Effects of the periodontal pathogens *Porphyromonas gingivalis* and *Tannerella forsythia* on platelets

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Effects of the Periodontal Pathogens *Porphyromonas gingivalis* and *Tannerella forsythia* on Platelets

Alexander M Andrews

A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy

December 2018
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<table>
<thead>
<tr>
<th>Name</th>
<th>Alexander Michael Andrews</th>
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<tbody>
<tr>
<td>Date</td>
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<td>Dr Prachi Stafford</td>
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Abstract

Cardiovascular disease (CVD) accounts for ~17.7 million deaths annually, worldwide. Although several key risk factors including smoking, diet and obesity have been identified, these account for only 40-50% of all cases. Historically, poor oral hygiene and the chronic oral condition, periodontitis, characterised by gum bleeding and tooth loss has been implicated in the aggravation of CVD. It has been postulated that in cases of periodontitis, oral bacterial pathogens including *Porphyromonas gingivalis* and *Tannerella forsythia* can access the vascular system during gum bleeding and inflammatory responses within the periodontal pocket and subsequently contribute to atherosclerosis though interactions with platelets. This study aims to determine how periodonto-pathogens interact with platelets and contribute to CVD, specifically atherosclerosis.

Here it is demonstrated that the CHRF-288-11 megakaryocytic-like cells can be utilised through a series of methodologies to study the pathogenic effects of periodontal pathogens on platelets and megakaryocytic cells. *In vitro* *P. gingivalis*, but not *T. forsythia*, is able to interact with platelets by inducing both platelet activation and aggregation. *P. gingivalis* is able to initiate platelet activation though intracellular calcium mobilisation that leads to the release of both alpha and dense granules, independent of bacterial outer membrane protein OMPA. *P. gingivalis* NCTC 11834, but not ATCC W50, actively associates and interacts with integrin α2β1 inducing strain dependant platelet aggregation and could suggest a role for bacterial fimbriae within platelet interactions.

Taken collectively, this data suggests that *P. gingivalis* could contribute to CVD and atherosclerosis through interactions with platelets following invasion of the vasculature. Interestingly, direct interactions between bacterial cells and platelets, as well as bacterial genetics and virulence, appear key to the extent of platelet response and could highlight novel risk factors for future patient treatments.
Acknowledgments

I would like to take this opportunity to thank Sheffield Hallam University for providing both the opportunity and funding to undertake this PhD project.

I would especially like to thank my supervisor Dr Prachi Stafford for her ongoing support and encouragement throughout the entirety of my time at Sheffield Hallam University. I would also like to thank Dr Sarah Haywood-Small and Professor Tom Smith for all their contributions.

A special mention to Joseph, Laura, Matthew, my parents John and Zoe, and especially Lucy who provide the small things that make the big things happen.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candidate declaration</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of figures</td>
<td>xi</td>
</tr>
<tr>
<td>List of tables</td>
<td>xv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xvi</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction Part I: Periodontitis and the oral microbiome</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 The oral microbiota</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2 Oral biofilm formation</td>
<td>3</td>
</tr>
<tr>
<td>1.1.3 Subgingival biofilm and periodontal disease</td>
<td>6</td>
</tr>
<tr>
<td>1.1.4 Pathogenic bacterial species</td>
<td>9</td>
</tr>
<tr>
<td>1.1.4.1 <em>Porphyromonas gingivalis</em></td>
<td>9</td>
</tr>
<tr>
<td>1.1.4.2 <em>Tannerella forsythia</em></td>
<td>15</td>
</tr>
<tr>
<td>1.2 Introduction Part II: Platelets</td>
<td>17</td>
</tr>
<tr>
<td>1.2.1 Megakaryocytes and platelet production</td>
<td>17</td>
</tr>
<tr>
<td>1.2.2 Platelet morphology and ultrastructure</td>
<td>19</td>
</tr>
<tr>
<td>1.2.2.1 The open canalicular system</td>
<td>19</td>
</tr>
<tr>
<td>1.2.2.2 The dense tubular system</td>
<td>21</td>
</tr>
<tr>
<td>1.2.2.3 Alpha granules, dense granules and lysosomes</td>
<td>21</td>
</tr>
</tbody>
</table>
1.2.3 Platelet receptors ................................................................. 23
1.2.4 Platelet integrins/glycoproteins .............................................. 26
  1.2.4.1 Integrin αIIbβ3/GPIIbIIIa .................................................. 28
  1.2.4.2 Integrin α2β1/GPIaIIa ...................................................... 30
  1.2.4.3 GPIb-IX-V ................................................................... 31
1.2.5 Platelets in haemostasis .......................................................... 33
  1.2.5.1 The intrinsic pathway ...................................................... 34
  1.2.5.2 The extrinsic pathway ...................................................... 35
  1.2.5.3 The common pathway ..................................................... 35
1.2.6 Platelet adhesion, aggregation and plug formation .................... 36
1.2.7 The roles of platelet within innate and adaptive immunity .......... 37
1.3 Introduction Part III: Periodontitis and cardiovascular disease ...... 38
  1.3.1 The systemic impact of periodontitis .................................... 38
  1.3.2 Periodontal infection and atherosclerotic vascular disease ....... 40
1.4 Research rationale and Thesis aims .......................................... 44

Chapter 2: Materials and methods ............................................... 45
  2.1 Materials ............................................................................... 46
  2.2 Antibodies ............................................................................ 47
  2.3 Bacterial Strains and Mutants ................................................. 49
  2.4 Bacterial Growth .................................................................. 50
  2.4.1 Long Term Bacterial Storage .............................................. 50
  2.5 Megakaryocytic Cell Culture and Differentiation ....................... 51
  2.5.1 Megakaryocyte Cell Line CHRF-288-11 ................................ 51
2.5.2 Cell Passaging .............................................................................. 51
2.5.3 Cryopreservation of Cells .............................................................. 51
2.5.4 CHRF-288-11 Differentiation ......................................................... 52
2.5.5 Cell Counting ................................................................................ 52

2.6 Platelet Isolation ............................................................................... 52
2.6.1 Blood collection ............................................................................. 53
2.6.2 Platelet rich plasma preparation .................................................... 53
2.6.3 Platelet poor plasma preparation ................................................... 53
2.6.4 Modified Tyrode's Buffer ............................................................... 53

2.7 Phenotypic Analysis Methods ............................................................ 54
2.7.1 Antibiotic Protection Assays .......................................................... 54
2.7.2 Immunofluorescent Microscopy ....................................................... 55
2.7.2.1 Phenotypic characterisation of CHRF-288-11 differentiation ...... 55
2.7.2.2 Phenotypic evaluation of bacterial challenge on differentiated CHRF-
288-11 cells ......................................................................................... 56
2.7.2.3 Visualisation of platelets challenged with perio-pathogens ........ 57
2.7.3 Electron microscopy ......................................................................... 58
2.7.3.1 2.8.3.1 White's Saline ................................................................. 58
2.7.3.2 Electron microscopy of platelet/perio-pathogen interactions ........ 58
2.7.4 Flow cytometry ............................................................................... 59
2.7.4.1 Relative expression of platelet markers by CHRF-288-11 cells ....... 59
2.7.4.2 Phenotypic analysis of platelet/perio-pathogen interactions ........ 60
2.7.5 Enzyme linked immunosorbent assays (ELISAs) .............................. 61
Chapter 2: Platelet Function Methodology

2.7.5.1 Development of a cell-based ELISA ............................................. 61
2.7.5.2 Recombinant protein-based ELISA ............................................ 62

2.8 Platelet Function Methodology ................................................................ 63

2.8.1 Platelet Aggregation ............................................................................. 63

2.8.1.1 Agonist preparation ........................................................................ 63
2.8.1.2 Platelet inhibitors ........................................................................... 64
2.8.1.3 Light transmission aggregometry ................................................... 64

