 2003).

### Effect of cell passage on the *P. gingivalis* invasion

In addition to the previous documented factors affecting *P. gingivalis* invasion in our study, we were concerned that cell phenotypic features could change with frequent passaging and these could affect invasion by bacteria. Indeed a considerable amount of data have shown that with increased cell passage number, significant cellular effects were reported compared to low passage cells. These include: decreased cell differentiation (Derubeis and Cancedda, 2004, Stenderup *et al.*, 2003) with abnormal differentiation behavior (Wall *et al.*, 2007), changes in cell morphology (Chen *et al.*, 2015, Donaldson and Shuler, 1998, Stenderup *et al.*, 2003), decreased proliferation rate (Chen *et al.*, 2015, Chennazhy and Krishnan, 2005, Fawdar, 2010), increased apoptosis (Chennazhy and Krishnan, 2005), diminished expression of functional markers/proteins (Chandrasekhar and Millis, 1980, Chandrasekhar *et al.*, 1983a, Chandrasekhar *et al.*, 1983b, Fawdar, 2010), decreased cell signaling activity (Lin *et al.*, 2004), and loss or diminished expression of potential receptors involved in the normal physiology of the cells (Ottino *et al.*, 2004). All of these findings, however, were reported with various normal/mesenchymal cells, and with different definitions of low and high passage cells. Here we used the term “early passaged cells” to describe those where the average of passage number was fairly low (p=56, p=57, and p=58), and “late passage cells” to those that had been passaged considerably more times (p.218, 219, and 220). A small increase in *P. gingivalis* uptake was observed in the early passaged cells (2.9%±0.2) compared to the late passage cells (2.3%±0.2) but this did not reach statistical significance (section 3.3.6).

In our studies we have used H357 oral keratinocytes, a squamous cell carcinoma (SCC)-derived cell line isolated from the tongue (Sugiyama *et al.*, 1993). Sugiyama *et al.* (1993) showed that there was no major alterations regarding integrin expression including  $\beta 1$  integrin when the passage number increased from p.20-p.40 in H357 and other 4 SCC cell lines. This might indicate that the integrin expression is stable when the passage number increased and explain the fact that the range of our invasion level was similar between p.57 to p.219.



Furthermore, several studies have shown that multiple cellular changes exist with carcinoma-derived cell lines of different passage number. For example, Anderle *et al.* (1998) showed that the expression and functionality of the MDRI protein, an efflux transporter involved in the protection of small intestine tissues from toxins, was significantly higher in low passage compared to high passage Caco-2 cells, which are derived from human colorectal carcinoma. In addition, it has been shown that the mER $\alpha$  receptor, which is involved in multiple systems such as cell proliferation, was expressed at low level in a high passage F10 cells, a rat pituitary tumor-derived cell line, compared to the higher expression in low passage cells (Cambell *et al.*, 2002), indicating the physiological cell changes are existed with different cell passages. It appears, however, that at least in regard to mediators of bacterial invasion, H357 cells may remain stable upto at least passage 220.

### 3.5 CONCLUSION

The bacterial internalization within the host cells is a complicated process that requires a cross talk between bacteria, cells, and the environment. A significant increased *P. gingivalis* invasion was reported when the incubation time and cell density were increased, and when a DNA extraction method to quantify the invaded bacteria compared to viable counting. This invasion not significantly different when stationary phase *P. gingivalis* were compared to the mid log phase, when saponin cell lysis was compared to distilled water lysis, and when early passage number H357 cells were compared to the later passage numbers.

The results obtained in this chapter highlight some factors which may contribute to variation in bacterial invasion assays. It is important to maintain consistency in regard to these variables to obtain the most reliable and reproducible results. The differences in the actual invasion level compared to the other similar investigations could be attributed to the variety of cell lines and *P. gingivalis* strains used.

**Chapter 4**  
**Cell response to**  
***P. gingivalis* stimuli.**

### 4.1 INTRODUCTION

The host cell response to the external stimuli including bacterial challenge is a dynamic process of host-bacterial interactions. In most instances, the host inflammatory response contributes to the elimination of causative microorganisms without inducing clinical signs of inflammation (Heumann and Roger, 2002). The detection of the bacterial outer membrane components by host cells is designed to promote bacterial killing and control infection. However, an uncontrolled cell response may aggravate the inflammatory response, resulting in increased bacterial colonization and tissue destruction.

An example of such a response is the recognition of *P. gingivalis* lipopolysaccharide (LPS) by toll-like receptors (TLRs). It has been shown that TLR-2, but not TLR-4, has a strong association with *P. gingivalis* LPS (Hirschfeld *et al.*, 2001, Martin *et al.*, 2001). The proposed LPS-induced activation of TLR-2 has several influences on cell surface receptors/molecules activation and signaling pathways, which are considered important as part of the cell response to external stimuli including bacterial challenge. These include: TLR-2-*P. gingivalis* fimbriae induced activation of CR3 which results in increased *P. gingivalis* invasion (Wang *et al.*, 2007, Zenobia and Hajishengallis, 2015), and TLR-2-induced activation of  $\beta 1/\beta 2$  integrins on the cell surface again with a resultant increase in *P. gingivalis* invasion involving cytoskeletal rearrangement (Burns *et al.*, 2006, Harokopakis and Hajishengallis, 2005). Therefore, the question was raised as to whether pre-treatment of H357 cells with *P. gingivalis* LPS has an effect on the *P. gingivalis* invasion, perhaps through pathways similar to those mentioned above.

It is not well investigated whether the physical state of the cells has an effect on the bacterial-cell interaction. It has been shown that incubating H357 cells with *S. aureus* in suspension resulted in a 27 fold higher invasion compared to incubation in monolayer culture. There was a concomitant increase in  $\alpha 5$ -integrin expression in cells in suspension, suggesting this integrin may be more available for bacterial binding and subsequent invasion under those conditions. Thus, the question is posed whether incubating H357 cells in suspension has a similar effect on *P. gingivalis* invasion compared to the monolayer culture, suggesting that the physical state of the cell may influence several cell surface molecules including integrin expression.

## Chapter 4 Cell response to *P. gingivalis* stimuli

A considerable amount of data has been obtained relating to *P. gingivalis* gene and protein profiles following host cell invasion. However, the post invasion gene characteristics of the eukaryotic cells following *P. gingivalis* invasion have not been well documented. Endothelin-1(ET-1) and urokinase plasminogen activator receptor (uPAR) are potentially important post-inflammatory mediators involved in variety of inflammatory reactions. As *P. gingivalis* infection of the epithelial cells showed significant increase in IL-1 $\beta$  and tumor necrosis factor alpha (TNF- $\alpha$ ) mRNA expressions compared to the unaffected cells (Sandros *et al.*, 2000), and cell stimulation with both cytokines has a significant impact on ET-1 mRNA expression (Fujioka *et al.*, 2003) and uPAR protein expression (Schwab *et al.*, 2004), the question then raised is whether infecting H357 cells by *P. gingivalis* has an influence on the ET-1 and uPAR mRNA expression in a time-dependent manner.

Finally, it has been shown that heterogeneity exists within cell populations at any one time in response to various stimuli, and different functional, physical, and phenotypic behaviours have been reported. For example, Micallef *et al.* (2010) showed that in the immortalized human keratinocyte, cell line HaCaT, a population with higher expression of the differentiation markers (HC-F1) had the lowest proliferative capability, while the lowest differentiated subpopulation (HC-F2) had the highest proliferative activity, suggesting the functional heterogeneity existed within this keratinocyte population. This was induced by Ca<sup>2+</sup>. The question then posed is whether heterogeneity exists in H357 oral keratinocytes in relation to their susceptibility to *P. gingivalis* invasion, which may be related to the differences in integrin expression or other cell characteristics related to *P. gingivalis* invasion.

Based on these mechanisms, this chapter investigates some of the cell responses to bacterial challenge, revealing some of the post invasion changes that occur.

### The aims of this part of the study were:

- 1- To investigate the influence of LPS pretreatment of H357 cells on *P. gingivalis* invasion efficiency.
- 2- To compare *P. gingivalis* invasion of cells in suspension compared to those in monolayer cultures.
- 3- To determine the effect of *P. gingivalis* infection on the cellular gene response particularly in ET-1 and uPAR mRNA expression
- 4- To characterize the pattern of *P. gingivalis*-H357 cell association.

## 4.2 METHODS

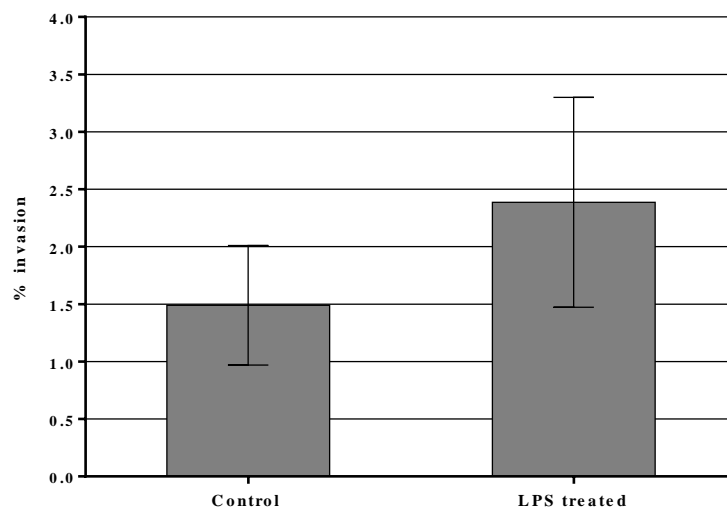
The following methods were used in this chapter:

- Epithelial cell culture (section 2.2)
- *P. gingivalis* culture (section 2.3)
- Antibiotic protection assay (section 2.4)
- Invasion assay of pretreated lipopolysaccharide (LPS) of *P. gingivalis* with H357 cell monolayer (section 2.11)
- Comparison of *P. gingivalis* invasion of adherent H357 cells to those in suspension (section 2.12).
- RNA extraction of H357 cell monolayers following exposure to *P. gingivalis* NCTC 11834 (section 2.14)
- Reverse transcriptase-Polymerase chain reaction (RT-PCR) (section 2.15).
- Real time- Polymerase chain reaction (qPCR) (section 2.16).
- ET-1 and uPAR mRNA expression of H357 cells exposed to *P. gingivalis* (sections 2.13, 2.14, 2.15, 2.16).
- *P. gingivalis* localisation within H357 cells (Immunocytochemistry of H357 cells invaded by *P. gingivalis* (section 2.23.1).

## 4.3 RESULTS

### 4.3.1 Influence of pre-treatment of H357 cells with LPS on the invasion efficiency of *P. gingivalis*.

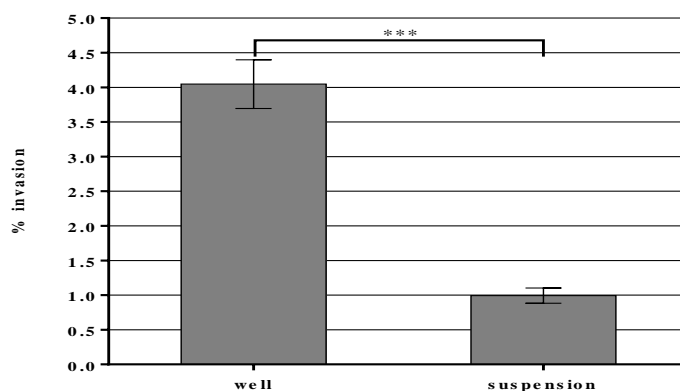
In order to address the question of whether LPS might activate cell TLR-2 and subsequently  $\beta 1$  integrin, the effect of pre-treating H357 cells with *P. gingivalis* LPS on the percentage of bacterial uptake was investigated. H357 cells were treated with *P. gingivalis* LPS and adjusted as described in section 2.11. A standard antibiotic protection assay was conducted as described in section 2.4 and the results were compared to control cells which were not exposed to LPS. The results showed that there was a small increase in *P. gingivalis* internalization between cells pretreated with LPS ( $2.21\% \pm 0.8$ ) and untreated control samples ( $1.4 \pm 0.45$ ) (figure 4.1) but this difference was not statistically different ( $p=0.2$ ).



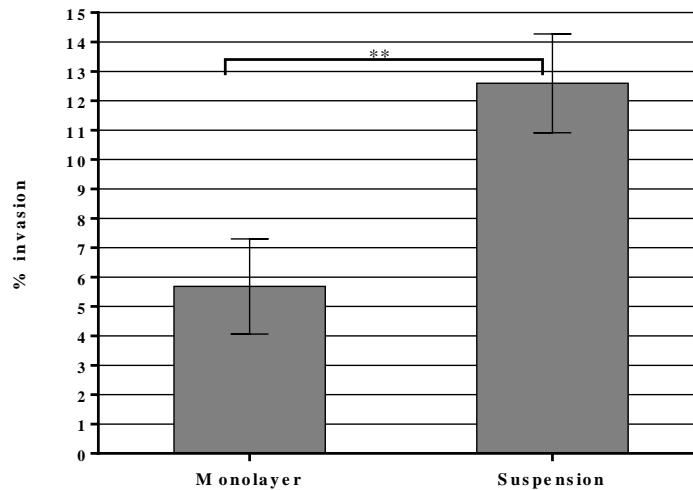
**Figure 4.1** Effect of *P. gingivalis* LPS pretreatment on the level of invasion of H357 cell monolayer by *P. gingivalis*. H357 cells were seeded in 24 well plate at  $1 \times 10^5$ /well for 24 hours at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , followed by medium replacement with KGM containing *P. gingivalis* LPS for the next day. Then the antibiotic protection assay was conducted as indicated previously. Viable counting of the invading bacteria was carried out and compared to the control samples without LPS. Results show mean $\pm$ SD of 3 independent experiments in triplicate.

### 4.3.2 Comparison of *P. gingivalis* invasion of adherent H357 cells to those in suspension.

As mentioned in section 2.12, a modified antibiotic protection assay was conducted to address the question as to whether the physical state of the cells has an influence on *P. gingivalis* uptake by comparing the infection of cells in suspension with infection of monolayer cultures using a standard antibiotic protection assay. Distilled water and 0.1% saponin (w/v) were used independently for cell lysis. When the infected H357 cells were lysed by distilled water, there was highly significant difference ( $p= 0.0001$ ) in the invasion percentage between monolayer cultures ( $4\% \pm 0.2034$ ) and cells in suspension ( $1\% \pm 0.06$ ) (figure 4.2). In contrast, when the infected cells were lysed with 0.1% saponin (w/v), there was significant difference ( $p=0.006$ ) in the invasion percentage between monolayer cultures ( $5.68\% \pm 0.9$ ) compared with higher invasion in cells with suspension ( $12.6\% \pm 0.97$ ) (figure 4.3). The *P. gingivalis* uptake in monolayer cultures (i.e. in wells) using both lysis methods are consistent with the results reported in section 3.3.3, graph 3.5. Although bacterial uptake within the same physical state of the cells (i.e. in wells) was similar between the two lytic systems, there was a clear difference in the internalization percentage of *P. gingivalis* between cells in wells and cells in suspension using distilled water and saponin independently.



**Figure 4.2 Comparison of *P. gingivalis* invasion between cells in suspension and cells on tissue culture plastic using distilled water.** H357 cells were prepared in suspension, infected with *P. gingivalis* for 90 minutes aerobically, and incubated with metronidazole to kill the adherent bacteria. The cells were lysed using distilled water on an end-over-end mixer and vortexed for releasing bacteria. The lysates were serially diluted and inoculated on FA blood agar prior to the viable counting of the bacteria. The results then compared to the cells lysed in monolayer cultures. The results represent the mean $\pm$ SD of 3 independent experiments in triplicate.



**Figure 4.3 Comparison of *P. gingivalis* invasion between cells in suspension and cells on well plate using 0.1% saponin.** The same experimental steps were conducted as described above in figure 4.2, but using 0.1% saponin. The results shown represent the mean±SD of 3 independent experiments in triplicate.

Cells in suspension were more difficult to lyse using distilled water than cells in monolayer culture. This may be attributed to the lack of ability to perform scraping and agitation of cells in suspension compared to cells attached to the tissue culture plastic wells. In contrast, cells in suspension lysed by 0.1% saponin (w/v) might have an advantage that the permeabilization effect of saponin in suspension is more efficient as there may be more surface area for interaction of the lysis buffer with cells. Therefore, invasion experiments with cells in suspension were performed with 0.1% saponin dissolved in PBS.

#### 4.3.3 Genetic characterisation of invaded H357 oral keratinocyte cells by *P. gingivalis*.

On the basis of a previous screen of genes upregulated in H357 cells following interaction with *P. gingivalis* (Whawell *et al.*, 2013) (Table 4.1), our study was conducted to determine and validate the effect of exposing H357 cells to *P. gingivalis* strain NCTC 11834 on Endothelin-1 (ET-1) and Urokinase Plasminogen Activator Receptor (uPAR) gene expression levels at different incubation times and to compare these results to control cells without *P. gingivalis* infection. This investigation is important to determine whether these genes were differentially expressed by H357 cells



## Chapter 4 Cell response to *P. gingivalis* stimuli

that are targeted by *P. gingivalis* or whether change in expression is in response to *P. gingivalis* invasion. The time course effect of *P. gingivalis* infection on the possible cellular genes involved in the pathogenesis of periodontal diseases is extremely important as it may correlates the expression of these genes with the signaling molecules involved in the bacterial invasion event.

**Table 4.1 List of up-regulated and down-regulated genes isolated following *P. gingivalis* infection with H357 cells for 90 minutes.** Eight genes including ET-1 and uPAR were over expressed >2.6 fold in cells associated with *P. gingivalis* compared to those that were not (Whawell *et al.*, 2013). ET-1 and uPAR were selected and validated with *P. gingivalis* invasion for 30 minutes, 90 minutes, 150 minutes, and 240 minutes in our experiments.