2.8.2 Platelet Calcium Mobilisation ............................................................. 65

2.8.3 Platelet Granule Release Quantitation by Enzyme-linked Immunosorbent Assay (ELISA) ................................................................. 66

2.9 Statistical Analysis .................................................................................... 66

Chapter 3: Characterisation of an in vitro cellular model to study platelet-periodontal pathogen interactions ........................................................................ 67

3.1 Introduction ............................................................................................. 68

3.2 Aims ......................................................................................................... 69

3.3 Results ..................................................................................................... 70

3.3.1 Optimisation of CHRF-228-11 differentiation ..................................... 70

3.3.2 Periodontal pathogen invasion and adhesion of CHRF-288-11 cells ..... 75

3.3.3 Analysis of CHRF-288-11 interactions with periodontal pathogens by immunofluorescent microscopy ......................................................... 82

3.3.3.1 Analysis of CHRF-288-11 mToR expression following incubation with periodontal pathogens ........................................................................ 82

3.3.3.2 Analysis of CHRF-288-11 integrin β3 expression following incubation with periodontal pathogens ...................................................................... 87
3.3.4 Flow cytometric analysis of megakaryocytic/platelet markers following *P. gingivalis* challenge .................................................. 92

3.3.5 Development of a cell-based ELISA for immunophenotyping of CHRF-288-11 cells following periodontal pathogen challenge .................................. 94

3.3.5.1 Is *P. gingivalis* association and interaction with CHRF-288-11 dependent on the multiplicity of infection? ........................................ 96

3.4 Discussion .................................................................................. 100

3.5 Summary ..................................................................................... 107

Chapter 4: Can periodontal pathogens interact with and induce platelet aggregation? ................................................................................. 108

4.1 Introduction ................................................................................. 109

4.2 Aims ........................................................................................... 110

4.3 Results ......................................................................................... 111

4.3.1 Platelet aggregation with wildtype *P. gingivalis* and *T. forsythia* ........ 111

4.3.2 Investigation of the effect of bacterial virulence on platelet aggregation in induced by *P. gingivalis* .................................................................. 113

4.3.3 Investigation of aggregation induced by *T. forsythia* mutants .......... 115

4.3.4 Investigations into the effect of bacterial viability on platelet aggregation ................................................................................................. 116

4.4 Discussion ................................................................................... 119

4.5 Summary ..................................................................................... 125

Chapter 5: Development and characterisation of a multicolour flow cytometry panel ............................................................................. 126

viii
5.1 Introduction ........................................................................................................127
5.2 Aims ..................................................................................................................129
5.3 Method development and gating strategy .........................................................130
  5.3.1 Panel selection ..............................................................................................130
  5.3.2 Flow cytometer instrument configuration ..................................................131
5.4 Results ...............................................................................................................136
  5.4.1 Antibody optimisation ................................................................................136
  5.4.2 Gating strategy ............................................................................................138
5.5 Adaptation of stimulated population ...............................................................140
5.6 Discussion .........................................................................................................144
5.7 Summary ............................................................................................................147

Chapter 6: The effect of periodontal pathogens on platelet function and protein expression .................................................................148
6.1 Introduction .......................................................................................................149
6.2 Aims ..................................................................................................................150
6.3 Results ...............................................................................................................151
  6.3.1 Analysis of platelet-\textit{P. gingivalis} interactions by immunofluorescence microscopy ..............................................................................................151
  6.3.2 Investigation of platelet aggregates by electron microscopy ......................157
  6.3.3 Analysis of platelet activation and bacterial association by flow cytometry ..............................................................................................161
  6.3.4 Induction of calcium mobilisation by periodontal pathogens .................167
6.3.5 Investigations into platelet degranulation by *P. gingivalis* ............... 171
6.3.6 Does the secretome of *P. gingivalis* induce platelet aggregation? .......... 173
6.3.7 Investigation of platelet receptor inhibitors on *P. gingivalis* induced platelet aggregation ............................................................................................................. 177
  6.3.7.1 The effect of Toll-like receptor inhibitors on *P. gingivalis* induced platelet aggregation ................................................................................................................. 177
  6.3.7.2 The effects of integrin $\alpha_3\beta_1$ and $\beta_3$ inhibitors on *P. gingivalis*-induced platelet aggregation .......................................................................................... 180
6.3.8 Development of a recombinant protein-based ELISA for the detection of *P. gingivalis*-platelet integrin interactions ......................................................... 186
6.4 Discussion ............................................................................................................. 188
6.5 Summary ................................................................................................................ 198

Chapter 7: General discussion and future directions .............................................. 199
  7.1 Introduction ........................................................................................................... 200
  7.2 The use of CHRF-288-11 cells as an *in vitro* platelet model ....................... 200
  7.3 Development of a multiparameter flow cytometry panel .................................. 201
  7.4 Periodontal pathogens and platelet interactions ............................................... 202
  7.5 Future Directions ............................................................................................... 203

Bibliography ............................................................................................................. 210
Ethical approval ....................................................................................................... 256
Copyright licences .................................................................................................... 257
List of figures

Figure 1.1 Tooth, a cross section of an adult human molar. ........................................... 2
Figure 1.2 Model of biofilm formation on the tooth surface............................................. 5
Figure 1.3 Periodontal disease progression................................................................. 7
Figure 1.4 Bacterial complexes of the oral microbiome............................................... 8
Figure 1.5 The cell lineage of megakaryocytes............................................................ 17
Figure 1.6 Representation of the 24 prototypical mammalian integrins..................... 27
Figure 1.7 The structure of integrin αIIbβ3................................................................. 29
Figure 1.8 The GPIb-IX-V complex............................................................................ 32
Figure 1.9 The intrinsic, extrinsic and common pathways of the coagulation cascade. 34
Figure 1.10 Schematic representation of the potential role of periodontal pathogens in atherosclerotic vascular disease progression......................................................... 42
Figure 3.1 CHRF-288-11 differentiation with 10 ng/ml of PMA........................................ 71
Figure 3.2 CHRF-288-11 differentiation using 50 ng/ml of PMA..................................... 72
Figure 3.3 Analysis of megakaryocytic protein expression following CHRF-288-11 differentiation........................................................................................................... 74
Figure 3.4 Invasion of CHRF-288-11 cells by *P. gingivalis* NCTC 11834.................... 76
Figure 3.5 *P. gingivalis* invades differentiated CHRF-288-11 cells............................ 77
Figure 3.6 CHRF-288-11 cellular invasion by *T. forsythia* ATCC 43037....................... 78
Figure 3.7 Invasion of differentiated CHRF-288-11 cells by *T. forsythia* ................... 79
Figure 3.8 Bacterial adhesion and invasion of CHRF-288-11 cell by periodontal pathogens....................................................................................................................... 81
Figure 3.9 The effect of *P. gingivalis* invasion on mToR expression in CHRF-288-11 cells. ......................................................................................................................... 83
Figure 3.10 Determination of mToR expression following exposure to *T. forsythia* in CHRF-288-11 cells. ................................................................................................................. 84

Figure 3.11 *P. gingivalis* decreases mToR expression in differentiated CHRF-288-11 cells. ......................................................................................................................... 85

Figure 3.12 Effect of *T. forsythia* invasion on mToR expression in differentiated CHRF-288-11 cells.............................................................................................................................................................. 86

Figure 3.13 The effect of *P. gingivalis* invasion on integrin β3 expression in CHRF-288-11 cells .......................................................................................................................... 88

Figure 3.14 Relative integrin β3 expression following exposure to *T. forsythia* ATCC 43037................................................................................................................................................ 89

Figure 3.15 The effect of *P. gingivalis* invasion on integrin β3 expression by differentiated CHRF-288-11 cells........................................................................................................ 90

Figure 3.16 *T. forsythia* increases β3 expression in differentiated CHRF-288-11 cells.. 91

Figure 3.17 Quantitation of megakaryocytic surface marker expression following challenge with *P. gingivalis* using flow cytometry. ................................................................. 93

Figure 3.18 Analysis of bacterial association and integrin expression by a cell-based ELISA................................................................................................................................................. 95

Figure 3.19 Specific periodontal pathogen interaction with differentiated CHRF-288-11 cells.. .............................................................................................................................. 97

Figure 3.20 The effect of MOI on periodontal pathogen induced integrin expression in differentiated........................................................................................................................................... 99

Figure 4.1 Induction of platelet aggregation by wildtype (WT) periodontal pathogens. ............................................................................................................................................................ 112

Figure 4.2 The effect of virulence associated proteins on platelet aggregation.. ...... 114

Figure 4.3 Platelet aggregation by *T. forsythia* WT strain and mutants..................... 115
Figure 4.4 The effect of bacterial viability on platelet aggregation. ........................................ 118
Figure 5.1 Reagent titrations. ........................................................................................................ 137
Figure 5.2 Platelet gating strategy .................................................................................................. 139
Figure 5.3 Representative revised gating strategy of platelet isolation ........................................ 140
Figure 5.4 Isolation of pathogen associated platelets ....................................................................... 142
Figure 5.5 Comparative histograms of platelet activation following exposure to *P. gingivalis*.) ................................................................................................................................. 143
Figure 6.1 *P. gingivalis* localises and associates with platelets ......................................................... 152
Figure 6.2 The effect of *P. gingivalis* on platelet integrin β1 expression ........................................ 153
Figure 6.3 The effect of *P. gingivalis* on platelet integrin β3 expression ......................................... 154
Figure 6.4 Platelet aggregate phenotyping by immunofluorescence microscopy ............................... 156
Figure 6.5 Phenotypic analysis of platelet aggregates by scanning electron microscopy (SEM) ........ 158
Figure 6.6 Visualisation of platelet aggregates by transmission electron microscopy (TEM) ........... 160
Figure 6.7 Detection of wild-type periodontal pathogen-platelet association by flow cytometry .... 162
Figure 6.8 Expression of platelet activation markers following association with *P. gingivalis*) ....... 163
Figure 6.9 The role of *P. gingivalis* outer membrane protein A (OMPA) in platelet association .......... 165
Figure 6.10 *P. gingivalis* outer membrane protein (OMP) deficient mutants induce platelet activation ................................................................. 166
Figure 6.11 Platelet calcium mobilisation is induced by wild type periodontal pathogens ................. 168
Figure 6.12 Platelet calcium mobilisation is induced by OMPA deficient *P. gingivalis* mutants. .......................................................... 170

Figure 6.13 Induction of platelet degranulation by wild type *P. gingivalis*. ................. 172

Figure 6.14 Platelet aggregatory response following exposure to *P. gingivalis* culture supernatant. .............................................................................................................. 174

Figure 6.15 *P. gingivalis* LPS as a platelet agonist. ......................................................... 176

Figure 6.16 The effect of Toll-like receptor inhibitors on *P. gingivalis* induced platelet aggregation................................................................................................................ 178

Figure 6.17 The effects of Toll-like receptor inhibitors on *P. gingivalis*-platelet interactions...................................................................................................................... 179

Figure 6.18 Representative aggregation plots following incubation with integrin β₃ inhibitor.................................................................................................................. 181

Figure 6.19 Representative aggregation plots following incubation with integrin α₂β₁ inhibitor.................................................................................................................. 182

Figure 6.20 Effects of integrin β₃ inhibitor on *P. gingivalis* NCTC 11834 induced platelet aggregation............................................................................................................. 184

Figure 6.21 Effects of integrin α₂β₁ inhibitor on *P. gingivalis* NCTC 11834 induced platelet aggregation........................................................................................................ 185

Figure 6.22 Detection of *P-gingivalis*-integrin binding by recombinant protein-based ELISA......................................................................................................................... 187

Figure 7.1 Circular representation of the *P. gingivalis* NCTC 11834, ATCC 381 and ATCC W50 genomes. .............................................................................................................. 207
List of tables

Table 1.1 Platelet granules and their primary functions.................................................. 22
Table 1.2 Major platelet receptors and their ligands/agonists........................................ 25
Table 1.3 Theoretical mechanisms proposed to how explain periodontal disease may have a systemic impact................................................................. 39
Table 2.1 Manufacturers and suppliers. ........................................................................ 46
Table 2.2 Bacterial specific antibodies used within this study........................................ 47
Table 2.3 Primary antibodies used throughout this study................................................ 48
Table 2.4 Secondary antibodies used throughout this study........................................... 48
Table 2.5 Bacterial strains and mutants used within this study........................................ 49
Table 2.6 Platelet inhibitors............................................................................................ 64
Table 5.1 Flow cytometer instrument configuration....................................................... 132
Table 5.2 Commercial reagents used.............................................................................. 133
Table 5.3 Representative compensation matrix. ............................................................ 134
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine diphosphate</td>
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<td>Brain heart infusion broth</td>
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<td>CFU</td>
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</table>
Chapter 1: Introduction
1.1 Introduction Part I: Periodontitis and the oral microbiome

1.1.1 The oral microbiota

During gestation, a human infant remains essentially sterile but during birth, immediately acquires microbial communities from the birth canal and surrounding environment (Perez-Muñoz et al., 2017). It is these pioneering bacterial colonies that form the human microbiome resulting in symbiotic relationships that inhabit various areas of the human body, including the skin, digestive tract and the mouth (Costello et al., 2012). The oral cavity, provides distinct microbial habitats including the tongue, cheek, lip and oesophagus, as well as specific localised areas such the gingiva and the enamel (Xu et al., 2015) (Figure 1.1).

Figure 1.1 Tooth, a cross section of an adult human molar. A structural, anatomical depiction of the human tooth, showing some areas of biofilm inhabitation such as the enamel and gingiva. (Encyclopaedia Britannica, 2013)
In healthy individuals, the human oral cavity is heavily colonised by a magnitude of microorganisms including bacteria, fungi, viruses and archaea (Winkelhoff et al., 1986; Mager et al., 2003; Wade, 2013). To date, around 700 bacterial species are known to form the complex structure of the human oral microbiota, although it has been hypothesized that a large number are yet to be identified due to lack of successful in vitro cultivation (Avila, Ojcius and Yilmaz, 2009). These diverse, polymicrobial, communities that inhabit the oral cavity exist predominantly as biofilms and function as a co-ordinated, fully metabolic society on every surface of the mouth including the teeth, gums, tongue and saliva (Marsh, 2004).

### 1.1.2 Oral biofilm formation

Biofilms can be defined as dynamic, complex biological systems that house multiple microorganisms providing a protected mode of growth, allowing cells to both survive in hostile environments and diverge to inhabiting new niches (Hall-Stoodley, Costerton and Stoodley, 2004). The first observation of biofilms was made by van Leeuwenhoek, who over 300 years ago described the vast array of 'animalcules' within dental plaques using primitive microscopy (Donlan, 2002). A general theory however, was not developed until 1978, stating that the majority of bacteria grow in biofilms that are adherent to surfaces, enclosed in an extra-cellular polymeric substance matrix and that differ profoundly from their planktonic (floating) counterparts (Costerton, Geesey and Cheng, 1978).