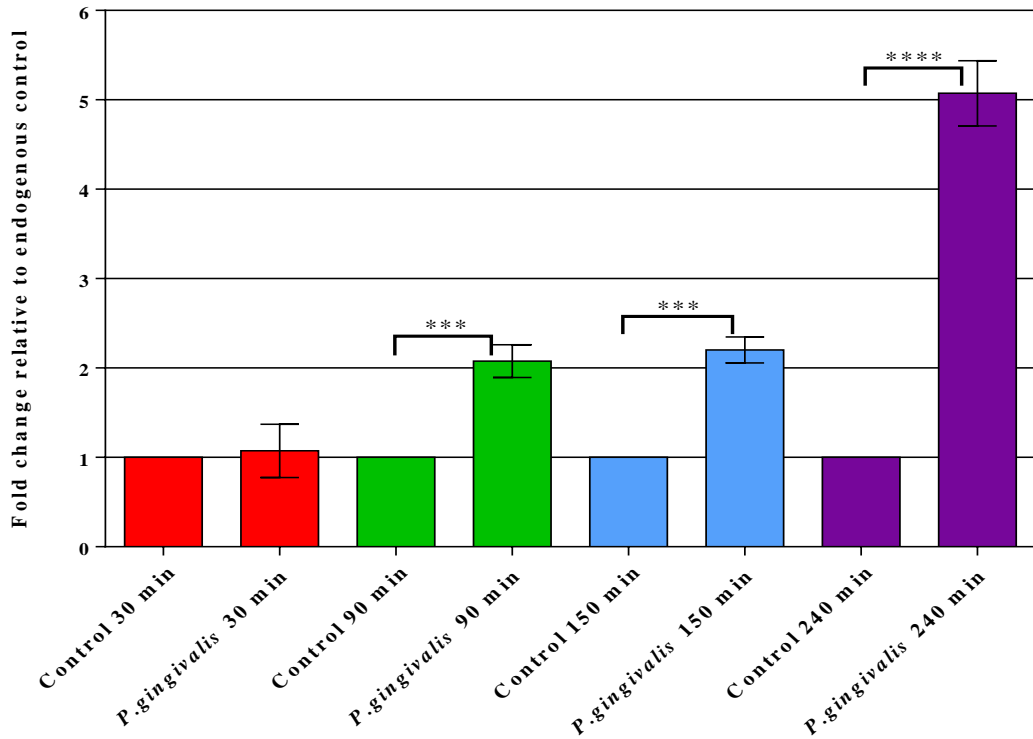
Up regulated	Fold change	Down regulated	Fold change
Grainyhead-like 3	5.85	Serpin peptidase inhibitor	-3.01
Zinc finger protein 750	5.00	Fibroblast growth factor receptor 3	-2.27
Keratin associated protein 2-4	4.48	F-box protein 32	-2.16
Small proline-rich protein 3	3.43	S100 calcium binding protein	-2.03
Endothelin-1	2.99	Keratin	-1.96
Plasminogen activator, urokinase receptor	2.73		
Claudin 4	2.63		
Follistatin	2.63		

### **4.3.3.1 ET-1 mRNA expression in H357 cells following exposure to *P. gingivalis* NCTC 11834.**

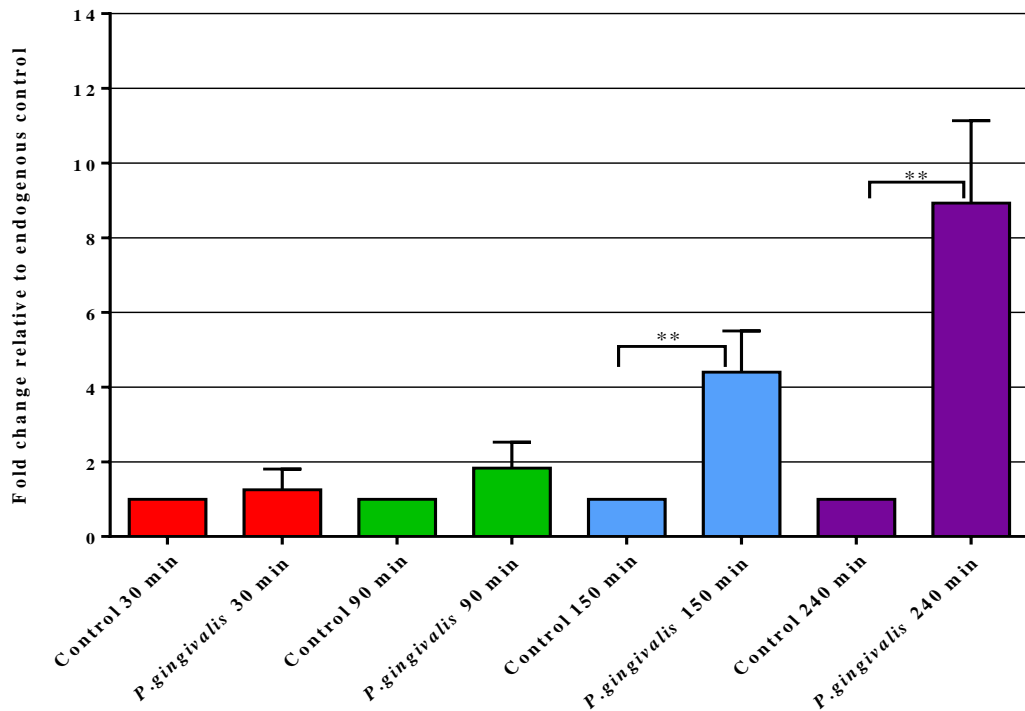
The results showed that ET-1 mRNA expression level was two fold higher by 90 and 150 minutes ( $P \leq 0.001$ ), and five fold higher by 240 minutes ( $P \leq 0.0001$ ) after incubation with *P. gingivalis* compared to the control cells. There was no difference in expression level of ET-1 between infected and control cells at 30 minutes post incubation ( $P \geq 0.05$ ) (figure 4.4). The latter may indicate that this short infection time was not long enough to influence ET-1 gene expression. The highly significant increase of ET-1 following 240 minutes of infection (5 fold) compared to 90 minutes and 150 minutes (2 fold) indicates that ET-1 requires this length of time with bacteria for changes in gene expression which may relate to the fact that this is mediated by other signalling molecules involved in the cell-bacterial interactions, such as post inflammatory cytokines (e.g. IL-1 $\beta$ ) as reported previously (Ansai *et al.*, 2002).

### **4.3.3.2 Urokinase plasminogen activator receptor (uPAR) expression in H357 cells following incubation with *P. gingivalis* NCTC 11834.**

Ogura *et al.* (1999) have reported that both uPA and uPAR expression were increased in gingival fibroblasts exposed to *P. gingivalis* LPS. However, the effect of the whole bacteria on the expression of the uPAR in oral keratinocytes with different incubation times has not been previously investigated. The effect of exposing H357 monolayer cultures to *P. gingivalis* on uPAR gene expression levels at 30 minutes, 90 minutes, 150 minutes, and 240 minutes was determined and compared with control cells without *P. gingivalis* infection. The results showed that uPAR mRNA expression was similar between control cells and cells infected with *P. gingivalis* for 30 minutes. Following 90 minutes of infection, a slight increase in uPAR gene level was observed which did not reach statistical significance ( $p \geq 0.05$ ). A marked increase in the uPAR mRNA expression was observed following 150 minutes (four fold increase) compared to the control cells, and the highest fold change increase was obtained following 240 minutes of infection (nine fold increase) ( $p \leq 0.01$ ) (figure 4.5). From our findings regarding ET-1 and uPAR gene expressions, it seems likely that the cells require time for the transcription machinery to express these genes when targeted by *P. gingivalis*.



**Figure 4.4 ET-1 mRNA expression by H357 cells at different incubation times following infecting with *P. gingivalis* NCTC 11834.** H357 monolayer cultures were grown on 6 well plate at  $2 \times 10^5$ /well for 48 hours, and incubated with *P. gingivalis* at MOI 1:100 for 30 minutes, 90 minutes, 150 minutes, and 240 minutes aerobically at 37°C. The cells were washed, trypsinized and RNA extracted from the cell pellet using a RNeasy Mini Kit (Qiagen). Following complementary DNA synthesis, ET-1 gene expression was performed using qPCR with SYBR green master mix (Applied Biosystems) and ET-1 specific primers using U6 as an endogenous control. This histogram shows the mean fold change in ET-1 expression relative to the uninfected control  $\pm$ SD of at least 3 independent experiments performed in triplicate. \*\*\*, \*\*\*\* Indicate statistically significant differences at ( $P \leq 0.001$ ), and ( $P \leq 0.0001$ ) respectively.

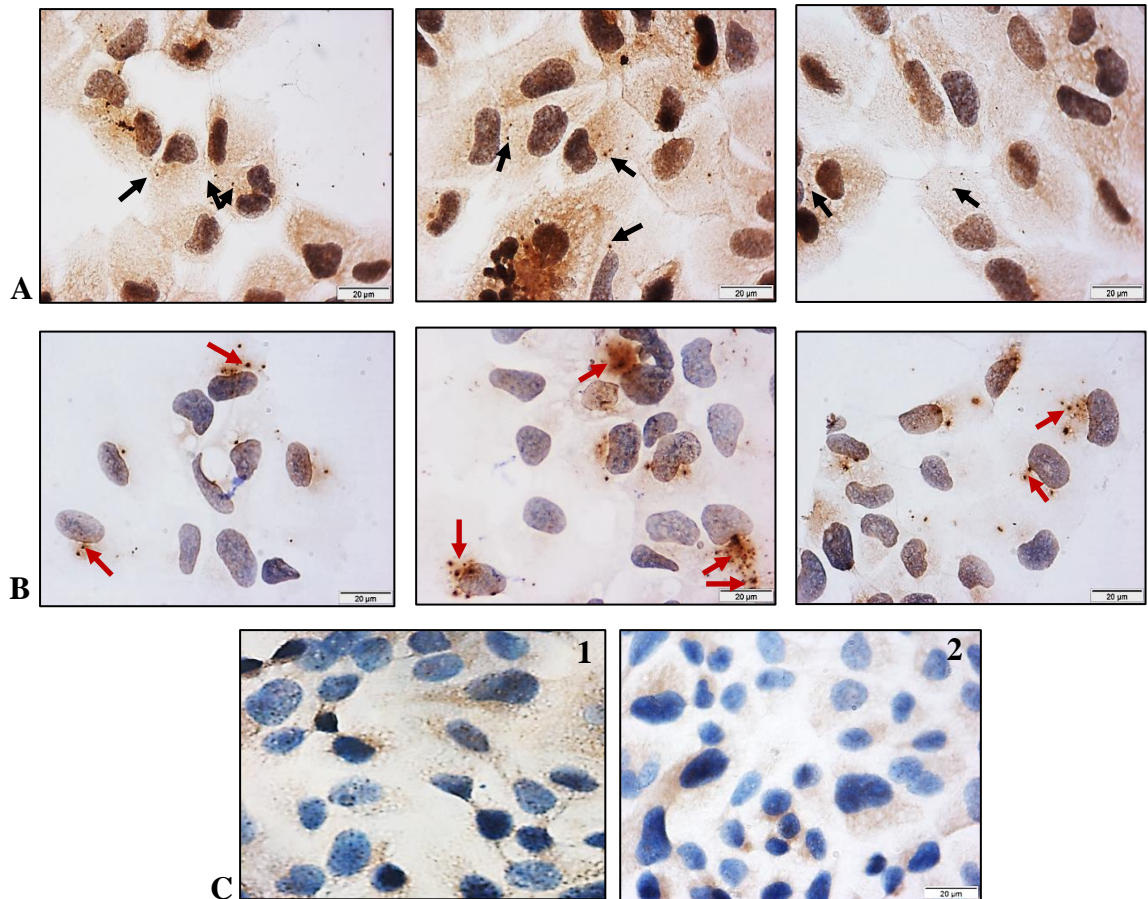


**Figure 4.5 uPAR mRNA expression following infecting H357 cells with *P. gingivalis* NCTC 11834 for different incubation times.** H357 cells were grown on 6 well plate at  $2 \times 10^5$ /well for 48 hours, and incubated with *P. gingivalis* at MOI 1:100 for 30 minutes, 90 minutes, 150 minutes, and 240 minutes aerobically at 37°C. The cells were washed, trypsinized and RNA extracted from the cell pellet using a RNeasy Mini Kit (Qiagen). Complementary DNA was synthesised and the quantification of uPAR gene expression was performed using qPCR with SYBR green master mix (Applied Biosystems) and uPAR specific primers using U6 as an endogenous control. This histogram shows the mean fold change in uPAR expression relative to the uninfected control  $\pm$ SD of at least 3 independent experiments performed in triplicate. \*\*, Indicate statistically significant differences at ( $P \leq 0.01$ ).

The results clearly show that an increasing time of incubation H357 oral keratinocytes with *P. gingivalis* is associated with increasing expression of both ET-1 and uPAR. Such observations could have an implication in the diagnosis and treatment of periodontal diseases as the level of expression of these genes may relate to the severity of disease or even provide a therapeutic target.

### 4.3.4 H357 Cell-*P. gingivalis* association.

Two primary *P. gingivalis* antibodies were used to identify the pattern of *P. gingivalis* invasion within H357 cells using immunocytochemical analysis as described in section 2.23.1, to determine which one can be used to identify the internalized *P. gingivalis*. MAb 1B5 monoclonal antibody (kindly provided by Professor Mike Curtis, Barts and The London School of Medicine), was raised against the *P. gingivalis* catalytic subunit of arginine-gingipain (RgpAcat) (Curtis *et al.*, 1999), and is also immunoreactive with *P. gingivalis* membrane type RgpAcat (mt-RgpAcat), membrane-type RgpB (mt-RgpB) and an anionic cell surface polysaccharide (APS). This antibody shows a few invaded bacteria within H357 cells in an unequal pattern of invasion (figure 4.6.A), indicating that some H357 cells are more susceptible to be invaded by *P. gingivalis* than others. This was confirmed when anti-whole *P. gingivalis* antibody (kindly provided by Dr. Graham Stafford, School of Clinical Dentistry, University of Sheffield), was used to determine the pattern of invasion. The results show more *P. gingivalis* associated with some cells than with others (figure 4.6.B), suggesting that certain cells in the population might be more vulnerable to the bacterial interaction. It was also observed that more *P. gingivalis* cells were visible using the anti-whole *P. gingivalis* antibody compared to that seen with the MAb 1B5 monoclonal antibody. To check for non-specific interactions, both primary antibodies were applied to control samples without *P. gingivalis* (figure 4.6.C1). Also, a control lacking primary antibody was included (figure 4.6.C2).



**Figure 4.6 Immunocytochemistry images of invaded H357 cells by *P. gingivalis* NCTC 11834.** H357 cells were grown on coverslips, infected with *P. gingivalis*, washed, fixed and quenched for the endogenous peroxidase activity. Cells were blocked with 100 % goat serum, washed and incubated separately with the two anti-*P. gingivalis* primary antibodies for overnight at 4°C. Cells were washed and incubated with biotinylated secondary antibody. Cells were then washed and incubated with Avidin Biotinylated enzyme Complex reagent. Cells then incubated with 3, 3'-diaminobenzidine substrate at room temperature. The coverslips then washed, counterstained with haematoxylin, and mounted onto microscope slide for visualization. Invaded *P. gingivalis* are shown as black dots using MAb 1B5 antibody (A) and anti-whole *P. gingivalis* antibody (B). Control samples consisted of non-infected cells (C.1) and infected cells without primary antibody (C.2).

### 4.4 DISCUSSION

#### **Invasion assay of H357 cell monolayer pre-treated with *P. gingivalis* LPS.**

An example of the cellular response to the bacterial stimuli is the recognition of bacterial LPS by cell TLRs. It has been shown that the LPS of most Gram-negative bacteria activate TLR-4 (Takeda and Akira, 2005), whereas *P. gingivalis* LPS has a strong association with TLR-2 and weak TLR-4 agonist activity (Hirschfeld *et al.*, 2001, Martin *et al.*, 2001). This discrepancy in the influence of LPS on TLRs activation could be attributed to structural and functional activity differences of *P. gingivalis* LPS (Jain and Darveau, 2010).

Our results showed that the pre-treatment of H357 cells with *P. gingivalis* LPS results in a slightly higher invasion by *P. gingivalis* ( $2.21\% \pm 0.8$ ) than seen without LPS treatment ( $1.4 \pm 0.45$ ) but this did not reach statistical significance (section 4.3.1). The LPS employed was ultrapure so there was no confounding effect of contaminants.

As a response to bacterial stimuli, it has been shown that TLR-2 is increased in expression and stimulated in various epithelial cells (Uehara *et al.*, 2007), including oral keratinocytes (Benakanakere *et al.*, 2009) and H357 cell line (Belfield, 2013). Therefore, it is likely that following *P. gingivalis* LPS stimulation, there was stimulation with clustering of TLR-2 on the surface of H357 oral keratinocytes used in our study.

Even though our data did not reach significance, there was a trend towards higher invasion obtained with cells pre-treated with LPS and this would support findings reported by others that TLR-2 activation could enhance *P. gingivalis* cell invasion. The proposed mechanism is that *P. gingivalis* FimA and minor fimbrial proteins interact with TLR-2 (Wang *et al.*, 2007) and the resultant interaction of TLR-2-FimA leads to inside-out signalling of the CD14/TLR-2 complex within the cell membrane lipid rafts. This in turn causes a conformational change in CR3 towards a high affinity structure (Wang *et al.*, 2007, Zenobia and Hajishengallis, 2015). The lipid raft mediated internalization of *P. gingivalis*, thus, could be explained partly through the significant role of the TLR-2 signalling pathway as well as through  $\beta$ 1-mediated lipid raft internalization (Mcgarrahy *et al.*, 2015, Tsuda *et al.*, 2008).

*P. gingivalis* LPS has some TLR-4 binding capability and along with TLR-2, it has been shown to be activated by two lipid A masses of *P. gingivalis* LPS, releasing TNF- $\alpha$  from the cells (Darveau *et al.*, 2004).

### **Comparison of *P. gingivalis* invasion of adherent H357 cells to those in suspension.**

Despite the reports of TLR-2 mediating events that could enhance invasion of cells by *P. gingivalis*, the main mechanism for internalization proposed in the literature is via interaction with  $\alpha 5\beta 1$  integrin. When cells adhere to a substrate they do so by integrin-mediated extracellular matrix (ECM) protein interactions which results in redistribution of integrins on the cell membrane with some becoming polarised and partially hidden from access by *P. gingivalis*. Consequently, we investigated whether the physical state of the cells has an influence on *P. gingivalis* invasion. As shown in section 4.3.2, a significant difference of approximately 3 fold higher invasion of *P. gingivalis* was obtained when H357 cells were in suspension (12.6% $\pm$ 0.97) compared to those adherent in a monolayer culture (5.68% $\pm$ 0.9) following lysis with saponin. This result is in agreement with Ridley *et al.* (2012), who showed that the invasion of H357 cells in suspension by *S. aureus* was considerably higher compared to the monolayer cultured H357 cells. It is presumed then that *P. gingivalis* preferentially invade non-adherent cells (in suspension) by exploiting the greater surface area of  $\alpha 5\beta 1$  integrin rather than the minimum space between the intimately associated cells as in monolayer culture cells.

### **ET-1 cellular response to *P. gingivalis* infection.**

Previous studies have been conducted to study the genes and proteins expression of *P. gingivalis* upon internalization into eukaryotic host cells. These related to virulence factors involved in the adhesion and invasion of *P. gingivalis* or to the maintenance of bacterial viability and function (Hosogi and Duncan, 2005), such as genes involved in the oxidative stress including superoxide dismutase (SOD), and heat shock genes including *groEL* (Andrian *et al.*, 2006). However, following *P. gingivalis* uptake, the post invasion gene profile of eukaryotic host cells has not been extensively studied. Therefore, our study was conducted to investigate whether such changes in the gene expression of H357 oral keratinocyte might exist as a response to the *P. gingivalis* invasion over different incubation times. ET-1 and uPAR molecules are examples of



## Chapter 4 Cell response to *P. gingivalis* stimuli

post inflammatory cellular markers involved in the inflammation signalling along with various inflammatory mediators including pro-inflammatory cytokines such as IL-1. Based on this, both markers were investigated in our study following infection of H357 cells with *P. gingivalis* at 30, 90, 150, and 240 minutes incubation to determine whether the mRNA expression corresponded to the length of time the cells were exposed to *P. gingivalis*.

Endothelin-1 (ET-1) is a 21 amino acid peptide with potent vasoconstrictive activity. It was originally isolated from porcine aortic endothelial cells and has multi-regulatory activities in a variety of tissues such as airway epithelium and adrenal cortex (Li *et al.*, 1999, Springall *et al.*, 1991, Yanagisawa *et al.*, 1988). It is associated with a variety of diseases such as hypertension, atherosclerosis, and inflammatory and sclerotic diseases (Inoue *et al.*, 1989). It is produced and expressed by a variety of cells including gingival epithelial cells, human periodontal ligament cells (Fujioka *et al.*, 2003, Liang *et al.*, 2014) and vascular endothelial cells (Chen *et al.*, 2000). Human gingival keratinocytes (HGK) showed the highest ET-1 expression compared to the other cells, however, there are no previous studies of the expression of ET-1 in oral keratinocyte cell lines such as H357 cells following infection with *P. gingivalis*.

There was a significant increase in ET-1 expression in H357 cells infected with *P. gingivalis* which increased over 240 minutes after infection. The results are in agreement with Yamamoto *et al.* (2003), who showed that the ET-1 mRNA expression level was higher in gingival epithelial cells infected with *P. gingivalis* compared to cells without bacterial stimulation, although they obtained their results following a 90 minutes incubation only. In addition, the trend for the time-induced mRNA expression of ET-1 is supported by Ansai *et al.* (2002), who showed that the ET-1 mRNA expression was increased steadily up to 16 hours of *P. gingivalis* infection.