Contrary to initial thoughts, oral biofilm formation is not a simple, uniform process but rather a sequence of complex developmental phases consisting of five stages (Hall-Stoodley, Costerton and Stoodley, 2004). Stages one and two are generally termed as
the association of transient bacteria which is followed by adhesion. Stages three and four are identified as the aggregation of cells into micro-colonies followed by their subsequent growth and maturation. The fifth stage describes a return to the mobile, transient phase following significant bacterial disturbance (Hall-Stoodley, Costerton and Stoodley, 2004). Within the mouth the initial stage which consists of transient bacteria, termed 'early colonisers', pioneer the colonisation process, in hopes of altering the habitat so that it is suitable for other species to populate (Socransky and Haffajee, 2005). These specific early colonisers have the unique ability to adhere to oral surfaces such as the host epithelial cells of the cheeks and gums, other established bacterium or bacterial fragments as well as the salivary pellicle; the latter being a selective, protective barrier between the tooth surface and the oral environment, consisting of host proteins, peptides and other organic molecules (Kolenbrander et al., 1993; Li et al., 2003; Black et al., 2004). A selection of early colonising bacteria (Figure 1.2), depicts the interactions between bacteria and specific adhesion receptors within the salivary pellicle including agglutinins, mucins, phospho-proteins, proline-rich proteins and specific enzymes such as alpha-amylase, known bacterial interactions within the biofilm are also highlighted (Kolenbrander, Andersen, David, et al., 2002). Of the early colonisers, between 47-90% of the cultivatable species consist of Streptococci with the remainder population constituting of other genera including Actinomyces, Veillonella and Neisseria (Palmer et al., 2003; Li et al., 2004).
Figure 1.2 Model of biofilm formation on the tooth surface. Early colonisers are shown to interact with receptors on the salivary pellicle. Co-aggregation between the early colonisers, *Fusobacterium nucleatum* as well as later colonisers such as *Porphyromonas gingivalis* and *Treponema denticola* are also illustrated. (Kolenbrander, Andersen, David, et al., 2002)

After initial colonisation, further bacterial species can become established within the biofilm through coaggregation; a process which allows cell-to-cell recognition and adhesion between genetically different bacteria, through facilitating mediators called adhesins (Figure 1.2) (Kolenbrander et al., 2006). One such example is the late coloniser *Fusobacterium nucleatum* which is able to coaggregate intergenetically with all known types of oral bacterial species (Gibbons and Nygaard, 1970; Kolenbrander et al., 2010).
1.1.3 Subgingival biofilm and periodontal disease

In healthy individuals, the oral microbiota generally consists predominantly of commensals or non-pathogenic bacteria such as Streptococci, Actinomyces and Veillonella (Jenkinson and Lamont, 2005). However, within a diseased state, the formation of a dysbiotic biofilm together with an increase in the pathogenic bacterial load leads to periodontal diseases, which encompasses any disorder of the tissues surrounding and supporting the teeth (the periodontium) and can affect up to 90% of the global population (Pihlstrom, Michalowicz and Johnson, 2005). Currently, whilst it is well-established that pathogenic bacteria are prevalent within periodontal diseases, the initial trigger or underlying cause which initiates this increase in pathogenic load and results in a diseased state remains to be elucidated. It is currently unclear whether this is a consequence of changes in one or more bacterial species, or whether it is initiated by the host, locally or systemically (Socransky and Haffajee, 2005).

Periodontal disease usually refers to the common inflammatory conditions of gingivitis and periodontitis. Gingivitis, the mildest form, is highly prevalent, effecting between 50-90% of the global population and is easily reversible through routine oral care (Albandar and Rams, 2002). Periodontitis, the chronic inflammatory form of the disease, is characterised by the destruction of the periodontium and surrounding connective tissues, with severe cases resulting in alveolar bone loss and loss of teeth (Lamont et al., 1995; Pihlstrom, Michalowicz and Johnson, 2005). The progression of gingivitis to advanced periodontitis is shown in Figure 1.3.
Figure 1.3 Periodontal disease progression. Progression of periodontal disease is measured by the level of inflammation, gum recedence and the progressive loss of both connective and bone tissue. The disease has two main classifications: the acute condition, gingivitis and the chronic condition, periodontitis (Hajishengallis, 2015).

Studies conducted by Socransky and collagues (1998) have shown that several bacterial complexes are associated with either periodontal health or disease. With regard to periodontitis, three bacterial species have been termed the 'red-complex' of perio-pathogens due to their involvement with the disease (Figure 1.4). These gram negative, anaerobic, red complex pathogens; Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola have been associated with periodontal disease with P. gingivalis being reported as the keystone pathogen in periodontitis (Lamont et al., 1995; Socransky et al., 1998; Darveau, 2010).
Regardless of the initial trigger, 'red complex' pathogens such as *P. gingivalis* are able to integrate within developing biofilms by utilizing certain levels of metabolic cooperation and compatible adhesins provided by early colonizing bacteria (Holt and Ebersole, 2005). It is the growing availability of these factors provided by localised tissue degradation and further bacterial colonisation that drives the increasing population and complexity of the gram-negative, anaerobic bacterial populations resulting in the bacterial community moving from a commensal environment and producing a phenotypical shift to a diseased state (Ruby and Barbeau, 2002; Jenkinson and Lamont, 2005). The progression of periodontal disease from a mild gingivitis state to the chronic, inflammatory state of periodontitis is the consequence of several cycles of tissue destruction caused by the combined impact of the now substantial pathogenic bacterial challenge and subsequent localised inflammatory response by the
host; with the bacterial challenge being supressed by the host immune response before the cycle repeating itself (Wilson and Henderson, 1995).

1.1.4 Pathogenic bacterial species

It is clear that the development of periodontal disease is reliant on a series of complex host/bacterial interactions with specific keystone pathogens implicated throughout the progression of the disease (Socransky et al., 1998; Holt and Ebersole, 2005). Of these fundamental pathogens, both *P. gingivalis* and *T. forsythia* have been reproducibly found in elevated numbers in patients with periodontitis (Byrne et al., 2009; Zhu et al., 2013) and although the pathological mechanisms of these bacteria are still under scrutiny, it is their unique virulence qualities that allows them to evade the host immune system and produce the destructive characteristics of the disease (Hernandez et al., 2011).

1.1.4.1 *Porphyromonas gingivalis*

*Porphyromonas gingivalis* is a rod shaped, Gram-negative, non-motile, asaccharolytic, black pigmented, anaerobic bacteria that can exist with or without fimbriae (Mayrand and Holt, 1988). Within the healthy oral flora, *P. gingivalis* can be found in low abundance contributing <5% of the total bacterial population (Forng et al., 2000), with this number increasing during the conversion from a symbiotic to a dysbiotic microbiota, through accumulation and proliferation of the bacterium within the dental plaque (Haffajee et al., 1998). Despite the aetiology of periodontitis remaining inconclusive, the underlying presence of *P. gingivalis* within periodontitis, coupled with the pathogen's ability to manipulate the oral bacterial community, has led to the classification of *P. gingivalis* as a 'keystone' pathogen within periodontal disease (Hajishengallis, Darveau and Curtis, 2012). As a survival strategy, *P. gingivalis* is known
to adhere to and invade host cells as well as produce a variety of virulence factors in order to acquire nutrients, proliferate and evade the active surveillance of the immune system (Papapanou et al., 1994; Holt et al., 1999). An overview of these bacterial virulence factors are detailed below.

1.1.4.1.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of Gram-negative bacterial cell walls which provides membrane stability and structural integrity to the bacteria (Ulevitch and Tobias, 1999). LPS is also able to stimulate host immune response through interactions with various receptors on the host's cells, initiating host cell activation and the production of innate host defence mediators such as proinflammatory cytokines, adhesion molecules and apoptosis (Caroff and Karibian, 2003; Dauphinee and Karsan, 2006). Bacterial LPS consists of three domains; a hydrophobic domain known as lipid A (or endotoxin), a non-repeating 'core' oligosaccharide and a distal polysaccharide O-antigen that interacts with the external environment (Ogawa and Yagi, 2010). The structural composition of LPS can however vary between bacterial species, for example changes within the fatty acid chain composition and charge, which can significantly vary the stimulation of host response. In the case of *P. gingivalis*, LPS released within outer membrane vesicles (Grenier and Mayrand, 1987) can penetrate periodontal tissues and stimulate innate immunity, thus contributing to the progression of periodontal disease (Moore, Wilson and Kieser, 1986; McCoy et al., 1987). Specifically *P. gingivalis* derived LPS has been shown to interact with various mammalian receptors including; toll-like receptor 2 and 4 within endothelial cells (Darveau et al., 2004), activation of monocytes through interactions with CD14 (Shapira et al., 1994) as well as acting as an antagonist for E-selectin supressing endothelial cell adhesiveness and promoting bacterial colonisation (Darveau et al., 1995). In addition, LPS derived from
*P. gingivalis* have been shown to vary in profile according to clinical isolates and can affect antibiotic resistance to polymyxin B and colony morphology (Díaz *et al.*, 2015). However, despite the plethora of research focussing on *P. gingivalis* LPS, the underlying roles of LPS as well as the basis of inter-stain LPS variation are yet to be fully established.

### 1.1.4.1.2 Gingipains

One of the most studied aspects of *P. gingivalis* virulence are a group of cysteine proteases belonging to the peptidase C25 family, termed gingipains (Eichinger *et al.*, 1999). *P. gingivalis* actively expresses three types of gingipain; the arginine specific gingipain A (RgpA), arginine specific gingipain B (RgpB) and a lysine specific gingipain (Kgp) (Curtis *et al.*, 1999). These ‘trypsin like’ enzymes are known to cleave polypeptides at the C-terminal of the respective arginine and lysine residues (Li and Collyer, 2011) and accounts for ~85% of the proteolytic activity of *P. gingivalis* (Potempa, Pike and Travis, 1997).

Functionally, the gingipains expressed by *P. gingivalis* have been implicated in a number of bacterial and eukaryotic cell interactions. For example, gingipain activity has been shown to facilitate binding of *P. gingivalis* to extracellular matrix proteins such as fibronectin and collagen (O’Brien-Simpson *et al.*, 2005) as well as bacterial adherence to both epithelial and endothelial cells (Pathirana *et al.*, 2007). All three gingipains have also been shown to be involved in nutrient acquisition through haemolysis (Smalley *et al.*, 2008; N. Li *et al.*, 2010), in haem uptake (Simpson, Olczak and Genco, 2004) and in the disruption of the coagulation system via the degradation of fibrinogen (McAlister *et al.*, 2009) and activation of thrombin and factor IX (Imamura *et al.*, 2001).
Previous work by several groups have also linked gingipain protease activity to the disruption of innate immune response through the degradation of multiple signalling peptides including complement proteins (Popadiak et al., 2007), cell surface receptors CD4 (Kitamura et al., 2002) and CD8 (Kitamura et al., 2002), intracellular adhesion molecule 1 (ICAM-1; Tada et al., 2003), as well as several cytokines including IL-1β (Stathopoulou et al., 2009), IL-6 (Banbula et al., 1999), IL-8 (Uehara et al., 2008) and TNF-α (Calkins et al., 1998).

1.1.4.1.3 Hemagglutinins

Hemagglutinins are a large class of virulence factors that are highly implicated with bacterial adhesion and nutrient acquisition (Han, Whitlock and Progulske-Fox, 1996; Lee, Hillman and Progulske-Fox, 1996; Shi et al., 1999). P. gingivalis is known to express at least eight hemagglutinins, of which a subset has been characterised and are known to be encoded by the group of hag genes, hagA-hagE (Connolly et al., 2017). Hemagglutinins are known to play key role in the absorption of heme, through erythrocyte binding, the facilitation of gingipain activity and agglutination (Lewis et al., 2006; Olczak et al., 2008), as well as been shown to mediate the adherence to both epithelial (Connolly et al., 2017) and endothelial (Song et al., 2005) cells. Further to this, work by Ito and colleagues (2010) identified that the hagA gene mediates coaggregation between P. gingivalis and Treponema denticola, suggesting a role for hemagglutinin domains within biofilm formation as well as mammalian cell adhesion.

1.1.4.1.4 Fimbriae

Fimbriae are thin, proteinaceous filaments that protrude from the outer membrane of bacterial cells (Jonson, Normark and Rhen, 2004) and were first identified on P. gingivalis by Yoshimura and colleagues (1984). P. gingivalis is known to express two
distinct types of fimbriae encoded by the \textit{fimA} gene, termed long/major fimbriae and the \textit{mfa1} gene, termed short/minor fimbriae (Enersen, Nakano and Amano, 2013). Major fimbriae of \textit{P. gingivalis} can be classified into six types; types I-V and Ib which are dependent on amino terminals and DNA sequences (Nakagawa \textit{et al.}, 2000, 2002). Interestingly the presentation of major fimbriae varies between strains of \textit{P. gingivalis}. Strains such as ATCC 381 and NCTC 11834 are highly fimbriated type I strains whereas strains such as ATCC W50 and W83 have much shorter, sparsely populated, type IV fimbriae (Sojar, Hamada and Genco, 1997). Despite the limited characterisation of \textit{mfa1} expression, both major and minor fimbriae are thought to contribute to both bacterial virulence and the progression of periodontal disease (Nakagawa \textit{et al.}, 2002).

The primary role of \textit{fimA} is to promote bacterial adherence to oral surfaces with \textit{fimA} deficient strains presenting a reduced capacity for both adherence (Hamada \textit{et al.}, 1994) and invasion (Njoroge \textit{et al.}, 1997; Weinberg \textit{et al.}, 1997) of oral epithelial cells. It is also thought that minor fimbriae contribute to the adhesive properties of \textit{P. gingivalis} through the coaggregation of bacterial species, the formation of micro-colonies and resistance to sheer forces (Lamont \textit{et al.}, 2002; Lin, Wu and Xie, 2006). Further to this, both the major (Ogawa, Uchida and Hamada, 1994) and minor (Amano \textit{et al.}, 2004) fimbriae have been shown to initiate immune response through the stimulation of inflammatory cytokine production by both monocytes and macrophages including interleukin 1β, interleukin 6, interleukin 8 and tumour necrosis factor α (Ogawa, Uchida and Hamada, 1994; Amano \textit{et al.}, 2004).
1.1.4.1.5 Outer membrane A-like proteins

Within *Escherichia coli* and other *Enterobacter* species, the outer membrane protein, OmpA, is a highly abundant protein responsible for several functions including acting as an adhesin, an invasin, an evasin as well as forming an immune target and contributing to biofilm formation (Smith et al., 2007). The two major outer membrane subunits which form the OmpA-like protein within *P. gingivalis* have been shown to share a high degree of similarity to that of the OmpA of *E. coli* and have been proposed to hold similar cellular functions (Nagano et al., 2005). Previous research by Iwami and colleagues (2007) identified that OmpA is crucial for membrane stability of *P. gingivalis* with further work by Murakami and colleagues (2014) suggesting that the individual subunits of OmpA could be responsible for glycoprotein interaction. Recently, the OmpA protein, specifically the A2 subunit, has been shown to contribute to both biofilm formation and host-cell interactions with epithelial cells (Naylor et al., 2016), with the *ompA* genes being upregulated in highly invasive populations of *P. gingivalis* (Suwannakul et al., 2010).

1.1.4.1.6 Adherence and invasion

As well as virulence factors, *P. gingivalis*, like any bacterial species, possess the ability to interact with cellular surfaces of the host cells to acquire necessary growth factors and ultimately to survive the harsh counter measures produced by the host (Stafford et al., 2012). As part of this survival strategy, *P. gingivalis* can adhere to and invade a variety of host cells and tissues by exploiting cellular pathways to evade the active surveillance of the immune system (Papapanou et al., 1994; Lamont et al., 1995; Deshpande, Khan and Genco, 1998; Dorn et al., 2000). After invasion, both pathogens and host cells remain viable for an extended period of time during which several cytological changes take place (Belton et al., 1999; Simin F Nakhjiri et al., 2001; Tribble
et al., 2006). These cytological changes include; remodelling of the tubulin and actin cytoskeleton (Yilmaz et al., 2003), prevention and manipulation of the apoptosis pathway through transcriptome alterations (Simin F Nakhjiri et al., 2001), secretion of an ATP hydrolysing enzyme (Yilmaz et al., 2008), dysregulation of cellular calcium signalling in the host cells (Izutsu et al., 1996), selective targeting of the MAP kinase pathway (Watanabe et al., 2001) and a down regulation of interleukin 8 expression at both the transcriptional and post-transcriptional level (Darveau et al., 1998).