The increase in ET-1 expression may occur as a result of stimulation by pro-inflammatory cytokines in H357 cells following *P. gingivalis* uptake. This assumption is based on Sandros *et al.* (2000) findings, who showed that the IL-1 $\beta$  and TNF- $\alpha$  mRNA expressions in the primary pocket epithelial cells were significantly increased following *P. gingivalis* infection for 90 minutes compared to uninfected cells. In addition, Fujioka *et al.* (2003) showed that upon treatment of HGK with IL-1 $\beta$ , ET-1

## Chapter 4 Cell response to *P. gingivalis* stimuli

mRNA expression (up to 24 hours) and protein expression (up to 48 hours) increased steadily in a time-dependent manner. In addition, TNF- $\alpha$  treatment has a similar effect on ET-1 protein expression, but ET-1 mRNA expression was highest at 1 hour then steadily decreased over the following 23 hours. Also, it has been shown that infection of epithelial cells with *P. gingivalis* induces both ET-1 and inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  or IL-8 and intercellular adhesion molecule-1 (Ansai *et al.*, 2002). Finally, stimulation of tracheal epithelial cells with pro-inflammatory cytokines, such as (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  results in upregulation of ET-1 following 24 hours stimulation (Endo *et al.*, 1992). The ET-1 expression levels seen in our study therefore may have been enhanced by stimulation by pro-inflammatory cytokines following *P. gingivalis* uptake and there is a reciprocal stimulatory effect between ET-1 and cytokine responses. This could result in establishment of a 'pro-inflammatory loop' independent of the original stimulating source (Mullol and Picado, 2000) and so if present in vivo may contribute to severe periodontal tissue destruction

Although our studies have been restricted to assessing gene expression, increased ET-1 protein levels may also be induced following increased cytokine expression and as a consequence of increased production of endothelin-converting enzyme (ECE-1) which leads to ET-1 production from its precursor, big ET-1 (Awano *et al.*, 1999). It has been shown that *P. gingivalis* PepO endopeptidase, an important peptidase required for invasion of HeLa cells by *P. gingivalis* ATCC 33277 (Ansai *et al.*, 2003), has a considerable homology with ECE-1 (Awano *et al.*, 1999), and this could provide an additional mechanism for continuous inflammatory stimulus. Therefore, it is possible with increasing time of *P. gingivalis* infection; continuous ET-1 production results from the ECE- like activity of *P. gingivalis* PepO endopeptidase, could be deleterious to periodontal health.

### **uPAR cellular response to *P. gingivalis* infection.**

In addition to ET-1, *P. gingivalis* infection leads to increased expression of urokinase plasminogen activator receptor (uPAR, CD87). uPAR is a highly glycosylated protein (55-60 kDa) linked to the cell membrane via a glucosylphosphatidylinositol (GPI) anchor (Møller, 1993). The expression of uPAR has been reported in different cell types such as neutrophils (Borregaard *et al.*, 1995), gingival fibroblasts (Ogura *et al.*, 1999), human chondrocytes (Schwab *et al.*, 2004) and several carcinoma-associated

## Chapter 4 Cell response to *P. gingivalis* stimuli

cells such as fibroblasts (Dublin *et al.*, 2000), macrophages (Pyke *et al.*, 1994), human prostate cell lines (Nalla *et al.*, 2010), gliomas and other metastatic models (Gondi *et al.*, 2004, Lakka *et al.*, 2003) and premalignant oral keratinocytes (Ghosh *et al.*, 2000, Ghosh *et al.*, 2006). uPAR plays a significant role in the activation of the plasmin activator (PA)-plasmin system, and it is considered as a dynamic regulator of the ECM degradation. Following binding with its substrates, urokinase plasminogen activator (uPA) and pro-uPAR, conversion of the zymogen plasminogen to the active protease plasmin occurs (Smith and Marshall, 2010). Plasmin then reciprocally cleaves and converts pro-uPA into active uPA in a positive feedback manner (Ogura *et al.*, 1999). The PA-plasmin system plays a significant role in the extra-vascular biological processes such as metastasis (Sumiyoshi *et al.*, 1992), wound healing (Schäfer *et al.*, 1994), inflammatory cell chemotaxis (Gyetko *et al.*, 1994), angiogenesis (Barnathan *et al.*, 1990), pro-enzyme activation (Thomson *et al.*, 1989), and degradation of several components of ECM (Alexander and Werb, 1991). It has been proposed that the continuous activation of the plasmin system has a significant role in ECM degradation either directly or by activation of matrix metalloproteases (MMPs), such as MMP3, MMP9, MMP12, and MMP13 (Carmeliet *et al.*, 1997, Møller, 1993, Smith and Marshall, 2010, Thomson *et al.*, 1989).

Despite the link between uPAR expression and tissue destruction, there are few data regarding its expression in response to microbial challenge. Here we show that there was a significant increase in uPAR expression upon infection of H357 cells with *P. gingivalis* (section 4.3.3.2), compared to control cells. This increase in uPAR expression could be attributed to the action of the *P. gingivalis* LPS (Ogura *et al.*, 1999) or as a result of the other virulence factors as we have used the whole bacteria in these experiments. This is supported by Grenier (1996), who showed that the strongest plasminogen activator/plasminogen activation response followed exposure to *P. gingivalis* protease (80 kDa protease) compared to other bacteria such as *A. actinomycetemcomitans* and *F. nucleatum*. In addition it has been reported that *P. gingivalis* is capable of suppressing anti-plasmin activity resulting in uncontrolled plasmin activity and massive ECM degradation, which provides an effective mechanism to promote pericellular degradation and bacterial invasion (Grenier, 1996). It has also been shown that *P. gingivalis* gingipain acts adjacent to the uPA-uPAR complex on the cell surface specifically through binding of Lysine-specific proteinase

## Chapter 4 Cell response to *P. gingivalis* stimuli

to vitronectin which is considered a ligand for uPAR (Mcalister *et al.*, 2009, Singh *et al.*, 2010). This offers a further mechanism for uPAR expression, activation and signalling with its substrate, uPA. Moreover, uPA/uPAR signalling has been found to promote osteoclastogenesis (Anaraki *et al.*, 2015), suggesting a possible role for uPAR in gingipain-induced alveolar bone loss observed in periodontitis.

As well as protease-driven activation a range of other stimuli that have been shown to induce the expression of the uPA/uPAR complex on the cell surface such as proinflammatory cytokines (Bechtel *et al.*, 1996, Hamilton *et al.*, 1991, Hasegawa *et al.*, 1997, Mochan *et al.*, 1988, Schwab *et al.*, 2004, Shetty *et al.*, 1996, Sirén *et al.*, 1999, Tewari *et al.*, 1994 Wu *et al.*, 2002).

Besides of all the above reported pathways of inducing uPAR expression, a significant body of evidence exists showing that uPAR has a close association and can form a stable complex with integrins on the cell surface. As uPAR is a GPI-anchored receptor which lacks an intracellular and transmembrane domain, uPAR must associate with other transmembrane receptors such as integrins to initiate various intracellular signalling pathways (Chaurasia *et al.*, 2006, Madsen *et al.*, 2007, Smith *et al.*, 2008, Wei *et al.*, 1996, Zhang *et al.*, 2003). Of the integrin family,  $\alpha 5\beta 1$  (Wei *et al.*, 2005) and  $\beta 1$  integrin (Aguirre, 2002, Chaurasia *et al.*, 2006, Liu *et al.*, 2002) have been reported to stimulate various uPAR-mediated intracellular signalling cascades, such as FAK and ERK activation, and acts as part of a feed-back loop activating  $\alpha 5\beta 1$  integrin functionality (Aguirre-Ghiso *et al.*, 2001, Ghiso *et al.*, 1999, Monaghan-Benson and Mckeown-Longo, 2006, Monaghan *et al.*, 2004, Wei *et al.*, 2005).

Thus, the increased level of uPAR within H357 may enhance the invasion efficiency of *P. gingivalis* through an activation of  $\alpha 5\beta 1$  and CR3 integrins on the surface of H357 cells. It is possible that clustering of uPA, uPAR and  $\alpha 5\beta 1$  integrin on the cell surface may activate the integrin, resulting in enhanced bacterial binding, induction of intracellular signalling pathways and cytoskeletal rearrangement leading to *P. gingivalis* uptake (Yilmaz *et al.*, 2002, Yilmaz *et al.*, 2003). Therefore, the highest *P. gingivalis* invasion obtained after 240 minutes incubation (section 3.3.1), could be partly related to the significant increase in uPAR expression (9 fold) which in turn activates more  $\alpha 5\beta 1$  and CR3 integrins to be exploited by *P. gingivalis* for binding and

## Chapter 4 Cell response to *P. gingivalis* stimuli

subsequent invasion. However, the significant difference with higher *P. gingivalis* invasion at 90 minutes (3.83%) compared to 30 minutes (0.33%) cannot be mediated by uPAR as the mRNA expression was only slightly increased at 90 minutes, suggesting involvement of other mechanisms such as  $\alpha 5\beta 1$  integrin-mediated effect, TLR-2-induced activation of CR3, lipid raft effect, and clathrin mediated endocytosis.

From our findings regarding ET-1 and uPAR gene expression, it is also possible that the cells may require time for the transcription machinery to express these genes when targeted by *P. gingivalis*. This is clearly observed when the maximum gene expression was reported following infection of H357 cells with *P. gingivalis* for 240 minutes compared to 30, 90, and 150 minutes.

uPAR could be used as a marker reflecting the severity of periodontitis to aid in the diagnosis and treatment of periodontal diseases. This assumption is based on previous studies showing inhibition of uPAR signaling through targeting uPAR-vitronectin interaction (Kjøller and Hall, 2001, Madsen *et al.*, 2007, Smith *et al.*, 2008, Wei *et al.*, 2008) and uPAR function in periodontal disease through its effects on integrin signaling. This may lead to reduced *P. gingivalis* adherence and invasion, and also decreased cellular proteolysis and ECM degradation.

### **Heterogeneous pattern of *P. gingivalis*-cell association in vitro**

To answer the question whether there is variation in the susceptibility of H357 cells to *P. gingivalis* invasion at any one time, direct immunocytochemical analysis of *P. gingivalis* cells inside H357 cells was investigated (section 2.23.1). The concept of cell heterogeneity in respect to cell subpopulation responses to various stimuli or different phenotypic and functional behaviour has been reported previously. For example, heterogeneity of neutrophil subsets which show different physical characteristics (Eggleton *et al.*, 1995), phenotypic characteristics (Fortunati *et al.*, 2009, Orr *et al.*, 2005), and functional properties (Fridlender *et al.*, 2009, Pillay *et al.*, 2012, Pillay *et al.*, 2010) to various stimuli was reported. This was supported by the findings of Elbim *et al.* (1993); who showed that following TNF priming, only half the neutrophils showed marked actin polymerization, H<sub>2</sub>O<sub>2</sub> production, and binding with biotinylated formyl-peptide. Other examples include the development of experimental cerebral malaria by emergence of a particular neutrophil subset with IgE receptors

## Chapter 4 Cell response to *P. gingivalis* stimuli

(Porcherie *et al.*, 2011), and the discovery of several subpopulations of bovine mammary gland phagocytes with varying phagocytic capability (Rivas *et al.*, 2002).

There is a significant body of work regarding fibroblast heterogeneity in the last decades. For example, several studies have reported phenotypic heterogeneity within the same tissue, whether healthy or diseased (Bertolami and Bronson, 1990, Bronson *et al.*, 1989, Hakkinen and Larjava, 1992, Harper, 1989, Vande *et al.*, 1989), and Irwin *et al.* (1994) provided evidence for intra and inter-site heterogeneity of fibroblast cells.

Keratinocyte heterogeneity has been the subject of numerous studies and has showed physical, phenotypic, and functional variations between different keratinocyte subpopulations. For instance, Micallef *et al.* (2010) showed that the most differentiated keratinocyte subpopulations had a reduced proliferative activity, and Izumi *et al.* (2007) showed the presence of a small oral keratinocyte subpopulation which contains a high percentage of progenitor/stem cells. However, whether such heterogeneity exists within cultures of H357 oral keratinocytes is not known. As shown in section 4.3.4, our results showed that certain H357 cells were more susceptible to *P. gingivalis* invasion than others. In addition, the results showed that the cell-associated *P. gingivalis* were extensively located in the peri-nuclear region of the cells. This is consistent with Belton *et al.* (1999), who showed similar association of *P. gingivalis* with the peri-nuclear area, and proposed that the peri-nuclear area of the oral keratinocytes contains several organelles which can be exploited by the invading *P. gingivalis* (e.g. mitochondria, endoplasmic reticulum, endosomes and bundles of keratin fibres). A similar phenomenon has been reported for other bacterial species too. Huang *et al.* (1998b), who showed that *S. typhi* internalization into human intestinal epithelial cells occurred in clusters of one to two bacteria per uptake event with subsequent bacterial engulfment occurring at the same entry point, suggesting that the entry points for *S. typhi* might be limited in number. This might partly explain our findings in that primary *P. gingivalis* invaders could facilitate the entry of other *P. gingivalis* bacteria through the same entry point, resulting in several *P. gingivalis* bacteria being observed within single H357 cells. As a further factor, it has been shown that heterogeneity also exists in a given population of *P. gingivlals*, with a highly cell invasive subset showing minimal protease activity compared to the lower invasive subset (Suwannakul *et al.*, 2010).

## Chapter 4 Cell response to *P. gingivalis* stimuli

However, the unequal pattern of *P. gingivalis* association with H357 cells observed in this study is contrary to the findings of Belton *et al.* (1999), who showed that upon infecting primary gingival epithelial cells (GECs) with *P. gingivalis*, over 90% of these cells were susceptible to *P. gingivalis* invasion to a similar degree, suggesting homogeneity of behavior was present among those cells. Such differences may be related to the functional heterogeneity and/or distribution of the receptors involved in *P. gingivalis* invasion within the H357 cell line compared to the primary GECs.

It is not well known whether cell surface expression of receptors varies within a population of host cells at any one time. If this heterogeneity of receptor expression exists, it would have a considerable impact on bacterial uptake such as *P. gingivalis* uptake through  $\alpha 5\beta 1$  integrin interaction (Tsuda *et al.*, 2008, Yilmaz *et al.*, 2008). However, we hypothesised that this heterogeneity in the susceptibility of H357 cells to *P. gingivalis* invasion at any one time might be related to the cell cycle phase that those cells are in. Therefore, the next chapter will discuss in detail the cell cycle characteristics of H357 cells in respect to *P. gingivalis* invasion

### 4.5 CONCLUSION

The data presented here in this chapter demonstrate that the cellular response to bacterial stimuli, such as *P. gingivalis* LPS did not significantly affect *P. gingivalis* invasion. The effect of invasion using cells in suspension was dependent on the method of cell lysis with saponin showing an increase whilst reduced invasion was seen when distilled water was used. This suggests that the availability and functionality of the receptors involved in *P. gingivalis* invasion, such as  $\alpha 5\beta 1$  integrin, may be influenced by the physical state of the cells, which could in turn be influenced by the type of cell lysis buffer employed. Incubating H357 cells with *P. gingivalis* induced significant increase in ET-1 and uPAR mRNA expression in a time dependent manner and this may occur as a direct response or through pro-inflammatory cytokine activation or  $\alpha 5\beta 1$  integrin cross talk. All of these cellular responses might occur in an unequal manner in response to the external stimuli and might explain the observed heterogeneity of invasion by *P. gingivalis* within a cell population. A better understanding of the cell response to the *P. gingivalis* challenge will augment our understanding of why some cells are more susceptible to the bacterial invasion than others at any one time.

**Chapter 5**  
***P. gingivalis*-cell cycle**  
**interaction.**



## 5.1 INTRODUCTION

*P. gingivalis* represents one of the key periodontal pathogens which are capable of targeting and invading gingival epithelial cells, residing in the cytoplasm and replicating and spreading to the neighbouring cells (Yilmaz *et al.*, 2006). However, within a given population, epithelial cells are not equally invaded, suggesting that there are differences in the susceptibility of cells to uptake of *P. gingivalis*, although the reason for this is unclear.

The host-bacterium interactions have a significant impact for bacterial establishment and survival at a site of infection. Several host cell receptors are shown to mediate cell-bacterial interaction, resulting in an intimate bacterial adherence and invasion into host cells (Chen *et al.*, 2001).  $\alpha 5\beta 1$  integrin is well documented example of a host cell receptor that mediates binding of *P. gingivalis* major fimbriae, resulting in stimulation of intra-cellular signalling transduction pathways with subsequent cytoskeletal rearrangement prior to *P. gingivalis* engulfment (Yilmaz *et al.*, 2002). On the cell membrane, urokinase plasminogen activator receptor (uPAR), an important part of plasmin system, has been thought to work in close association with  $\alpha 5\beta 1$  integrin, regulating its function (Wei *et al.*, 2005). In addition, high expression of uPAR on the surface of gingival fibroblasts has been reported following *P. gingivalis* infection (Ogura *et al.*, 1999). *P. gingivalis*-cell interactions may disrupt cell integrity and impair immune surveillance through inducing/inhibiting cell cycle progression and apoptosis (Inaba *et al.*, 2009, Pischon *et al.*, 2009).

The mammalian cell cycle is a conserved process, which is precisely controlled and regulated by a complex of interconnected pathways that ensures each step progresses in the proper sequence and time (Meloche and Pouyssegur, 2007, Nakayama and Nakayama, 2006). This dynamic process consists of two main cycles. The chromosome cycle, in which the replication of the genomic component takes place during S-phase, is followed by segregation of copied chromosomes to the daughter cells during M-phase. Secondly, the growth cycle, which consists of gap phases that connects M-phase to the beginning of S-phase through G1-phase, and the gap which connects S and M phases, known as G2-phase (Shaw *et al.*, 2010, Sherr, 1994, Van Den Heuvel, 2005). The transition from G1-phase until the accomplishment of cell mitosis and cytokinesis is

controlled through the interaction of cyclins and their dependent kinases (CDKs) (Pan *et al.*, 2014). Recently, it has been reported that the cell cycle of gingival epithelial cells (Kuboniwa *et al.*, 2008) and the proliferation of immortalized human gingival epithelial cells (Pan *et al.*, 2014) were greatly accelerated following infection with *P. gingivalis*. However, the influence of the host cell cycle on *P. gingivalis* interactions has not been previously studied. The previous chapter showed that there is heterogeneity in the susceptibility of H357 cells to invasion by *P. gingivalis* in a given population, with some cells being highly invaded. We hypothesise that at any one time within a culture, cells will be at different phases of the cell cycle and that this may influence their ability to be targeted by *P. gingivalis*. This chapter explores the cell cycle of H357 cells in relation to *P. gingivalis* interactions.

### **The aims of this part of the study were:**

- 1- To investigate the cell cycle profile of H357 cells following different times of serum synchronization.
- 2- To determine the effect of cell cycle synchronization on *P. gingivalis* invasion and adhesion.
- 3- To study the co-localisation of *P. gingivalis* with cells in S-phase.
- 4- To characterize the molecular mechanisms thought to mediate *P. gingivalis* invasion by comparing the mRNA and surface expression of  $\alpha 5$ -integrin and uPAR between serum synchronized and non-synchronized cells.

## 5.2 METHODS

The following methods were used in this chapter:

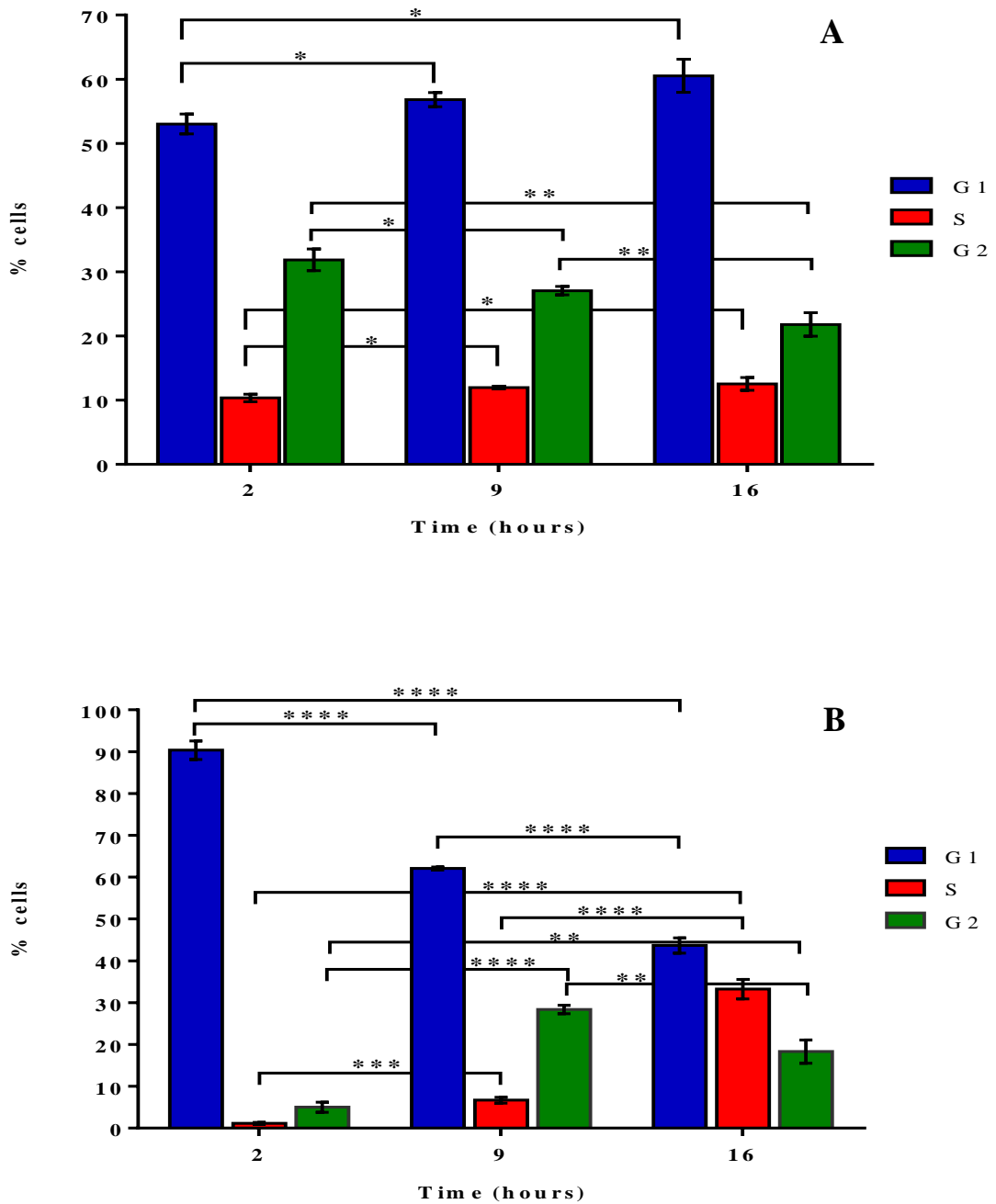
- Epithelial cell culture (section 2.2)
- *P. gingivalis* culture (section 2.3)
- Antibiotic protection assay (section 2.4)
- Cell cycle characterization of H357 oral keratinocytes by flow cytometry (section 2.18)
- Immunofluorescence of serum synchronized cells using 5-bromo-2-deoxyuridine (BrdU) staining (section 2.22)
- Effect of cell cycle phases on the bacterial association (section 2.19).
- Effect of cell cycle synchronization on the *P. gingivalis* invasion efficiency (section 2.20)
- Effect of serum synchronization on cell surface proteins:
  - Effect of serum synchronization on cellular gene expression (section 2.21.1).
  - Effect of serum synchronization on cell surface protein expression (section 2.21.2).
- Double immunofluorescence of oral keratinocytes and invasive *P. gingivalis* (section 2.23.2).

## 5.3 RESULTS

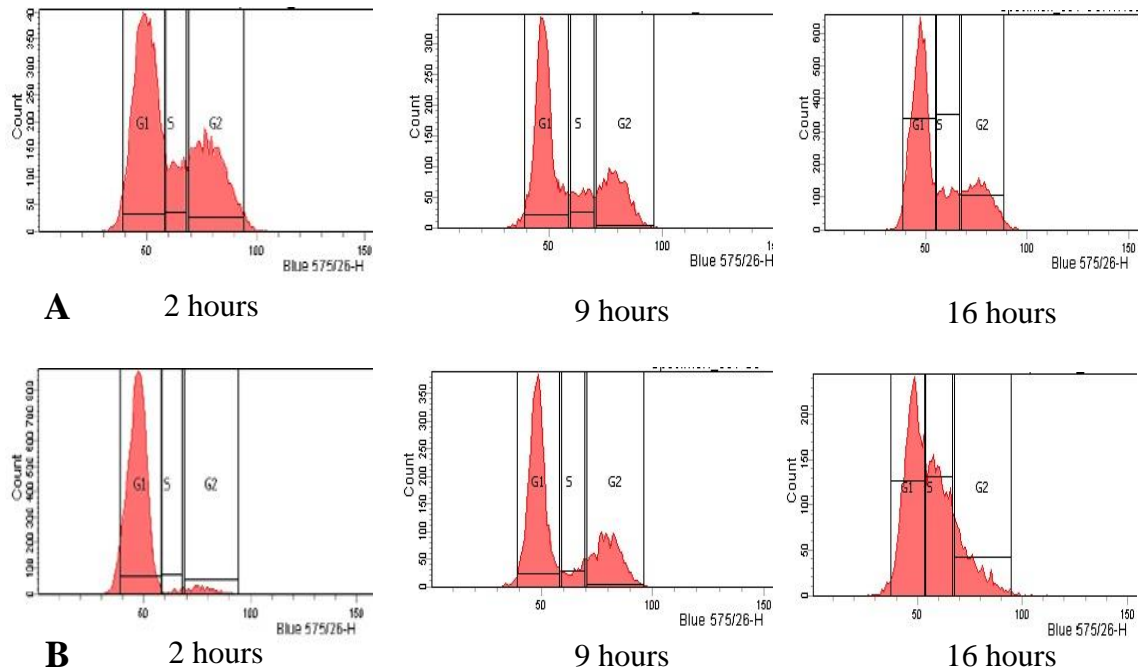
### 5.3.1 Cell cycle characterization of H357 oral keratinocytes by flow cytometry and immunofluorescence techniques.

#### *Flow cytometry.*

Cell cycle analysis was performed using propidium iodide (PI) DNA staining and flow cytometry following serum starvation to synchronize cells at G<sub>0</sub>-phase, then serum was re-introduced to the cells for a period of 2, 9, and 16 hours. The number of cells in G<sub>1</sub>, S, and G<sub>2</sub>-phases was based on the DNA content which is stained by propidium iodide. The serum synchronized cells were compared with unsynchronised control cells. We observed, as shown in figures 5.1.A, 5.2.A, that the percentage of control cells at G<sub>1</sub>-phase was slightly increased with increasing time of cell incubation with KGM to have 53%±1.5, 56.8%±1.1, and 60.5%±2.5 at 2, 9, and 16 hours of cell incubation respectively. In a similar way, percentage of cells in S-phase was slightly increased from 10.3%±0.5 at 2 hours, 11.9%±0.2 at 9 hours and 12.5%±0.9 at 16 hours. The percentage of cells at G<sub>2</sub>-phase was slightly decreased with increasing time of incubation being 31.8%±1.7, 27%±0.6, and 22.3%±1.5 at 2, 9, and 16 hours respectively (fig. 5.1.A). All of these results were compared with cells which underwent serum starvation and re-supplemented with serum at 2, 9, and 16 hours. Interestingly, we demonstrated, as shown in figure 5.1.B, 5.2.B, that the highest number of cells in G<sub>1</sub>-phase was shown after 2 hours of serum re-supplementation (90.3%±2.2), and this was significantly reduced ( $P \leq 0.0001$ ) with increasing time of serum incubation to 62%±0.7 and 43.6%±1.8 at 9 and 16 hours of re-introducing serum, respectively. Conversely, cells in S-phase showed a significant increase ( $P \leq 0.001$ ,  $P \leq 0.0001$ ) with increasing time of serum re-introduction from 1.13%±0.2 to 6.7%±1, and 33.2%±2.3 at 2, 9, and 16 hours, respectively. Serum synchronized cells in G<sub>2</sub>-phase showed fluctuant increase and decrease ( $P \leq 0.01$ ,  $P \leq 0.0001$ ) in proportion being 5%±1.2, 28.3%±1, and 18.3%±2.7 at 2, 9, and 16 hours respectively (figure 5.1.B).



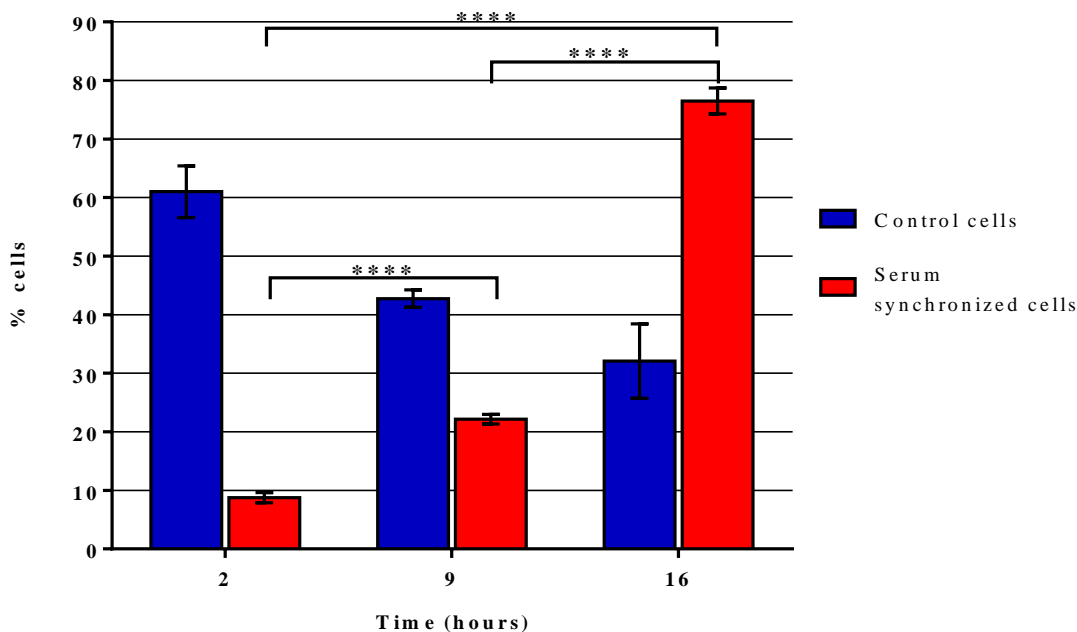
**Figure 5.1 Cell cycle analysis of H357 oral keratinocytes** following 2, 9, and 16 hours of re-introducing serum (**B**), compared to the control cells which did not undergo serum replacement (**A**). Cells were serum starved for 24 hours, followed by re-introduction of serum for 2, 9 and 16 hours. Cells were washed, trypsinised, fixed with 70% ethanol (v/v), and incubated with PI and RNase. Cells were analysed using LSRII flow cytometer with BD FACS DIVA software. Cell percentage is expressed as mean±SD of at least 3 independent experiments. \*, \*\*, \*\*\*, \*\*\*\* Indicate statistically significant differences at ( $P \leq 0.05$ ), ( $P \leq 0.01$ ), ( $P \leq 0.001$ ), and ( $P \leq 0.0001$ ) respectively.



**Figure 5.2 DNA content of control (A) and serum synchronized (B) H357 oral keratinocytes** divided into G1, S, G2-phase peaks. Cells were serum starved for 24 hours, followed by re-introduction of serum for 2, 9 and 16 hours. Cells were washed, trypsinised, fixed with 70% ethanol (v/v), and incubated with PI and RNase. Cells were analysed using LSRII flow cytometer with BD FACS DIVA software. Gates were drawn on each graph, and the cell number was automatically counted. The percentage of cells were calculated as mean $\pm$ SD in triplicate at least 3 independent experiments.

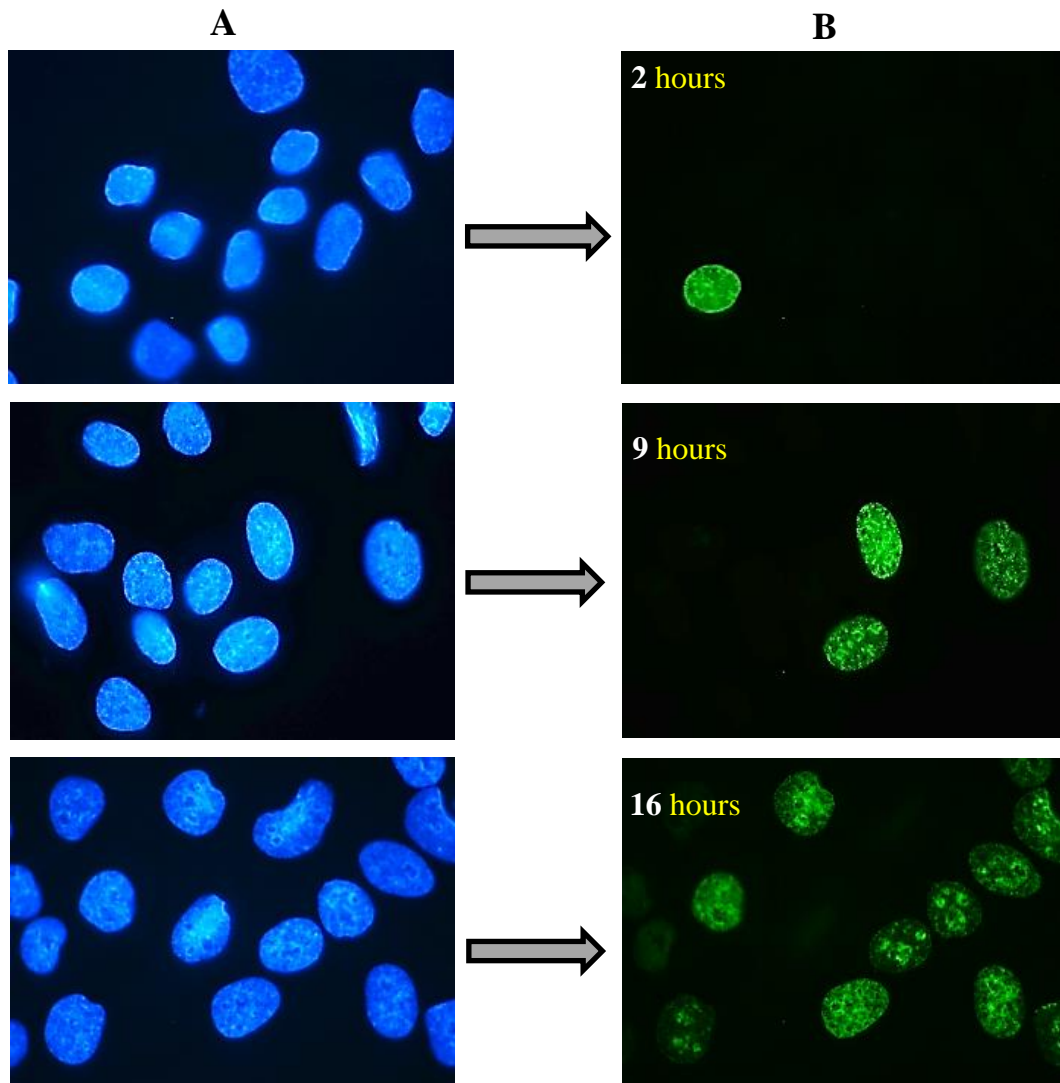
***Immunofluorescence.***

The percentage of cells in S-phase in particular was investigated using BrdU staining, which acts as thymidine analogue and is incorporated within the DNA structure during replication. Thus, this technique distinguishes cells in S-phase from the non-BrdU incorporated cells which could be in early G1 or G2/M-phase. The percentage of cells in S-phase was calculated by dividing the number of cells stained with BrdU by the total number of cells stained by 4', 6-diamidino-2-phenylindole (DAPI) multiplied by 100. Our results show, as shown in figure 5.3, that the percentage of BrdU-positive cells was highly significantly increased with increasing time of re-introducing serum (2 hours:  $8.7\% \pm 0.8$ , 9 hours:  $22.1\% \pm 0.8$ , 16 hours:  $76.5\% \pm 2.2$ ). This was accompanied by a gradual decrease in the percentage of BrdU-positive cells in unsynchronized (control) samples with increasing time of KGM incubation (2 hours:  $61\% \pm 4.4$ , 9 hours:  $42.7\% \pm 1.4$ , 16 hours:  $32\% \pm 6.3$ ). Interestingly, our results showed a high degree of consistency with this technique and the data obtained using flow cytometry regarding the pattern of increasing percentage of cells in S-phase in synchronized samples. However, the immunofluorescence method showed a relatively higher number of cells at each time point compared to the flow cytometry method, this could be attributed to the differences in the quantification between both methods. In flow cytometry, the percentage of cells is expressed following automated counting by the Diva software based on gates drawn between the cell cycle phases, while the percentage of these cells incorporating BrdU was calculated visually in relation to the total number of cells stained by DAPI as indicated in figure 5.4.



**Figure 5.3 Histogram showing the percentage of BrdU-positive cells in synchronized versus unsynchronized samples measured using immunofluorescence.** Cells were seeded on coverslips at  $5 \times 10^4$ /well for 24 hours, followed by serum starvation for 24 hours, and re-introducing serum for 2, 9 and 16 hours. Cells were then incubated with BrdU labelling solution, fixed with 4% formaldehyde (v/v), washed and permeabilized with 0.1% Triton® X-100 (v/v). Cells were then treated sequentially for 10 minutes with 1M HCl, 2M HCL, and phosphate/citric acid, washed with 0.1% Triton (v/v), and incubated with primary mouse monoclonal BrdU antibody. Cells were then washed with 0.1% Triton (v/v), incubated with goat anti-mouse IgG polyclonal secondary antibody, mounted with anti-fade DAPI mountant on microscope slides, and visualized by fluorescence microscopy. The percentage of cells in S-phase (BrdU+ve cells) was calculated by dividing the number of cells stained with BrdU by the total number of cells stained by DAPI. Cell percentage is expressed as mean $\pm$ SD of at least 3 independent experiments. \*\*\*\*, indicate statistically significant differences at  $P \leq 0.0001$ .