1.1.4.2 Tannerella forsythia

Tannerella forsythia, an anaerobic, gram negative bacterium of the Cytophaga-Bacteroides family, was originally isolated from the oral cavity and described as Bacteroides forsythus by Tanner and colleagues (1986).

To date, whilst the presence of T. forsythia in periodontitis is well documented through clinical studies, the virulence factors of the bacterium are not fully characterised (Sharma, 2010). Studies have shown that T. forsythia adheres to and invades epithelial cells both in vivo (Rudney, Chen and Sedgewick, 2005; Colombo et al., 2007) and in vitro (Sabet et al., 2003; Inagaki et al., 2006; Sakakibara et al., 2007). In addition, T. forsythia is known to induce enzymatic activity that both protect the bacterium from the innate immune system, whilst also digesting host proteins as a molecular resource (Sharma, 2010). Specifically T. forsythia is able to trigger an up-regulation of host metalloproteinases (including MMP- 2, 3, 8, 7, 13 14 (Tervahartiala et al., 2000; Kiili et al., 2002), induce neutrophil recruitment, increase inflammatory response (Jusko et al., 2012; Garred et al., 2016) and degrade host proteins such as collagen, elastin, gelatin and casein (Ksiazek, Mizgalska, Eick, et al., 2015). The pathogen also expresses
endolases which can digest fibronectin and induce pro-inflammatory cytokine production (IL-1β, 6, 8 and TNF-α) (Lee et al., 2015). Furthermore, *T. forsythia* expresses sialidase enzymes, which cleave the sialic acids contained within host’s surface glycoproteins (Sharma, 2010). As well as acting as a sources of macronutrients, this process also aides bacterial adhesion to mammalian cells and promotes biofilm development (Godoy et al., 1993; Thompson et al., 2009; Honma, Mishima and Sharma, 2011). *T. forsythia* has also been shown to directly affect host cell transcription by upregulating the expression of an array of host genes (Bakthavatchalu et al., 2011). Taken together, all of these factors ultimately result in cytopathogenic consequences (Nakajima et al., 2006).
1.2 Introduction Part II: Platelets

1.2.1 Megakaryocytes and platelet production

Megakaryocytes constitute a highly specialized subset of the haematopoietic stem cell lineage, that through proliferation and terminal differentiation give rise to circulatory platelets (Deutsch and Tomer, 2006). Usually residing within red bone marrow, immature megakaryocytes undertake a series of maturation stages (Figure 1.5) which result in the expansion of cytoplasmic mass, measuring >50-100 nm and polyploidization up to 128N (Tomer, Harker and Burstein, 1988; Patel, Hartwig and Italiano, 2005).

Figure 1.5 The cell lineage of megakaryocytes. Diploid megakaryocyte progenitor cells, blast forming units (BFU) and colony forming units (CFU), undertake several genetic replications, through endomitosis before reaching maturity. Adapted from Italiano and Hartwig (2007).
Until recently, the bone marrow was thought to be the only location for platelet maturation (Levine et al., 1993). Studies have since demonstrated that megakaryocytes have the ability to migrate out of the red marrow and enter the circulatory system before translocating to the extravascular space of the lung tissue where they undertake platelet biogenesis (Lefrançais et al., 2017). However despite these novel findings, the precise understanding of mass platelet production remains to be elucidated.

It was initially hypothesized that platelets stem from long cytoplasmic protrusions, which extend from megakaryocytes, to form what is termed 'pro platelets' (Radley and Scurfield, 1980) and although other mechanisms were proposed, a modified version of the proplatelet hypothesis provides the most reliable model both in vivo and in vitro (Italiano et al., 1999; Johnson, Fletcher and Morgan, 2016; Ru et al., 2016). This modified model proposes that during the formation of pro platelet, cytoplasmic extensions act as a cellular highway on which megakaryocytes transfer a plethora of cellular structures and proteins to form the anucleate platelets (Deutsch and Tomer, 2006). For example, platelet proteins such as fibrinogen receptors and von Willebrand factor are synthesised by the megakaryocytes before being conveyed to either the newly formed platelet outer-membrane or are packaged into specialised secretory granules (Richardson et al., 2005). Individual organelles such as alpha/dense granules, lysosomes and mitochondria also migrate from the main cellular body of the megakaryocyte, with ~30% of organelles being in motion at any one time (Richardson et al., 2005). During this process, the pro-platelets extend into the sinusoidal space, where they fragment and detach into individual platelets (Machlus and Italiano, 2013). However the underlying mechanism of how platelets are packaged with the necessary organelles and granules remains to be fully understood.
1.2.2 Platelet morphology and ultrastructure

Platelets are small, discoid, anucleate cells, measuring 2-3 µm that hold a fundamental function within haemostasis (Thon and Italiano, 2012). An adult human maintains approximately one trillion platelets, of which two-thirds are in circulation, with the remaining third reversibly sequestered in the spleen (Thon and Italiano, 2010). Circulatory platelets are maintained for 7-10 days, and as well as being anucleate, platelets contain several distinguishing organelles including the open canalicular system, the dense tubular system, as well as alpha and dense granules (Heijnen and Korporaal, 2017).

1.2.2.1 The open canalicular system

The surface connected open canalicular system (OCS) comprises of a complex series of intracellular membrane channels that form an interconnected membrane store with specific function (Heijnen and Korporaal, 2017). As platelets are anucleate and have a limited RNA stores, platelets have restricted capacity for protein synthesis and rely on protein uptake from the surrounding plasma (Zucker-Franklin, 1981). Proteins such as fibrinogen (Harrison et al., 1989) and tissue factor (Escolar et al., 2008) are endocytosed via the OCS and distributed to the relevant organelles, here the alpha granules within the platelet.

The OCS also functions as a cellular delivery system. During platelet activation both alpha and dense granule stores fuse with the OCS and plasma membrane, allowing their content to be trafficked to the cellular surface which does not only facilitate granule release but is also thought to potentiate the activation process (White and Krumwiede, 1987; Fogelson and Wang, 1996).
As well as acting as a cellular transportation network, the OCS is crucial in maintaining the concentration of adhesion receptor levels on the outer membrane (Sixma, 1986; Escolar, Leistikow and White, 1989), by acting as a storage site for glycoprotein adhesion receptors. For example the glycoproteins GPIIbIIIa (Cramer et al., 1990) and GPIb (Cramer et al., 1990), are found to be evenly expressed between the outer membrane and the OCS. It is hypothesised that the reserves of adhesion molecules contained within the OCS are trafficked to the membrane during activation to upregulate adhesion receptors available during thrombus formation (Escolar, Leistikow and White, 1989). Despite the OCS not being classified as a completely separate cellular component, it is thought that this intracellular plasma membrane (Escolar, Leistikow and White, 1989) as well as secretory alpha granules (Peters, Michelson and Flaumenhaft, 2012) facilitate the membrane remodelling process during platelet spreading and filapodia formation. Whether the OCS and plasma membrane are able to fuse remains to be fully understood but it has been suggested that the OCS is crucial for platelet activation acting as both cellular storage and enabling trafficking of both granule stores and glycoproteins, regulating platelet adhesion and aggregation (Selvadurai and Hamilton, 2018).
1.2.2.2 The dense tubular system

Circulatory platelets do not contain ribosomal endoplasmic reticulum. To compensate for this, platelets contain the dense tubular system (DTS) which is derived from the parent endoplasmic reticulum of megakaryocytes (White, 1972). The DTS is primarily thought to regulate and store intracellular calcium in a similar way to that of the sarcoplasmic reticulum of striated muscle cells (White, 1972). During platelet stimulation the DTS has been shown to rapidly discharge stored calcium ions and raise cytoplasmic calcium concentrations (Dean, 1984; Ware et al., 1986) thereby facilitating multiple aspects of platelet activation including granule centralisation (Kroll and Schafer, 1989) and regulation of aggregation (Nesbitt et al., 2003).