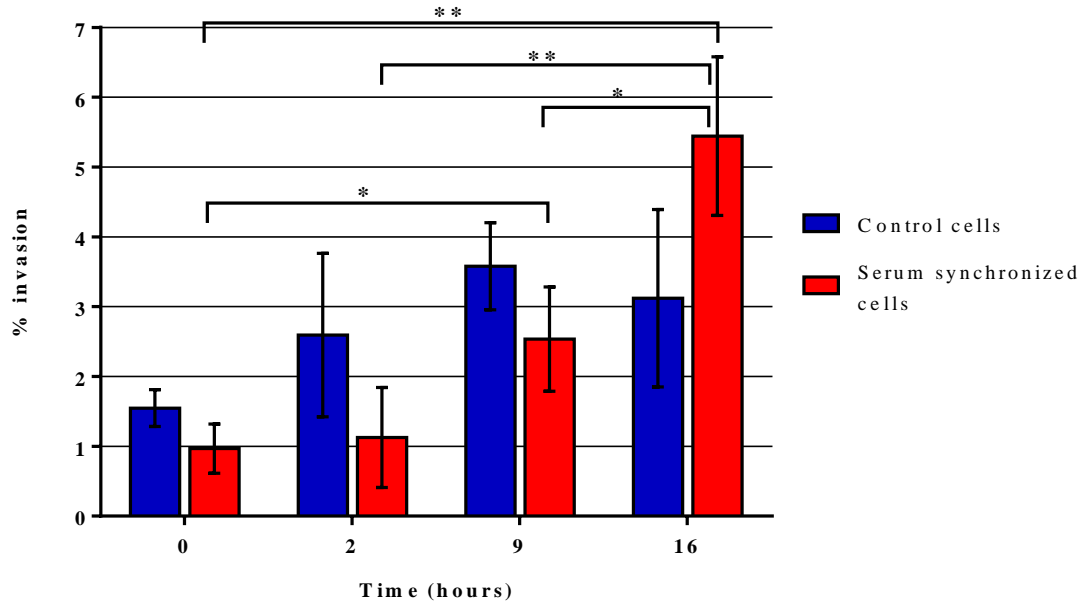




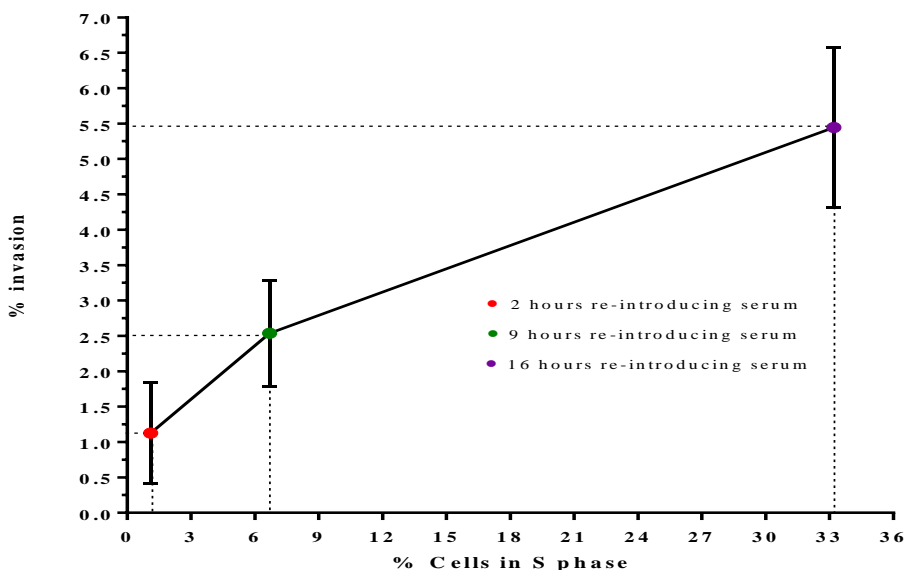
**Figure 5.4 Immunofluorescence images of H357 cells stained with DAPI (A) (total cells) and their correspondent BrdU content (B) (S-phase cells).** H357 cells were treated with BrdU as described in section 2.22. Cells were incubated with primary mouse monoclonal antibody MoBU-1, followed by goat anti-mouse IgG polyclonal secondary antibody (Alexa Fluor® 488). The total number of cells were stained with DAPI stain included within the anti-fade mountant, and visualized through the DAPI fluorescence channel (A), while the BrdU cells were quantified by switching to the fluorescein 5(6)-isothiocyanate (FITC) channel within the fluorescence microscope (B). For each fluorescent channel, 10 fields were captured with x100 magnification, analysed and selected from 3 individual experiments.

### 5.3.2 Effect of cell cycle synchronization on *P. gingivalis* invasion efficiency.

To investigate the effect of serum synchronization on the level of *P. gingivalis* invasion compared to unsynchronized cells, the antibiotic protection assay was performed following 0, 2, 9, and 16 hours of re-introducing serum, and the viable counting was performed to show the percentage of the invaded bacteria as described in section 2.4. Figure 5.5 shows that the percentage of invaded *P. gingivalis* was significantly increased following 9 hours of serum synchronization ( $2.5\% \pm 0.7$ ) ( $p \leq 0.05$ ), and reached the highest level of  $5.4\% \pm 1.1$  ( $p \leq 0.01$ ) after 16 hours of re-introducing serum compared to the relatively similar lower invasion percentage at time zero ( $0.9\% \pm 0.3$ ), and 2 hours ( $1.1\% \pm 0.7$ ). This indicates that the bacterial invasion was closely correlated to the time of serum incubation, which could be due to the higher number of cells in S-phase as shown in figure 5.1.B. When the level of *P. gingivalis* invasion was plotted against the percentage of cells in S-phase following 2, 9, and 16 hours of refeeding H357 cells with serum, a strong positive correlation was observed ( $R=0.98$ ) as shown in figure 5.6, suggesting cells with S-phase of the cell cycle could be more vulnerable to *P. gingivalis* invasion than other phases of the cell cycle.



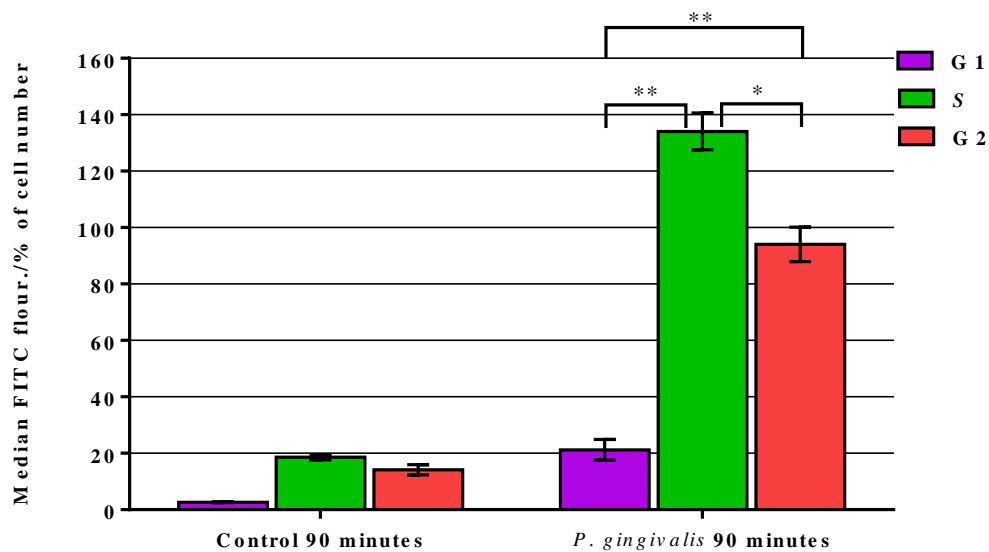
**Figure 5.5** The percentage of *P. gingivalis* invasion in serum synchronized H357 cells compared to control cells. H357 cells were serum starved for 24 hours and serum re-introduced with serum for 0, 2, 9, and 16 hours. Cells were then washed with PBS and infected with *P. gingivalis* NCTC 11834 for 90 minutes at a MOI 100:1. Cells were treated with metronidazole for 1 hour to kill the adherent bacteria, and then lysed with distilled water for 30 minutes to release the invaded bacteria. The lysate was serially diluted and inoculated in duplicate on FA blood agar. Viable counting was performed following 5 days of incubation under anaerobic conditions. Graphs show mean $\pm$ SD of 3 independent experiments performed in triplicate. \*, \*\* Indicate statistically significant differences at ( $P \leq 0.05$ ) and ( $P \leq 0.01$ ) respectively.



**Figure 5.6** Correlation graph showing the percentage of *P. gingivalis* invasion in serum synchronized cells versus the percentage of cells in S-phase. Graphs show mean $\pm$ SD of 3 independent experiments performed in triplicate (R=0.98).

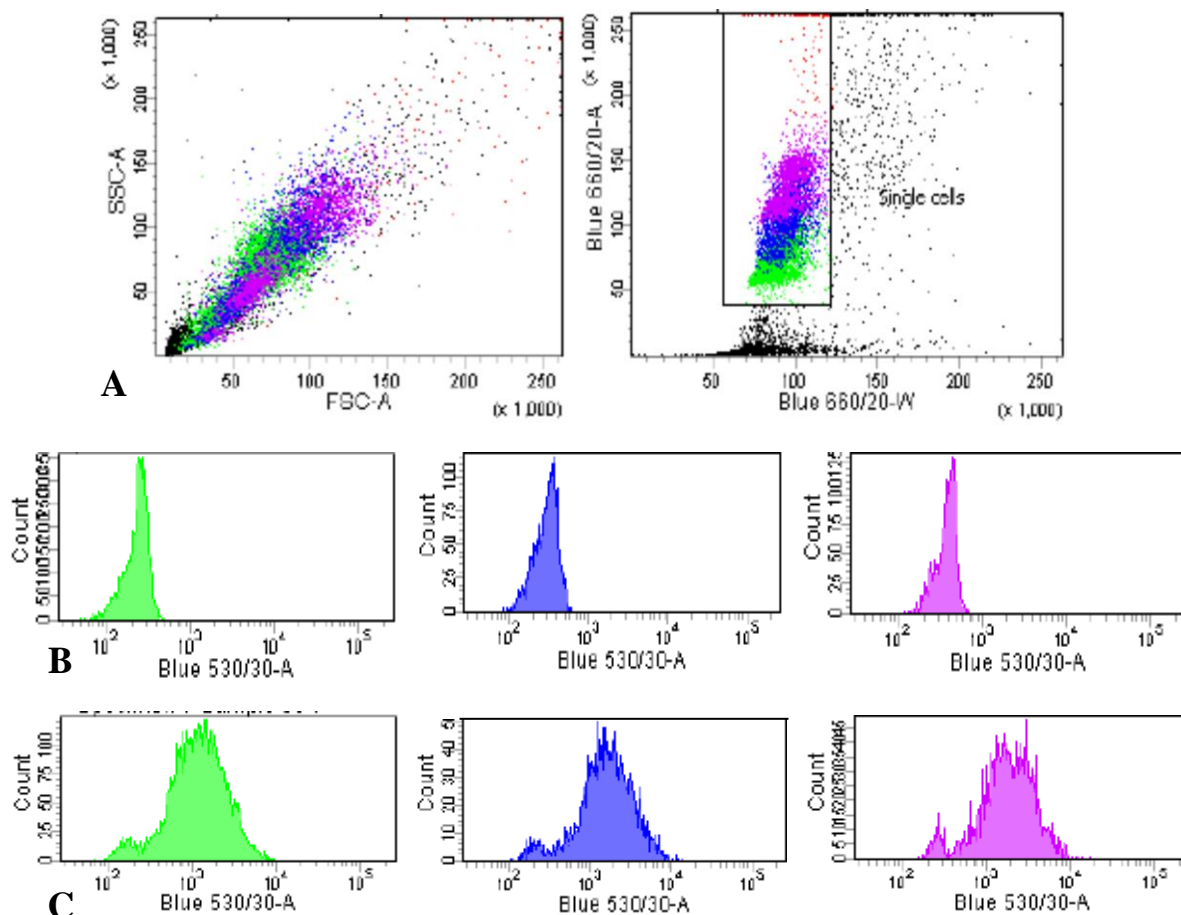
### 5.3.3 Effect of cell cycle phases on the bacterial association.

To explore whether bacteria preferentially adhere to H357 cells in any particular phase of the cell cycle rather than specifically invade, cells were infected with FITC labelled *P. gingivalis* for 90 minutes, stained with PI, and analysed by flow cytometry to investigate the percentage of cells in each phase and the number of these associated with bacteria. The fluorescent expression index is calculated as the percentage of the median fluorescence in each cell cycle phase determined using PI. Our results showed that the highest *P. gingivalis* association was observed in S-phase ( $134 \pm 6.5$ ), followed by G2-phase ( $94 \pm 6$ ), and the lowest association was found in G1-phase ( $21.2 \pm 3.68$ ) (figure 5.7) with significant differences between all phases. This indicates that the time of DNA replication may be a favorable point for *P. gingivalis* interaction with host cells. This supports our findings regarding the correlation of *P. gingivalis* invasion with cells in S-phase. In comparison, the control uninfected cells showed a much reduced and similar pattern of fluorescence expression at all phases of the cell cycle, with cells in S-phase ( $18.6 \pm 0.8$ ), followed by G2-phase ( $14.1 \pm 1.7$ ), and the lowest association was found around G1-phase ( $2.6 \pm 0.1$ ).



**Figure 5.7 Effect of cell cycle on *P. gingivialis* association.** H357 cells were infected with FITC labelled *P. gingivalis* for 90 minutes, fixed with ethanol, and treated with PI+RNase prior to cell cycle analysis by flow cytometry. The fluorescent expression index was calculated as a percentage of the median fluorescence in each cell cycle determined phase using PI. Data are shown as mean $\pm$ SD of at least 3 independent experiments. \*, \*\* Indicate statistically significant differences at ( $P\leq 0.05$ ) and ( $P\leq 0.01$ ) respectively.

Data presented in figure 5.8.A show that there are 3 populations of H357 cells represented by cells in G1 (green), cells in S-phase (blue), and cells in G2 (purple). The fluorescence expression in each cell cycle phases is clearly shifted and increased when cells interact with *P. gingivalis* (figure 5.8.C) compared to the relatively low fluorescence expression in control cells without infection (figure 5.8.B).



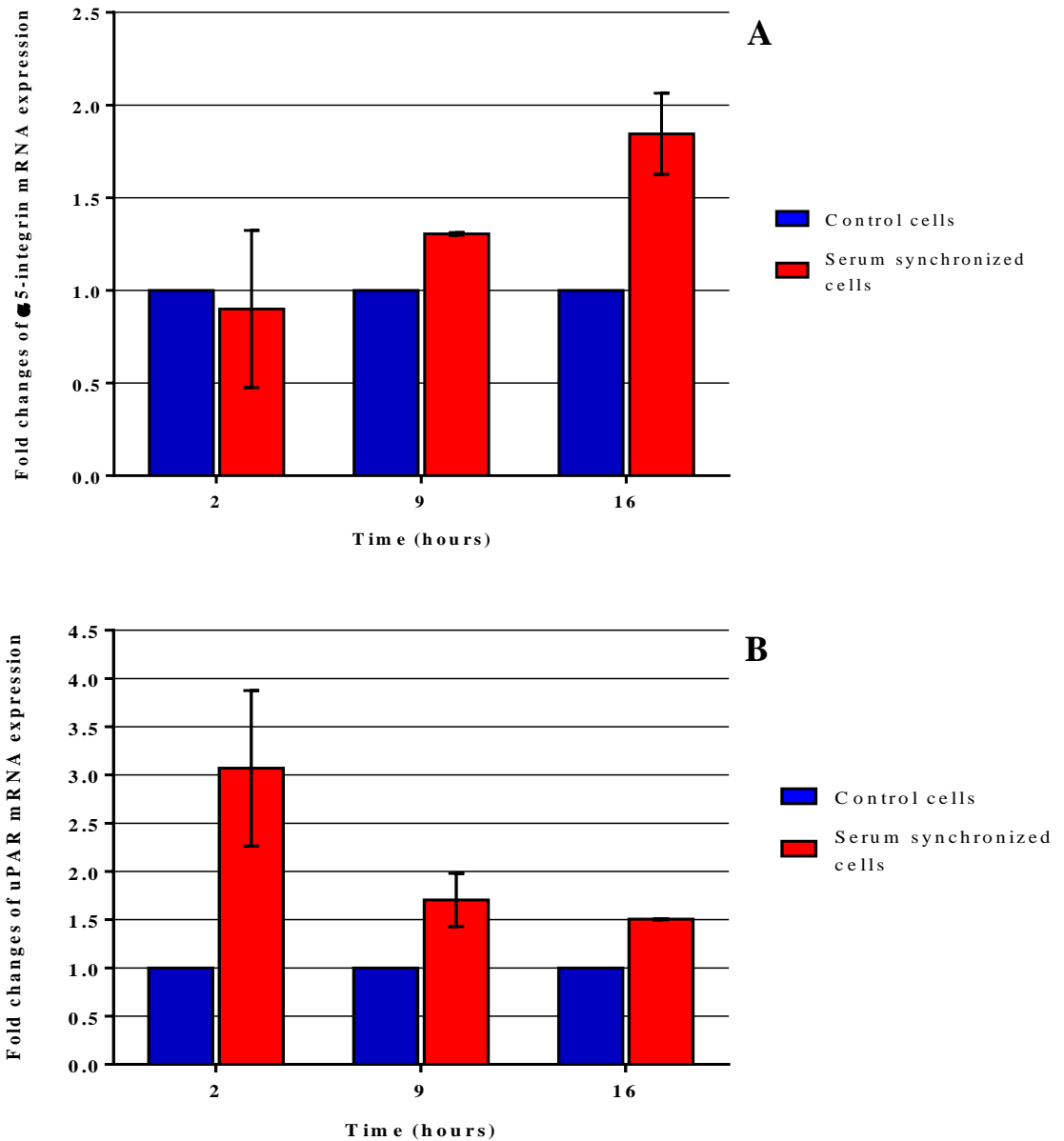
**Figure 5.8** Flow cytometry analysis of control and *P. gingivalis*-associated H357 cells. (A) Representative dot plots of H357 cells infected with FITC labelled *P. gingivalis* for 90 minutes. Cells were washed, fixed with ethanol, and treated with PI+RNase prior to cell cycle analysis by flow cytometry. The forward scatter (FSC)/side scatter (SSC) shows populations of cells in G1, S, and G2-phases. These were gated out for the analysis with the PI detector (blue 660/20). (B) The histograms represent the fluorescence expression from G1 (green) to S (blue) to G2 (purple) within control H357 cells without *P. gingivalis* infection. (C) The histograms show a significant increase of the fluorescence expression in all populations of the *P. gingivalis*-associated H357 cells within their correspondent G1, S, and G2-phases of the cell cycle.

**5.3.4 Effect of serum synchronization on the H357 oral keratinocyte-protein expression.**

To determine the possible mechanisms underlying the increased association of *P. gingivalis* with cells in S-phase, we investigated the gene and cell surface protein expression levels of two potential host mediators of this interaction,  $\alpha 5$ -integrin and uPAR following 2, 9, 16 hours of re-introducing serum to H357 cells compared to control cells.

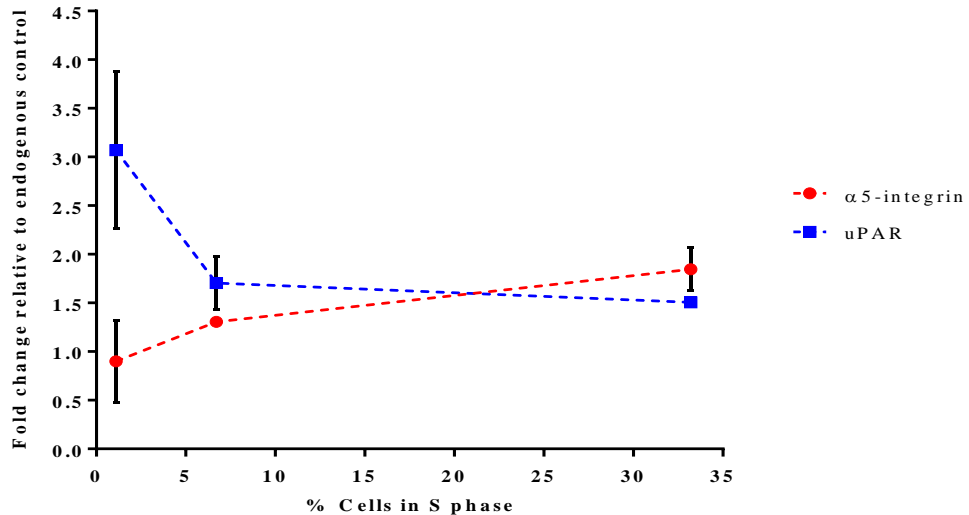
**5.3.4. 1 Effect of serum synchronization on the cellular  $\alpha 5$  integrin and uPAR mRNA expression.**

Our results showed that the mRNA expression level of  $\alpha 5$ -integrin was similar between serum synchronized and control cells after 2 hours of re-introducing serum (figure 5.9 A). This gradually increased, however by approximately 1.5 fold at 9 hours; and 2 fold at 16 hours of serum incubation compared to the control cells although the difference was not significant. The pattern of uPAR mRNA expression was gradually decreased with time of serum synchronization, from 3 fold at 2 hours, 1.7 fold at 9 hours, and 1.5 fold at 16 hours, compared to control cells (figure 5.9 B) although again these differences were not statistically significant. A correlation graph showed that there was a strong positive correlation of  $\alpha 5$ -integrin mRNA expression and the percentage of cells in S-phase ( $R=0.96$ ), while the opposite negative correlation was found regarding uPAR gene expression ( $R=-0.7$ ) (figure 5.10).



**Figure 5.9 mRNA expression of  $\alpha 5$ -integrin and uPAR in serum synchronized and control H357 cells.** H357 cells were serum starved for 24 hours and serum re-introduced for 2, 9, and 16 hours. The cells were washed, trypsinized, centrifuged and the cell pellet was lysed. RNA extracted, complementary DNA was synthesised and the quantification of  $\alpha 5$  integrin and uPAR gene expression was performed using qPCR with  $\alpha 5$ -integrin and uPAR specific primers. This histogram shows the mean fold change in  $\alpha 5$ -integrin (**A**) and uPAR (**B**) expression within serum synchronized H357 cells relative to the unsynchronized control cells  $\pm$ SD of at least 3 independent experiments performed in triplicate.

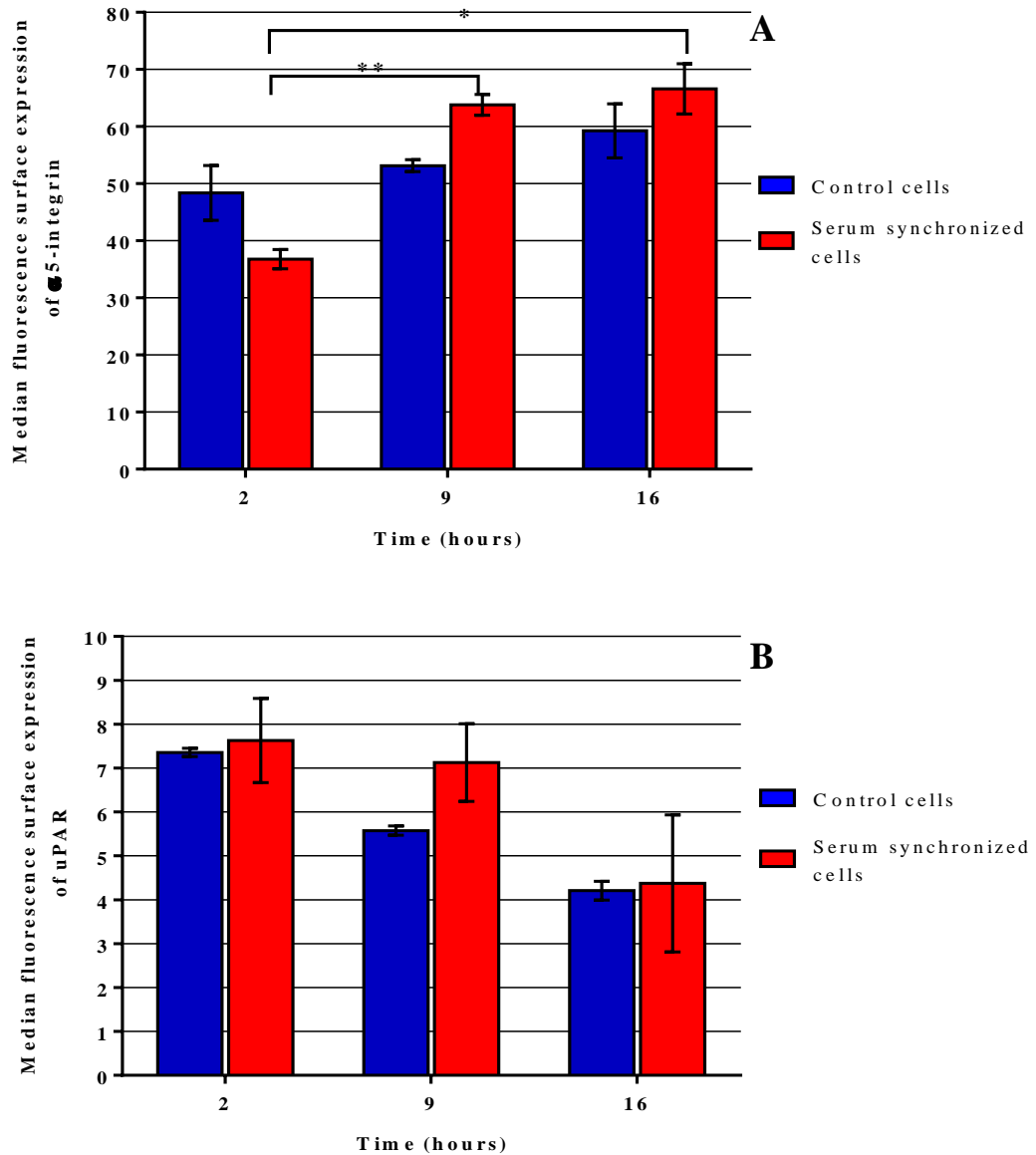




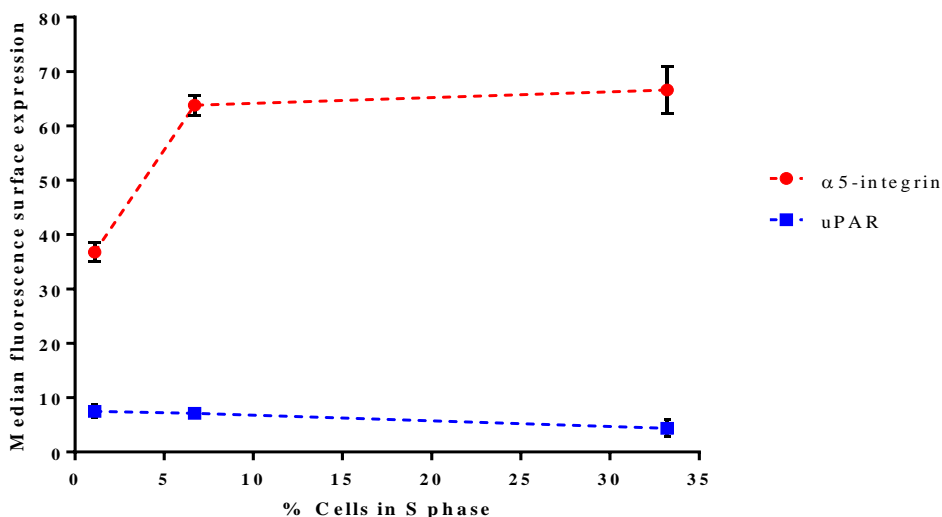
**Figure 5.10 Correlation of the mRNA expression of  $\alpha 5$ -integrin and uPAR with the percentage of cells in S-phase.** The fold change relative to control of the mRNA expression of both  $\alpha 5$ -integrin and uPAR gene was correlated with the percentage of cells in S-phase following 2, 9, and 16 hours of serum re-introduction to H357 cells. Data show the mean $\pm$ SD of at least 3 independent experiments.

#### 5.3.4.2 Effect of serum synchronization on the cell surface expression of $\alpha 5$ integrin and uPAR.

The cell surface expression of  $\alpha 5$  integrin and uPAR was investigated to determine whether levels correlate with gene transcription. Flow cytometry was used following 2, 9, 16 hours of re-introducing serum to H357 cells. Interestingly, the surface expression levels of  $\alpha 5$ -integrin and uPAR were very similar to their transcription levels.  $\alpha 5$ -integrin surface expression in H357 cells showed a significant increase in expression 9 hours after serum was re-introduced with a median fluorescence value of  $63.8\pm 1.3$ , and at 16 hours with a median value of  $66.6\pm 3.1$ , compared with a value of  $36.7\pm 1.2$  at 2 hours (figure 5.11 A). On the other hand, decreased surface expression of uPAR was found with increasing time of serum synchronization. There was non-significant decrease in expression following 16 hours of serum incubation ( $4.3\pm 1.1$ ), compared to 9 hours ( $7.1\pm 0.6$ ) and 2 hours ( $7.6\pm 0.6$ ) (figure 5.11 B). A correlation graph was plotted between median surface expression of both markers and the percentage of cells in S-phase. The results showed a strong positive correlation between the surface expression of  $\alpha 5$ -integrin and the percentage of cell in S-phase ( $R=0.6$ ), a negative correlation was found regarding uPAR ( $R=-0.9$ ) (figure 5.12).

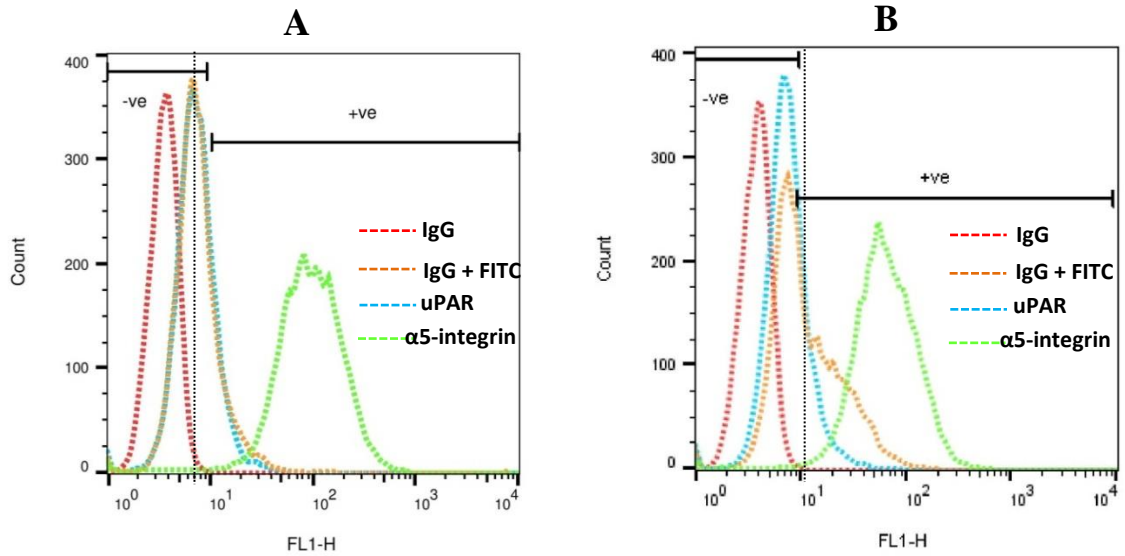


**Figure 5.11 Comparison of surface expression of  $\alpha 5$ -integrin and uPAR between serum synchronized and control H357 cells.** H357 cells were serum starved for 24 hours and serum re-introduced for 2, 9, and 16 hours. Cells were washed and adjusted at  $1 \times 10^6$  cells/ml. 100  $\mu$ l of cell suspension was incubated with primary monoclonal mouse antibodies against  $\alpha 5$ -integrin and uPAR. Cells were centrifuged, washed and incubated with FITC-goat anti-mouse IgG secondary antibody in 10% PBS containing serum (v/v). After re-washing surface expression was determined by FACS Calibur. This histogram shows the mean median fluorescence expression of  $\alpha 5$ -integrin (**A**) and uPAR (**B**) on serum synchronized H357 cells relative to the unsynchronized control cells  $\pm$ SD of at least 3 independent experiments performed in triplicate. \*, \*\* indicate statistically significant differences at ( $P \leq 0.05$ ) and ( $P \leq 0.01$ ) respectively.



**Figure 5.12 Correlation of the surface expression of  $\alpha 5$ -integrin and uPAR with the percentage of cells in S-phase.** The median fluorescence expression of  $\alpha 5$ -integrin and uPAR gene was correlated with the percentage of cells in S-phase following 2, 9, and 16 hours of serum re-introduction to H357 cells. Data show the mean $\pm$ SD of at least 3 independent experiments.

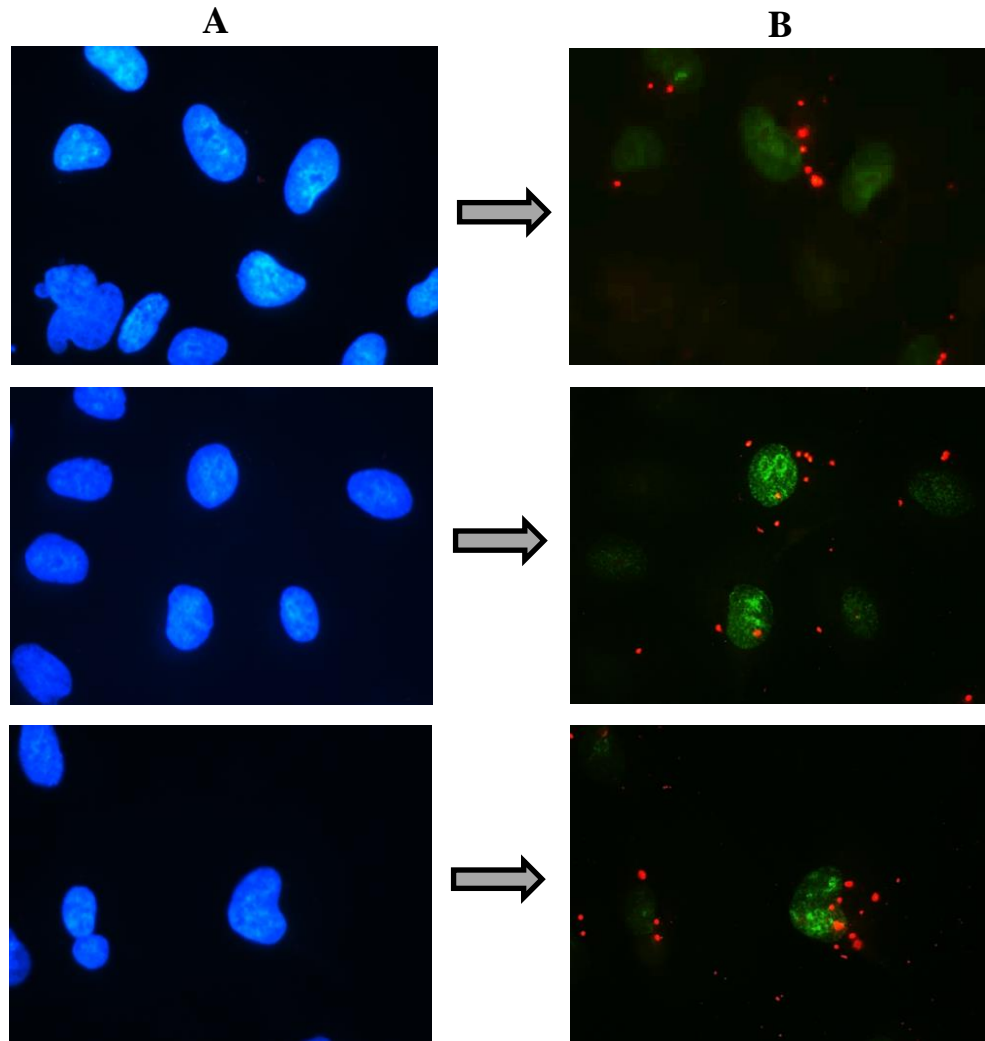
Data presented in figure 5.13 shows the fluorescence intensity of  $\alpha 5$ -integrin and uPAR at 2 hours after re-introduction of serum compared to the unsynchronised cell population. A large increase in the FITC fluorescent intensity associated with  $\alpha 5$ -integrin expression was observed compared to those expressing uPAR in unsynchronised cell population (figure 5.13 A). A similar pattern of fluorescent intensity relationship was observed in serum synchronised samples regarding cells with  $\alpha 5$ -integrin expression compared to those with uPAR expression (figure 5.13 B). Data shown here suggest that uPAR was expressed at low levels in both unsynchronised and synchronised cells compared to  $\alpha 5$ -integrin as it is located within the same band of control isotype IgG population fluorescent intensity. A similar pattern of fluorescent relationship of  $\alpha 5$ -integrin compared to uPAR and control group was observed in H357 cells when the time of serum synchronisation was increased to 9 hours and 16 hours, providing an additional evidence that uPAR expression was reduced during increasing time of serum synchronization. An example is shown below.



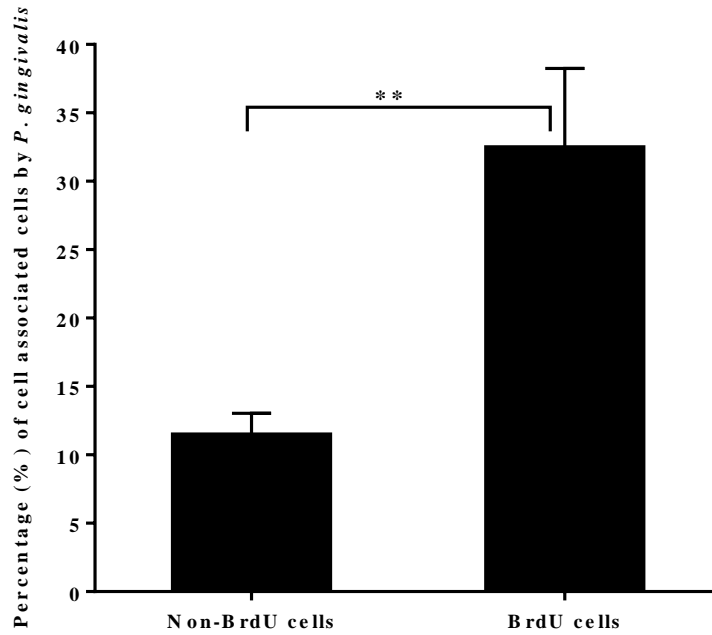
**Figure 5.13** Surface expression of uPAR and  $\alpha 5$ -integrin 2 hours after re-introduction of serum. H357 cells were serum starved for 24 hr and serum re-introduced for 2 hr. Cells were washed and adjusted at  $1 \times 10^6$  cells/ml. 100  $\mu$ l of cell suspension was incubated with primary monoclonal mouse antibodies against  $\alpha 5$ -integrin and uPAR. Cells were centrifuged and washed with PBS and incubated with FITC-goat anti-mouse IgG secondary antibody. Cells were washed, centrifuged, and re-suspended with PBS containing 10% serum (v/v). The surface expression was determined by FACS Calibur. A representative histogram of fluorescence (FL1-H) against the number of cells is shown. FITC fluorescence due to  $\alpha 5$ -integrin expression compared to uPAR and control isotype IgG groups is shown in unsynchronised cells (A) compared to serum synchronised cells (B). The graph represents one of at least 3 independent experiments. IgG= isotype control; IgG+FITC= isotype control antibody plus free FITC; uPAR= anti-uPAR antibody;  $\alpha 5$ -integrin= anti- $\alpha 5$ -integrin antibody.