1.2.2.3 Alpha granules, dense granules and lysosomes

One of the defining characteristics of platelet haemostatic activity is their large granular storage; namely the alpha granules, dense granules and lysosomes which contain a number of biologically active molecules (Rendu and Brohard-Bohn, 2001).

Alpha granules, the most abundant of the granules within platelets (40-80 per platelet), contain both soluble proteins which are released extracellularly, and membrane bound proteins that become expressed on the plasma membrane (Blair and Flaumenhaft, 2009). This proteinous payload is highly involved in platelet adhesion as well as wound healing, vascular remodelling, immune response and inflammation (Heijnen and van der Sluijs, 2015). Dense granules are the second most abundant of the platelet granules and house a variety of haemostatically active molecules that are predominantly responsible for recruiting further platelets to the site of vascular insult (Flaumenhaft, 2013). Further to this, platelets are also known to contain few primary and secondary lysosomes. Although their function is not well studied, it has been
suggested that their enzymatic content is involved in endosomal digestion as observed in nucleated cells (Flaumenhaft, 2013). Key alpha granules, dense granules and lysosomes are detailed in Table 1.1.

<table>
<thead>
<tr>
<th>Granule</th>
<th>Compound</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>α granule</td>
<td>Platelet factor 4</td>
<td>Neutralises heparin effect/cytokine</td>
</tr>
<tr>
<td></td>
<td>β thromboglobulin</td>
<td>Promotes fibroblast chemotaxis</td>
</tr>
<tr>
<td></td>
<td>Platelet derived growth factor</td>
<td>Mitogen for fibroblast and smooth muscle cells. Chemotaxin for neutrophils</td>
</tr>
<tr>
<td></td>
<td>von Willebrand factor</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td></td>
<td>Thrombospondin</td>
<td>Promotes platelet-platelet interaction</td>
</tr>
<tr>
<td></td>
<td>Fibronectin</td>
<td>Adhesion of platelets and fibroblasts</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen</td>
<td>Promotes adhesion and coagulation</td>
</tr>
<tr>
<td></td>
<td>Factor V</td>
<td>Promotes coagulation</td>
</tr>
<tr>
<td></td>
<td>vascular endothelial growth</td>
<td>Vascular remodelling</td>
</tr>
<tr>
<td>factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dense granules</td>
<td>ADP</td>
<td>Activation of platelets</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>Source of ADP</td>
</tr>
<tr>
<td></td>
<td>Serotonin</td>
<td>Vasoconstriction</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>Coagulation</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>β-galactosidase</td>
<td>Glycoside hydrolase</td>
</tr>
<tr>
<td></td>
<td>Acid phosphatase</td>
<td>Hydrolyses acid phosphatases</td>
</tr>
<tr>
<td></td>
<td>Hexosaminidase</td>
<td>Hydrolyses hexosamines</td>
</tr>
</tbody>
</table>

Table 1.1 Platelet granules and their primary functions. Adapted from Pallister and Watson (2011).
1.2.3 Platelet receptors

Platelets, like many cell types express a multitude of surface receptors in order to sense and interact with their environment. The major platelet receptors as well as their agonist or ligands are summarised in Table 1.2. Platelet integrins/membrane glycoproteins specific to this thesis are reviewed in more detail within Section 1.2.4.
<table>
<thead>
<tr>
<th>Receptor Classification</th>
<th>Platelet Receptor</th>
<th>Ligand/Agonist</th>
<th>Physiological Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrins</td>
<td>α2β1 (GPIIIa, CD49b/61)</td>
<td>Collagen</td>
<td>Platelet adhesion and thrombus stabilisation</td>
</tr>
<tr>
<td></td>
<td>α5β1 (CD49e/61)</td>
<td>Fibronectin</td>
<td>Platelet adhesion</td>
</tr>
<tr>
<td></td>
<td>α6β1 (CD49f/61)</td>
<td>Laminin</td>
<td>Platelet adhesion</td>
</tr>
<tr>
<td></td>
<td>αLβ2 (CD102, ICAM-2))</td>
<td>Leukocyte function-associated antigen 1 (LFA-1)</td>
<td>Leukocyte adhesion</td>
</tr>
<tr>
<td></td>
<td>αIIbβ3 (GPIIb/IIIa, CD41/61)</td>
<td>Fibrinogen, fibrin, von Willebrand factor, fibronectin, vitronectin and thrombospondin</td>
<td>Promotion of platelet aggregation and platelet-platelet binding</td>
</tr>
<tr>
<td></td>
<td>αVβ3 (CD51/61)</td>
<td>Vitronectin, fibrinogen, von Willebrand factor, prothrombin and thrombospondin</td>
<td>Platelet adhesion</td>
</tr>
<tr>
<td>Selectins</td>
<td>P-selectin (CD62P, GMP-140)</td>
<td>P-selectin glycoprotein ligand-1 (PSGL-1), GPIb-IX-V</td>
<td>Expressed during platelet activation through alpha granule release. Binds to neutrophils and monocytes.</td>
</tr>
<tr>
<td></td>
<td>C-type lectin-like receptor-2 (CLEC-2)</td>
<td>Podoplanins and rhodocytin</td>
<td>Platelet aggregation</td>
</tr>
<tr>
<td>Leucine-rich repeat receptors</td>
<td>GPIb-IX-V</td>
<td>von Willebrand factor, thrombin, P-selectin, factor XI, factor XII</td>
<td>Platelet adhesion and aggregation</td>
</tr>
<tr>
<td></td>
<td>Toll-like receptor 2 (TLR2)</td>
<td>Identify various products of bacteria, viruses, protozoa and fungi</td>
<td>Innate immune response and platelet activation</td>
</tr>
<tr>
<td></td>
<td>Toll-like receptor 4 (TLR4)</td>
<td>Identify various products of bacteria, viruses, protozoa and fungi</td>
<td>Innate immune response and platelet activation</td>
</tr>
<tr>
<td>Receptor Classification</td>
<td>Platelet Receptor</td>
<td>Ligand/Agonist</td>
<td>Physiological Role</td>
</tr>
<tr>
<td>-------------------------</td>
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<td>---------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Transmembrane receptors</td>
<td>P2X₁</td>
<td>Adenosine triphosphate (ATP)</td>
<td>Extracellular calcium influx, platelet activation and aggregation</td>
</tr>
<tr>
<td></td>
<td>P2Y₁₂</td>
<td>Adenosine diphosphate (ADP), P2Y₁</td>
<td>Calcium mobilisation, platelet spreading, granule release, thromboxane production, αIIbβ3 activation and platelet aggregation</td>
</tr>
<tr>
<td></td>
<td>P2Y₁</td>
<td>Adenosine diphosphate (ADP), P2Y₁₂</td>
<td>Calcium mobilisation, platelet spreading, granule release, thromboxane production, αIIbβ3 activation and platelet aggregation</td>
</tr>
<tr>
<td>Immunoglobulin receptors</td>
<td>GPVI</td>
<td>Collagen</td>
<td>Intracellular calcium mobilisation, cytoskeletal rearrangement, granule release, GPIIbIIIa activation</td>
</tr>
<tr>
<td></td>
<td>FcγRIIA (CD32)</td>
<td>Binding of GPVI</td>
<td>Intracellular calcium mobilisation, cytoskeletal rearrangement, granule release, GPIIbIIIa activation</td>
</tr>
<tr>
<td>Prostaglandin receptors</td>
<td>Thromboxane A₂/prostaglandin H₂</td>
<td>Thromboxane A₂</td>
<td>Calcium mobilisation and platelet activation</td>
</tr>
<tr>
<td></td>
<td>Prostacyclin receptor (PGI₂)</td>
<td>Prostacyclin</td>
<td>Platelet inhibitor, retains platelet resting state</td>
</tr>
<tr>
<td></td>
<td>Thromboxane A₂/prostaglandin H₂</td>
<td>Thromboxane A₂</td>
<td>Calcium mobilisation and platelet activation</td>
</tr>
<tr>
<td></td>
<td>Prostacyclin receptor (PGI₂)</td>
<td>Prostacyclin</td>
<td>Platelet inhibitor, retains platelet resting state</td>
</tr>
</tbody>
</table>