**5.3.5 Association of *P. gingivalis* with cells in S-phase using double immunofluorescence.**

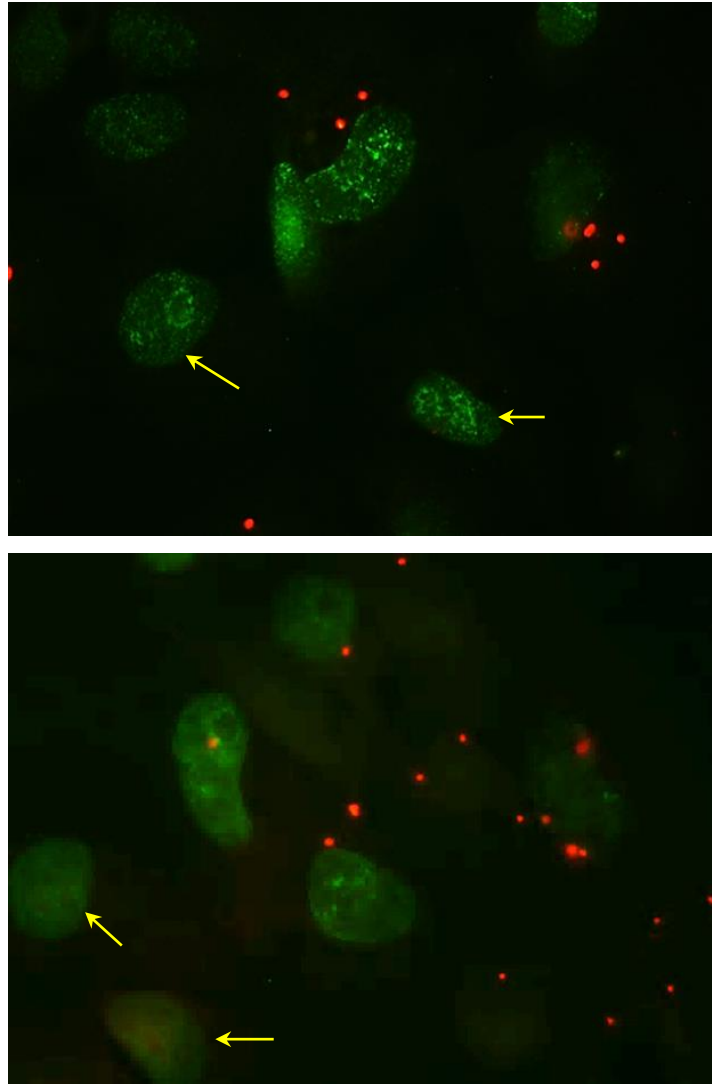
To identify any co-localisation between cells in S-phase and the internalized *P. gingivalis*, a double immunofluorescence technique was conducted using different fluorescent secondary antibodies to label S-phase cell nuclei and the associated *P. gingivalis*. As mentioned in section (2.23.2) and following the primary antibody incubation step, BrdU-stained cells were labelled using fluorescent goat anti-mouse secondary antibody (Alexa Fluor® 488 conjugate), the cell-associated *P. gingivalis* were stained using goat anti-rabbit secondary antibody (Alexa Fluor® 594 conjugate, texas red stain), and the total cell population was stained using DAPI. The results showed that there was co-localisation between *P. gingivalis* and BrdU-positive cells. From the total cell population, the BrdU-labelled cells were 3 times more likely to be associated with *P. gingivalis* cells (32.5%±5.8) compared with BrdU-negative cells (11.4%±1.5;  $P \leq 0.01$ ) (figure 5.14, 5.15), suggesting that cells in the S-phase may be more favorable for *P. gingivalis* adhesion and invasion. However, we found that not all BrdU-positive cells had detectable *P. gingivalis*; approximately 63%±5.3 of the BrdU-positive cells did (figure 5.16). Interestingly, BrdU-positive cells had 10 times more bacteria associated with them than the BrdU-negative cells ( $P \leq 0.0001$ ), which provides further evidence for the specificity of this interaction between cells in S-phase and *P. gingivalis* (figure 5.17).



**Figure 5.14 Association of *P. gingivalis* with cells in S-phase.** H357 cells were grown on coverslips and infected with *P. gingivalis* for 90 minutes. Cells were washed, incubated with BrdU, and both anti-rabbit whole *P. gingivalis* and BrdU mouse monoclonal primary antibodies at room temperature overnight. Cells were washed and incubated with both secondary antibodies against BrdU and *P. gingivalis* (Texas Red). Cells were washed and coverslips was mounted onto slides using ProLong® diamond anti-fade mounting with DAPI. The images were captured and merged for double staining analysis and examples are shown. (A) Cells stained with DAPI, (B) BrdU-positive cells (green nuclei) and their associated *P. gingivalis* (red dots) were quantified by switching to the FITC and Texas Red channels respectively. For each fluorescent channel, 10 images were captured using x 100 magnification and analysed from 3 individual experiments.

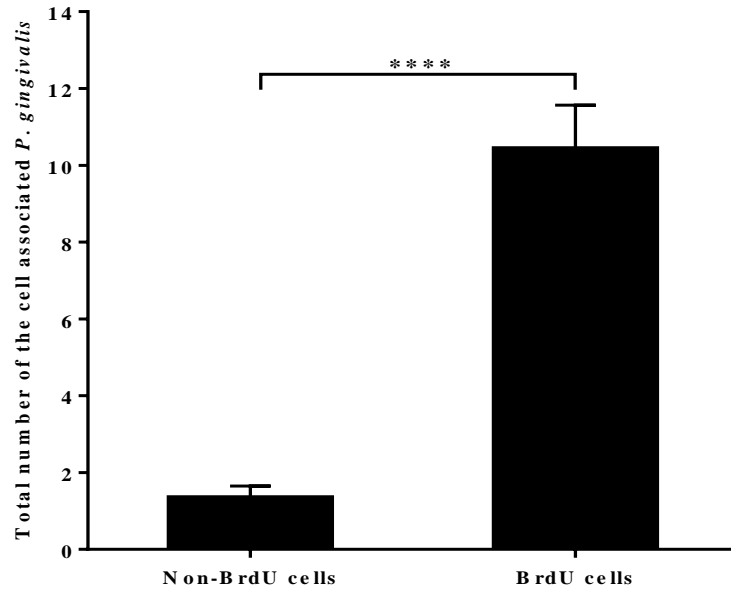


**Figure 5.15 Histogram showing the percentage of BrdU-positive and negative cells with associated *P. gingivalis*.** H357 cells were infected with *P. gingivalis* for 90 minutes using an antibiotic protection assay. Cells were then incubated with BrdU followed by incubation with anti-BrdU and anti-whole *P. gingivalis* primary antibodies at room temperature over night. Cells were then incubated with secondary antibodies against both BrdU cells (AlexaFluor 488) and invaded *P. gingivalis* (Texas red). Visualization was performed using fluorescence microscopy. Graphs show mean $\pm$ SD of 3 independent experiments performed in triplicate. \*\* Indicate statistically significant differences at ( $P\leq 0.01$ ).



**Figure 5.16 Example of unequal *P. gingivalis* association among BrdU-positive cells.** This figure shows that not all BrdU-positive cells (yellow arrows) had associated *P. gingivalis* (red dots). The BrdU-positive cells were quantified by switching to the FITC channel within the fluorescence microscope. 10 images were captured using x100 magnification and analysed from 3 individual experiments.





**Figure 5.17 Histogram of the number of *P. gingivalis* cells associated with BrdU-positive and negative cells.** H357 cells were infected with *P. gingivalis* for 90 minutes using an antibiotic protection assay. Cells were then incubated with BrdU followed by incubation with anti-BrdU and anti-whole *P. gingivalis* primary antibodies at room temperature over night. Cells were then incubated with secondary antibodies against both BrdU (AlexaFluor 488) and *P. gingivalis* (Texas red). Visualization was performed using fluorescence microscopy. Graphs show mean $\pm$ SD of 3 independent experiments performed in triplicate. \*\*\*\* Indicate statistically significant differences at ( $P\leq 0.0001$ ).

## 5.4 DISCUSSION

*P. gingivalis*, like other virulent pathogens, has the ability to modify the host cell cycle to promote bacterial survival, colonization and dissemination at the site of infection. Several studies have shown that disruption of the cell cycle can significantly impact immune surveillance and inflammation and so contribute to periodontal tissue destruction. The data reported here add to our understanding of how *P. gingivalis* can colonise epithelial cells at a periodontal site, which may have implications for the diagnosis and management of periodontal diseases.

The previous chapter reported that not all H357 cells in a given population at any one time were equally associated with *P. gingivalis*. We proposed that this phenomenon might be associated with the phase of the cell cycle that the cells are in. The majority of earlier investigations studied the influence of internalised *P. gingivalis* on the progression of the cell cycle itself but did not determine whether the bacteria showed a greater affinity for cells in any given phase of the cell cycle. The aim of the work reported in this chapter was to address this.

Kuboniwa *et al.* (2008) showed that the proliferation rate of the gingival epithelial cells (GECs) was accelerated through the S-phase of the cell cycle following *P. gingivalis* infection, and that this was mediated through an activation of cyclin A, Cdk 2,4,6, and PI3K, along with downregulation of p53 (which control cell apoptosis). *P. gingivalis* may thus affect gingival turnover/integrity *in vivo*. In addition, Pan *et al.* (2014) showed that the proliferation of the immortalized human gingival epithelial cells was significantly enhanced by *P. gingivalis* by inducing G1/S transition through activation of cyclin D and cyclin E. On the other hand, there are contradictory reports in the literature. For example, the proliferation rate of oral cancer cells has been shown to be inhibited by *P. gingivalis* through inducing G1 arrest, decreased expression of cyclin D1 and its dependent kinase (cdk4), and upregulation of p21 (Cdk inhibitor) (Cho *et al.*, 2014). Such G1 arrest was also documented with osteoblast cells infected with *P. gingivalis* and this was thought to be mediated by Lysine-specific proteinase and Rgp gingipains (Kato *et al.*, 2008).

Despite this controversy, what is clear is that bacteria have the ability to modulate the cell cycle and this could be to their benefit in terms of staying within the cells or exiting from them into the extracellular compartment.

### **Cell cycle characterization of H357 oral keratinocytes by flow cytometry and immunofluorescence techniques.**

For hypothesis testing, a well-described method of cell culture synchronisation by serum starvation was used (Attardi *et al.*, 2004, Langan and Chou, 2011, Rosner and Hengstschläger, 2011). As indicated in section 2.18, H357 cells were serum starved for 24 hours to arrest the cell growth and bring cells into a quiescent state at G0 (Lyublinskaya *et al.*, 2015), and by re-introducing serum cells then enter the cell cycle simultaneously (Cooper, 1998, Hossain *et al.*, 1997, Xiong *et al.*, 2012). It has been shown that serum starvation for 24 hours of human dermal fibroblasts showed no significant difference in cell morphology and apoptosis compared to unsynchronised control cells (Chen *et al.*, 2012), thus 24 hours of serum starvation was employed in our study.

The cell cycle characterisation, as investigated by flow cytometry using PI to detect DNA content, showed that cells in S-phase were significantly increased in proportion with increasing serum stimulation time to reach the highest level at 16 hours (33%) (figure 5.1B). Our results are in agreement with Chen *et al.* (2012), who showed that following re-feeding human dermal fibroblasts with serum for 20 hours, a clear increase in the S-phase cell population was obtained compared to the unsynchronised control cells. In addition, Cai and Dynlacht (1998) showed that WI38 human fibroblasts entered S-phase following 16-20 hours serum stimulation and reached the maximum level after 24 hours. Therefore, the highest percentage of H357 cells in S-phase might be expected if the time of serum re-introduction was increased to 24 hours.

The characterisation of cells in S-phase was additionally confirmed using BrdU (Gratzner, 1982). BrdU is a synthetic thymidine analogue that incorporates into newly replicated DNA during S-phase of the cell cycle (Darzynkiewicz and Zhao, 2014). Labelling H357 cells with BrdU and examination by fluorescence microscopy showed a similar pattern as that obtained by flow cytometry (highest proportion of cells in S-phase (76%) at 16 hours).

Although the actual proportion of cells in S-phase found by each method differed, both showed highest S-phase after 16h. The reasons for this could be that in flow cytometry, counting of cells according to the cell cycle phases was based on gates being drawn around each phase in the fluorescence expression histogram, then the final percentage of each phase is analysed by LSRII flow cytometer. This technique is subject to some variation in the way the gates are drawn between individual experiments. In contrast, the immunofluorescence technique, counting cells in S-phase is mainly dependent on the visual detection of fluorescently labelled BrdU-positive cells. Counting BrdU-labelled cells through randomly selected images captured by immunofluorescence microscope under x100 magnification inevitably analysed fewer than the 10,000 cells measured by flow cytometry.

### **Effect of cell cycle synchronization on *P. gingivalis*-cell association.**

It has been shown that through overexpression of cyclin D1 and E1, the transition of cell cycle phase G1 to S-phase was enhanced upon infecting immortalized gingival epithelial cells with *P. gingivalis* ATCC 33277 from 10 hours (24%) to 12 hours (32%) (Pan *et al.*, 2014). This was supported by another study that showed gingival epithelial cells in S-phase were increased from 33% at 6 hours to 64% following 20 hours of infecting cells with *P. gingivalis* in a FimA-dependent manner. This was associated with increased expression of cyclin A and Cdk 4 and 6 and reduced expression of cyclin D (Kuboniwa *et al.*, 2008). However, in our study it is unlikely that *P. gingivalis* would have been able to drive the cell cycle in such a way, as the bacterial infection was only for 90 minutes. Indeed our data showed that several hours after serum re-introduction were required to significantly increase the number of cells in S-phase.

For the first time, we demonstrate that the level of *P. gingivalis* invasion was proportionally related to the time of serum synchronisation, with the highest invasion percentage (5.4%) being obtained 16 hours after serum re-introduction (figure 5.5). In addition, this increase in invasion was positively correlated ( $R=0.98$ ) with the proportion of cells in S-phase (figure 5.6), suggesting that *P. gingivalis* cells are more readily internalised by cells in S-phase compared to other phases of the cell cycle.

It is well known that serum starvation represents the most extensively described condition that results in cellular autophagy. The depletion of the nutrient and vital amino acids required for cellular growth results in degradation of the cytoplasmic organelles inside lysosomes with autophagosome formation (Cuervo, 2004, Klionsky *et al.*, 2008, Yorimitsu and Klionsky, 2005, Yue *et al.*, 2002). Similarly, it has been shown that *P. gingivalis* induces cellular autophagy with subversion as a replication niche using numerous host proteins resulting from cellular degradation. This is an important source of nutrients for asaccharolytic *P. gingivalis*, facilitating bacterial survival within the host cells (Dorn *et al.*, 2001, Mysak *et al.*, 2014). Thus, it is possible in our study that starving cells from serum for 24 hours may stimulate autophagic pathways, which could possibly trigger intracellular trafficking with eventual bacterial engulfment. While there is support for this from previous studies which showed that following invasion *P. gingivalis* is located within autophagosome-like vacuoles and that these are exploited for bacterial replication and persistence in the host cell (Dorn *et al.*, 1999, 2001), the interaction between *P. gingivalis* and the autophagy pathways seems to be cell specific. Bacterial localization within autophagic vacuoles has been found in human coronary artery endothelial cells, whereas *P. gingivalis* cells were found to be free in the perinuclear area within the cytoplasm of gingival epithelial cells and KB epithelial cells (Belton *et al.*, 1999, Houalet-Jeanne *et al.*, 2001), which is relevant to our study. However, under the stress conditions of serum starvation and using H357 oral keratinocytes, there may be more exploitation of autophagosomes by *P. gingivalis* NCTC 11834, and it is possible that they could induce cytoskeletal rearrangement leading to enhanced uptake of other bacteria. This requires further work to confirm. A similar association of *P. gingivalis* cell-association with cells in S-phase was also found in non-serum starved cells following co-localization of *P. gingivalis* with BrdU-positive cells. In addition, a significant difference in *P. gingivalis* invasion was found between 9 hours and 16 hours of serum re-introduction, which are both considered to be long enough for full cell recovery. The preferential interaction or invasion of *P. gingivalis* with cells in S-phase is additionally supported by the experiment in which labelled *P. gingivalis* were incubated with PI-labelled cells. Analysis by flow cytometry showed that the highest association of bacteria was with cells in S-phase, followed by G2/M-phase, then G1-phase (figure 5.7).

### **Effect of serum synchronization on the mRNA and surface expression of receptors thought to mediate *P. gingivalis* invasion.**

It has been well documented that inter-cellular interactions, stimulation of the cellular proliferation and differentiation and cell signalling with the microenvironment are commonly mediated by host cell integrins (Fournier *et al.*, 2008, Hynes, 1992). In addition,  $\alpha 5\beta 1$  integrin, plays a pivotal role in mediating the adherence and subsequent invasion of several bacteria by host cells including *S. aureus* (Agerer *et al.*, 2005), group A streptococcus (GAS) (Ozeri *et al.*, 2001), *E. coli* (Plancon *et al.*, 2003), and *P. gingivalis* (Nakagawa *et al.*, 2005, Nakagawa *et al.*, 2002a, Tsuda *et al.*, 2008, Yilmaz *et al.*, 2002).

The  $\alpha 5$ -integrin subunit (Ridley *et al.*, 2012) is thought to be involved in the internalization of GAS through binding the GAS-associated fibronectin binding protein in a transforming growth factor beta 1 (TGF- $\beta 1$ )-induced manner (Wang *et al.*, 2006). On the other hand, uPAR has been found to form a firm complex and close association with  $\alpha 5\beta 1$  integrin (Wei *et al.*, 2005), with multi-regulatory functions such as binding with fibronectin (Monaghan *et al.*, 2004, Wei *et al.*, 2005) and consequent signalling (Monaghan-Benson and Mckeown-Longo, 2006). We therefore investigated whether  $\alpha 5$ -integrin and uPAR mRNAs and surface expression levels varied in relation to cell cycle phase. Our results demonstrated that the mRNA and surface expression of  $\alpha 5$ -integrin gradually increased to its highest level by 16 hours after re-introduction of serum. This increased expression of  $\alpha 5$ -integrin was positively correlated with the proportion of cells in S-phase and thus might explain the high *P. gingivalis* invasion obtained at the same time (16 hours -5.4%) (figure 5.9A and 5.11A). It has been shown that TGF- $\beta$  has a significant influence on expression of  $\alpha 5\beta 1$  integrin and it was highly expressed in HeLa cells during the mid-S-phase of the cell cycle (Van Der Meijden *et al.*, 2002). This therefore might support the notion that since high expression of  $\alpha 5$ -integrin occurs in S-phase this could mediate higher invasion of cells in S-phase by *P. gingivalis*.

While uPAR has been reported to regulate the activity of  $\alpha 5\beta 1$  integrin (Wei *et al.*, 2005), our result showed that the mRNA and surface expression levels were gradually decreased with increasing time of serum re-introduction, suggesting that the serum might have a negative regulatory effect on uPAR expression (figure 5.9B and 5.11B).

In addition, uPAR expression was negatively correlated with cells in S-phase (figure 5.10 and 5.12), indicating that not all cellular genes are highly expressed during S-phase. However, this reduced expression of uPAR following serum re-introduction for different times is different from our previous results that showed the mRNA expression of uPAR was significantly increased with increasing time of *P. gingivalis* invasion (figure 4.5). The effect of different stimuli on uPAR expression may explain this observation, as uPAR expression by bacterial stimuli might be different from those in cells following serum starvation.

### **Association of *P. gingivalis* with cells in S-phase using double immunofluorescence.**

Approximately 63% of the total BrdU-positive cells had associated *P. gingivalis*, and there were 10 times more bacteria associated with BrdU-positive cells than with BrdU-negative cells (figure 5.17). This might be attributed to enhanced expression of receptors on the cell surface that are involved in the adherence and invasion of these bacteria, such as  $\alpha 5\beta 1$  integrin, which would support our suggestion that *P. gingivalis* preferentially binds to and invades cells in S-phase (figure 5.16). It is true that not all cells in S-phase were equally associated with *P. gingivalis*, so there are still other variable factors that govern *P. gingivalis* cell-association and these need further exploration.

## **5.5 CONCLUSION**

For the first time, we report that cells in S-phase of the cell cycle were preferentially invaded by *P. gingivalis*, and this may explain why the susceptibility of oral keratinocytes in culture to *P. gingivalis* invasion is unequal. The upregulation of  $\alpha 5$ -integrin but not uPAR provides evidence of a possible mechanism by which this may occur. These data have implications for the understanding of *P. gingivalis* virulence and possibly of other periodontal pathogens, through targeting stable dividing cells. Thus, *in vivo*, *P. gingivalis* may target and invade cells that turn over rapidly such as junctional epithelial cells, which might explain the bacterial persistence at deep sub-gingival sites with subsequent periodontal tissue destruction.

# **Chapter 6**

## **General discussion and conclusion**



### 6.1 GENERAL DISCUSSION

Periodontitis is an irreversible progressive inflammatory disease affecting the supporting tissues of the tooth, and globally, it represents the major cause of tooth loss (Choi and Seymour, 2010). Although the aetiology of periodontitis has been shown to be the result of changes to the complex microbiological environment, *P. gingivalis* is considered one of the pivotal microorganisms that is involved in the initiation and progression of periodontal disease (Curtis *et al.*, 2011). Through a wide range of *P. gingivalis* virulence factors, such as fimbriae (Yilmaz *et al.*, 2002) and gingipain proteases (Stafford *et al.*, 2013), oral epithelial cells and other cells can be invaded *in vitro* (Lamont *et al.*, 1995) and *in vivo* (Rautemaa *et al.*, 2004) by *P. gingivalis*. This invasion strategy is significant for bacterial evasion of immune surveillance, providing the bacteria with a rich source of nutrients, modulating the host cell response, and aiding the bacteria to reside and replicate intracellularly. All of these factors could contribute to prolonged colonisation and re-infection at the target sites. Evidence in the literature shows that bacterial invasion is a complex process requiring cross-talk between bacteria, host cells and the environment. This study has explored the factors that might influence *P. gingivalis* invasion and the host cell response to that invasion.

*P. gingivalis* strain NCTC 11834 is a hyper-invasive strain compared to other strains of *P. gingivalis* (Dorn *et al.*, 2000, Suwannakul *et al.*, 2010), and so was used in this study to ensure sufficient sensitivity following the various cell manipulations. For this work we used the epithelial cell line, H357, (SCC-derived oral keratinocytes of the tongue) and others have shown that this did not differ significantly from normal oral keratinocytes (NOKs) in term of susceptibility to *P. gingivalis* invasion (Pinnock, 2012, Suwannakul *et al.*, 2010). One advantage of using the cell line was its consistency between experiments, whereas NOKs could only be kept growing for a limited number of passages and so would be a source of variation in experiments. Another advantage is its relatively high turn-over rate, which is also a feature of junctional epithelial cells (Newman *et al.*, 2014, Shimono *et al.*, 2003). In addition, the effect of cell passage number on *P. gingivalis* invasion was studied by comparing early passage cells (average of p=57), to late passage cells (average of p=219). Results showed no difference between both cell sets, which might be attributed to the minimal

variation in  $\beta 1$  integrin expression with increasing cell passage number (Sugiyama *et al.*, 1993).

The antibiotic protection assay was used in our study to investigate the internalisation of *P. gingivalis* into H357 cells, as this assay is the most commonly used (Lamont *et al.*, 1995). Our results demonstrated that the proportion of internalised bacteria significantly increased with increasing incubation time. It is possible that the higher bacterial engulfment rate with increased infection time may result from the existence of one or more possible mechanisms. These include: prominence of cellular microvilli that encapsulate and engulf bacteria (Njoroge *et al.*, 1997), cytoskeletal rearrangement becoming highly organised and stimulated (Yilmaz *et al.*, 2003), enhancement of intracellular bacterial replication (Lamont *et al.*, 1995), and the opportunity of host-bacterial contact occurring being higher, leading to more stimulation of cellular receptors involved in bacterial uptake, such as  $\alpha 5\beta 1$  integrin (Nakagawa *et al.*, 2002a, Yilmaz *et al.*, 2002).

The antibiotic protection assay is one which relies on recovery and enumeration of viable bacteria from the host cells. Therefore, the method of host cell lysis, whilst not compromising *P. gingivalis* viability, is an important consideration. We found that there was no difference in the percentage of recovered bacteria when using distilled water or 0.1% saponin (w/v) to lyse H357 monolayers. Distilled water exerts its lytic effects through osmosis increasing the cell volume until the cells burst (Guilak *et al.*, 2002). Saponin induces pore formation in the cell membrane leading to long lasting increases in cell permeability (Seeman, 1974). Although widely diverse effects have been reported using distilled water and for different times (Crowston *et al.*, 2004) and using different concentrations of saponin (Dourmashkin *et al.*, 1962, Hedman, 1980, Jalal *et al.*, 1992), others have used distilled water for 20-30 minutes or 0.1% saponin (w/v) successfully for invasion studies of *P. gingivalis* (Lamont *et al.*, 1995, Wayakanon *et al.*, 2013). As no difference in *P. gingivalis* invasion was observed between the two methods, and because distilled water was convenient, it was the standard that was used throughout the study, except when studying invasion of H357 cells in suspension.

## Chapter 6 General discussion and conclusion

*P. gingivalis* invasion in gingival epithelial cells has been shown to be higher in the exponential bacterial growth phase compared to the stationary phase, it is thought that the higher metabolic activity and *de novo* synthesis of potential proteins involved in invasion might explain this observation (Lamont *et al.*, 1995). However, the results in this thesis showed that there was no difference in *P. gingivalis* invasion between stationary phase and exponential phase cells. Support for this comes from the fact that *P. gingivalis* gingipains which enhance fimbriae-fibronectin binding (Kontani *et al.*, 1997), contribute to fimbrial maturity (Nakayama *et al.*, 1996), and fimA gene expression is not influenced by bacterial growth phase (Muthiah *et al.*, 2013, O'Brien-Simpson *et al.*, 2001, Tokuda *et al.*, 1998, Vanterpool *et al.*, 2005, Xie *et al.*, 1997).

While the antibiotic protection assay is the most commonly used method for studying bacterial invasion of cells as mentioned above, we also tested whether alternative methods would be easier but as effective. Some workers have quantified bacteria by measuring the bacterial DNA concentration by real time PCR (Asai *et al.*, 2002, Boutaga *et al.*, 2005, Nonnenmacher *et al.*, 2004). This has been shown to be a rapid, reliable and sensitive technique to detect even small bacterial populations (Boutaga *et al.*, 2007). However, the method cannot differentiate between live and dead bacteria but more importantly it cannot differentiate between bacteria that are adherent to the cell membrane but not internalised from the bacteria that have been internalised (Ammann *et al.*, 2013). Therefore, the culture-based method was used in our study for determining viable invaded *P. gingivalis*. Possibly a combination of fluorescence microscopy with live/dead staining of bacteria along with real time PCR could be a useful approach in future studies.

The number of bacteria contacting the host cell surface is likely to influence the degree of bacterial invasion achieved in experimental systems. Indeed, here we showed that *P. gingivalis* invasion was significantly higher when cells were seeded at  $7.5 \times 10^4$  cells/well than at either  $5 \times 10^4$  or  $2.5 \times 10^4$  cells/well. It is assumed that this is because the opportunity to interact with receptors such as  $\alpha 5 \beta 1$  integrin (Yilmaz *et al.*, 2002), TLRs (Wang *et al.*, 2007), and cytokeratins (Sojar *et al.*, 2002) would be greater. Moreover we wondered whether the physical state of the cell when contacting the bacteria would affect bacterial invasion. Specifically, when cells are anchored onto the substratum there is a polarisation of molecules involved in adhesion (e.g. the integrin

## Chapter 6 General discussion and conclusion

$\alpha 5\beta 1$ ) such that they are relatively hidden on the underside of the cells from the bacteria (Ridley *et al.*, 2012). We therefore compared invasion of cells that were not anchored down with cells in conventional monolayer. To release the bacteria effectively from cells in suspension we had to use saponin lysis and we found a 3 fold higher invasion of *P. gingivalis* of those cells compared monolayer cultures. It seems likely then that this could be explained by more receptors being available for bacterial attachment when in suspension (Hauck, 2002). Since cells grown in suspension may increase expression of intercellular junction-related molecules, such as intercellular adhesion molecule-1 ICAM-1 in an attempt to make contact with the adjacent cells, *P. gingivalis* might not be limited to the integrins for cell binding but could use ICAM-1 (Tamai *et al.*, 2005). In addition, molecules such as E-cadherin could be degraded by *P. gingivalis* proteases, leading to cell barrier destruction and enhanced entry of bacteria (Choi *et al.*, 2013).

Investigating the cell responses to external stimuli including bacterial challenge is as important as studying the bacterial factors that affect invasion. Exploring such response may reveal some of the underlying mechanisms involved in bacterial pathogenicity. A question was raised whether cells that had previously contacted *P. gingivalis* lipopolysaccharides (LPS) would change the expression of surface receptors and so influence *P. gingivalis* internalisation. *P. gingivalis* LPS, has been reported to interact with TLR-2 (Martin *et al.*, 2001), but here we showed that there was no difference in *P. gingivalis* invasion of cells that had been pre-incubated with LPS compared to control cells. Several studies however showed that activated TLR-2 has a considerable impact on *P. gingivalis* invasion. These include its effect on stimulation of  $\beta 1$  integrins (Harokopakis and Hajishengallis, 2005, Yilmaz *et al.*, 2002) and the activating effect on CR-3, which acts as a binding receptor for *P. gingivalis* fimbriae and both of which have been reported to lead to increased *P. gingivalis* invasion (Wang *et al.*, 2007, Zenobia and Hajishengallis, 2015). Although we did not observe an obvious effect of LPS stimulation on *P. gingivalis* invasion in our study, it is possible that there is cell-to-cell variation in response and so some cells within a population could become more invaded than others. Changes initiated by LPS on the cell membrane may be subtle and induce membrane changes that allow subsequent bacteria to enter cells via the same

port of entry. Although this has not yet determined and so requires further investigations.

The post invasion gene profile of H357 cells following infection with *P. gingivalis* for different times was investigated as part of exploring the cell response to bacterial stimuli. As important inflammatory mediators, the mRNA expression of Urokinase plasminogen activator receptor (uPAR) and Endothelin-1 (ET-1) molecules were investigated following infection of H357 cells with *P. gingivalis* for 30, 90, 150, and 240 minutes. Significantly increased mRNA expression of ET-1 and uPAR was found with increasing *P. gingivalis* time of infection from 30 to 240 minutes. Since *P. gingivalis* infection has been shown to significantly increase the mRNA expression of IL-1 $\beta$  and TNF- $\alpha$  (Sandros *et al.*, 2000), and IL-1 $\beta$  enhances the expression of ET-1 (Fujioka *et al.*, 2003) and uPAR (Schwab *et al.*, 2004), it is likely that the pro-inflammatory cytokines have a vital role in the expression of both mediators. In addition, *P. gingivalis* might have a direct role in stimulating the expression of cellular ET-1 and uPAR genes. Since ET-1 is produced from its precursor, big ET-1, by the action of endothelin-converting enzyme (ECE-1), increased activity of ET-1 could be attributed to the ECE- like activity of *P. gingivalis* PepO endopeptidase (Awano *et al.*, 1999). On the other hand, *P. gingivalis* LPS (Ogura *et al.*, 1999) and gingipain complex (Mcalister *et al.*, 2009, Singh *et al.*, 2010) have been shown to increase uPAR mRNA expression. There is an intimate association between  $\alpha$ 5 $\beta$ 1 integrin and uPAR, in which  $\alpha$ 5 $\beta$ 1 can regulate uPAR function and induce its expression (Bass and Ellis, 2009) and uPAR can influence  $\alpha$ 5 $\beta$ 1 integrin conformation and function (Wei *et al.*, 2005). As *P. gingivalis* adherence and invasion was shown to be mediated by  $\alpha$ 5 $\beta$ 1 integrin, the effect of this integrin on the increased uPAR mRNA expression with increasing time of *P. gingivalis* infection needs to be considered.

In terms of determining the association of *P. gingivalis* with the host cells, it is not well known whether host cell susceptibility to bacterial invasion is homogenous or heterogeneous although the concept of cell heterogeneity with existence of physical, phenotypic, and functional differences amongst various cell populations in response to external stimuli has been previously reported. The results showed here that upon infecting H357 cells with *P. gingivalis*, some cells showed more associated *P. gingivalis* than others and that the bacteria appeared to be located in the perinuclear

## Chapter 6 General discussion and conclusion

area. It is thought that the existence of organelles such as mitochondria and endosomes in the perinuclear area could be exploited by *P. gingivalis* following invasion (Belton *et al.*, 1999), thus more bacteria are found near to the cell nucleus. In addition, it has been suggested that there are a limited number of microbial entry points to invade host cells (Huang *et al.*, 1998b) and changes to such entry points induced on the cell membrane by bacteria could be utilized by other bacterial cells so increasing the number of internalised bacteria (Edwards *et al.*, 2006). These changes, together with intracellular changes induced following *P. gingivalis* adherence to the host cells, (e.g. calcium ion flux levels, activation and phosphorylation of MAPKs and PI3K), may explain the higher invasion of *P. gingivalis* into some cells than others.

Although such invasion was found to be homogenous within gingival epithelial cells (Belton *et al.*, 1999), our heterogeneous *P. gingivalis*-cell association might be attributed to the heterogeneity of receptor distribution and/or function involved in *P. gingivalis* invasion of the H357 cell line compared to their gingival epithelial cells. As there is heterogeneous expression of potential receptors involved in *P. gingivalis* invasion such as  $\alpha 5\beta 1$  integrin (figure 5.13), this may explain the unequal internalisation of *P. gingivalis* within host cells at any one time. However, we hypothesised that this unequal cell susceptibility to *P. gingivalis* invasion might be attributed to the phase of the cell cycle that the cells were in.

While the majority of previous studies have investigated the influence of *P. gingivalis* on progression of the cell cycle in those cells it has invaded, our approach was to examine the effect of the cell cycle phases on the cell's interaction with *P. gingivalis*. Following arrest of H357 cell growth for 24 hours by serum starvation, cells were synchronised in their cell cycle by the re-introduction of serum and restarting growth. We found that a high proportion of cells were in S-phase 16 hours after re-introduction of serum. *P. gingivalis* invasion positively correlated with the percentage of cells in S-phase, which suggests that something about cells in that phase encourages uptake of the bacteria. However, serum starvation is also known to induce cellular autophagy, with degradation of cytoplasmic organelles and formation of autophagosomes (Cuervo, 2004). It is possible, therefore, that cellular autophagy may provide *P. gingivalis* with an advantageous environment for growth. This is supported by studies showing that *P. gingivalis* exists within autophagosome-like vacuoles, which are thought to be used

## Chapter 6 General discussion and conclusion

as a replication niche for the bacterium in the host cells (Dorn *et al.*, 1999, 2001). On the other hand, the increased *P. gingivalis* invasion observed with increasing time of serum re-introduction could be attributed to the cell recovery accompanied by increased expression of appropriate cell receptors for *P. gingivalis* and that may lead to greater cell invasion/cell association. Although we have not addressed this, a similar association of *P. gingivalis* with cells in S-phase was found in non-serum starved cells since from the total cell population, 11% BrdU-negative, 33% BrdU-positive, and 63% of BrdU-positive cells had cell-associated *P. gingivalis*. Furthermore, there were 10 times more *P. gingivalis* cells associated with BrdU-positive cells than with BrdU-negative cells. However, the ability of *P. gingivalis* to increase cells in S-phase with increasing infection time has been reported by others (Kuboniwa *et al.*, 2008, Pan *et al.*, 2014), and although not specifically addressed in this study, it is unlikely that *P. gingivalis* infection would induce cell cycle phase progression within the time frame of the bacterial interaction (90 minutes) since several hours of serum re-introduction are required to significantly increase the number of cells in S-phase. Thus, *in vivo*, rapid turnover cells, such as junctional epithelial cells (Newman *et al.*, 2014, Shimono *et al.*, 2003), may be targeted preferentially by *P. gingivalis* and these cells could act as the ‘Achilles heel’ for local periodontal tissue destruction.

To further investigate the potential mechanisms involved in interaction of *P. gingivalis* with cells in S-phase, the mRNA and surface expressions of  $\alpha 5$ -integrin and uPAR were changed with time after serum synchronisation. Both receptors have been shown to be associated with each other and to be involved in *P. gingivalis* invasion. The gradual increase in mRNA and surface expression of  $\alpha 5$ -integrin observed with increasing time of serum re-introduction might explain why *P. gingivalis* invasion was significantly enhanced with increasing time of serum synchronisation. This could also be attributed to the influence of TGF-  $\beta 1$  as it has been shown to influence the expression of  $\alpha 5\beta 1$  integrin (Moir *et al.*, 2008), and is highly expressed in mid-S-phase (Van Der Meijden *et al.*, 2002). Data presented here also reports that increasing  $\alpha 5$ -integrin expression was positively correlated with the number of cells in S-phase. Conversely, uPAR mRNA decreased as did its surface level with increasing time after serum re-introduction. This clearly shows that not all cellular genes had increased expression in S-phase and serum starvation has a negative effect on uPAR expression. However, it seems that uPAR may be differentially expressed on the cell surface

depending on the nature of stimuli they receive because we previously found that there was an increase in the mRNA expression of uPAR following increasing *P. gingivalis* infection time (figure 4.5).

### 6.2 GENERAL CONCLUSION

Several factors attributed to host cells, bacteria, and the environment, have been shown in the literature to work in concert and influence bacterial engulfment by host cells. We have demonstrated that when factors such as bacterial infection time and host cell density are increased, then significant increases in *P. gingivalis* invasion occur. In addition, growing cells in suspension significantly increased *P. gingivalis* uptake by H357 cells when saponin was used for cell lysis compared to distilled water, suggesting that the physical state of the cells may influence the receptors for bacterial invasion. In contrast bacterial growth phase, cell passage number and pre-treatment of cells with LPS were without effect. The specific cell line and *P. gingivalis* strain used in our study may explain why the levels of *P. gingivalis* invasion were different in comparison to the similar published studies. As a cell response to *P. gingivalis* infection, ET-1 and uPAR, as inflammatory mediators, were significantly increased at the gene level with increasing infection time, suggesting that these mediators could be used as potential markers to reflect the severity of periodontal inflammation, and might be used in diagnosis and management of periodontitis. All of these factors, in addition to those that were previously investigated in the literature, may in combination with variations in the host cell susceptibility to bacterial attack have an influence on the preference of bacteria to associate with some cells than others. This may explain the observed unequal *P. gingivalis* invasion at a given cell population at any one time. This heterogeneous host cell susceptibility to bacterial invasion was shown to be cell cycle phase specific, with the highly invaded cells being shown to be in S-phase. We have also demonstrated that targeting cells in S-phase of the cell cycle appeared to be mediated by  $\alpha 5$ -integrin, providing a possible mechanism for this unequal invasion. It is possible that, *in vivo*, the preference of *P. gingivalis* to colonise deeply in sub-gingival tooth surfaces could be aided by greater opportunity to target rapidly dividing cells, such as junctional epithelial cells. Although further investigations are required to characterise the association of *P. gingivalis* to cell cycle phase, our data have suggested that the interaction of *P. gingivalis* with their target host is highly organised, yielding new insights to understand the mechanisms involved in the pathogenicity of



periodontitis, and may provide useful tools for the diagnosis and treatment of periodontal disease.

### 6.3 FUTURE WORK

- 1- Although our *in vitro* work acts as a useful model to study *P. gingivalis* invasion, other cell types could be used, such as junctional epithelial cells as they might resemble *in vivo* conditions more closely. Also, other *P. gingivalis* strains including recent clinical isolates should be studied to check our data are generalizable.
- 2- Exploring the factors affecting cell invasion by other periodontopathogens (*T. forsythia*, *T. denticola*, *A. actinomycetemcomitans*) and the host cell response to these bacterial stimuli would be worthwhile. In particular it would be interesting to determine whether these also target cells in S-phase.
- 3- Examining the mRNA expression of other cellular genes showed in table 4.1, following *P. gingivalis* infection for varying times rather than the single 90 minutes time point used by (Whawell *et al.*, 2013). This would determine whether there is consistency with the observed increase in ET-1 and uPAR mRNA expression. This may also indicate whether these or others could be used as markers reflecting disease severity.
- 4- Designing antimicrobial agents/antagonists that could interfere with targeting cells in S-phase by *P. gingivalis*, which might then be used as a therapeutic agent against bacterial adherence and invasion, decreasing the severity of periodontal diseases.

### Publications

Al-Taweel FB, Douglas CW & Whawell SA (2016). The periodontal pathogen *Porphyromonas gingivalis* preferentially interacts with oral epithelial cells in S phase of the cell cycle. *Infect Immun* **84**: 1966-1974.

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