Table 1.2 Major platelet receptors and their ligands/agonists. Adapted from Saboor and colleagues (2013) and Watson, Morgan and Harrison (2015).
1.2.4 Platelet integrins/glycoproteins

Glycoproteins are a family of transmembrane adhesion and signalling proteins, which within platelets are fundamental to haemostasis and thrombus formation (Clemetson and Polgár, 1997). Many of the glycoproteins expressed by platelets belong to the supergene family of integrins (Saboor et al., 2013). Mammalian integrins are expressed as heterodimers of non-covalently associated α and β subunits (Hynes, 2002). There are 18 α subunits and eight β subunits that construct the 24 separate heterodimers shown in Figure 1.6.
Figure 1.6 Representation of the 24 prototypical mammalian integrins. The integrin family of proteins are constructed of 24 heterodimers, each containing an α and β subunit. (Barczyk, Carracedo and Gullberg, 2010).
Platelets are known to express six integrins; namely three β1 associated integrins (α2β1, α5β1 and α6β1), a further two β3 associated integrins (αIIbβ3 and αVβ3) as well as integrin αLβ2 which are summarised in Section 1.2.3 (Table 1.2). In addition to integrins, platelets are also known to express other glycoproteins such as the von Willebrand factor receptor complex, GPIb-IX-V and the collagen receptors GPIV and GPVI (Saboor et al., 2013). Glycoproteins relevant to the work described in this thesis are reviewed in detail, below.

1.2.4.1 Integrin αIIbβ3/GPIIbIIIa

Expressing 80,000-100,000 copies, integrin αIIbβ3 (GPIIbIIIa, CD41/61) is the major integral plasma membrane protein of platelets, accounting for ~17% of the total membrane mass (Wagner et al., 1996a). Like all integrins, αIIbβ3 is a heterodimer and consists of a 1008 amino acid αIIb subunit and a 762 amino acid β3 subunit. Each of the subunits consist of a large extracellular region, a single transmembrane spanning region and a short cytoplasmic tail (Bennett, 2005). The extracellular domain of the alpha subunit consists of the N-terminal β-propeller domain, the thigh domain and two calf domains (Xia et al., 2004). The extracellular domain of the β3 subunit is composed of an A domain, PSI domain (plexin/semaphorin/integrin), hybrid domain, four EGF domains and a membrane-proximal βTD domain (Xiong et al., 2001; Xia et al., 2004). These 12 domains assemble as two cytoplasmic 'tails' and a globular 'head' that is formed by the interaction of the seven-bladed β-propeller of the α-subunit and the βA domain (Figure 1.7; Takagi et al., 2002; Xia et al., 2004).
Located between the thigh and calf domains is a bending point, referred to as a 'genu' or 'knee'. This genu allows both subunits of the αIIbβ3 integrin to exist in two extreme conformations; a bent resting state or an activated extended state (Takagi et al., 2002). In the bent conformation, the ligand binding site of αIIbβ3 is partially sequestered resulting in a low affinity phenotype, but during platelet activation, this inactive bent integrin extends upwards in a 'switch blade' like motion, opening the head piece into a high affinity state for the ligand (Shimaoka, Takagi and Springer, 2002; Takagi et al., 2002).

αIIbβ3 is known to recognise and bind to the amino acid sequence Arg-Gly-Asp (RGD) present on many extracellular matrix proteins including fibrinogen, fibronectin, vitronectin and von Willebrand factor (Bennett, 2005). Although αIIbβ3 is known to contribute to platelet adhesion through interactions with extracellular matrix proteins
such as von Willebrand factor, this integrin holds a predominant role in facilitating platelet aggregation (George, 2000). Following activation and conformational changes in the αIIbβ3 integrin structure, the receptor is able to bind to soluble circulatory fibrinogen, which then acts as bridging molecules between two αIIbβ3 receptors on adjacent platelets (Refaai and Laposata, 2002).

The conformational change undertaken by integrins following platelet activation are often attributed to the process of inside-out' signalling. Despite this process not being fully understood, it is known that integrin ligand binding by an external stimulus such as collagen or ADP, induces a series of cellular signalling pathways including activating the protein kinase C pathway and calcium mobilisation (Takagi et al., 2002). This in turn then stimulates integrin activation through 'inside-out' signalling, increasing ligand affinity thus driving platelet aggregation and thrombus formation (Bennett, 2005; Bledzka et al., 2013).

1.2.4.2 Integrin α2β1/GPIaIIa

The α2β1 integrin (GPIaIIa, CD49b/61), expressed at 2000-4000 copies per platelet, is often hailed as the second most important platelet receptor after integrin αIIbβ3 (Clemetson and Clemetson, 2007). The importance of α2β1 within coagulation was first identified in patients lacking in α2β1 expression, who present with a mild bleeding disorder and a defective aggregatory response following collagen stimulation (Nieuwenhuis et al., 1985).

The activity of α2β1 is often discussed with the other major platelet collagen receptor GPVI. Historically it was believed that α2β1 was solely responsible for collagen adhesion, with GPVI acting as a regulatory mediator of the subsequent platelet aggregation (Jung and Moroi, 1998). However recent advancements have found that
binding of GPVI to collagen generates further inside-out signalling, activating integrin α2β1 allowing it to also bind to the collagen ligand (Jung and Moroi, 1998, 2000). Interestingly agonists specific to αIIbβ3-induced platelet aggregation have also been shown to activate integrin α2β1 increasing its affinity and initiating collagen binding (Jung and Moroi, 2000). Further to this, under sheer flow the α2β1 collagen receptor is not able to initiate platelet adhesion independently (Sarratt et al., 2005). In vitro, platelets depend on the initial tethering of GPIb of the GPIb-IX-V complex, to initiate adhesion before secondary binding activity is undertaken by further collagen binding proteins such as α2β1 and GPVI, further emphasising the complexity of platelet thrombus formation (Clemetson and Clemetson, 2007).

1.2.4.3 GPIb-IX-V

The GPIb-IX-V complex is a platelet surface receptor with unique properties regarding structure, function and signalling pathways (López, 1994). The history of GPIb-IX-V begins with the discovery of the congenital bleeding disorder, Bernard-Soulier syndrome (Bernard and Soulier, 1948), in which patients present with an increased bleeding time, thrombocytopenia and extremely large platelets, which was later attributed to a deficiency of GPIb-IX-V expression (López et al., 1998). GPIb-IX-V consists of the following subunits; GPIbα (CD42bα) disulphide-linked to GPIbβ (CD42bβ/CD42c), noncovalently complexed with GPIX (CD42a) and GPV (CD42d) all of which contain leucine-rich repeat regions and are expressed in a ratio of 2:2:2:1 respectively (Figure 1.8) (Andrews, Lopez and Berndt, 1997; López et al., 1998; Berndt et al., 2001).
Figure 1.8 The GPIb-IX-V complex. GPIb-IX-V is constructed of the following subunits; GPIbα disulphide-linked to two GPIbβ subunits which are noncovalently associated with GPIX and GPV. The N-terminal houses the ligand binding site and leucine-rich repeat regions. (Hadjkacem, Gargouri and Gargouri, 2011).

The 282 amino acid residues within the N-terminal of GPIbα house the binding site for the ligands von Willebrand factor, macrophage-1 antigen (Mac-1), P-selectin, α-thrombin, clotting factors XI and XIIa, as well as circulatory high-molecular weight kininogen (HMWK; Berndt et al., 2001).

During haemostasis, the predominant function of GPIb-IX-V is adhesion to von Willebrand factor under high sheer flow, after which, several signalling pathways are initiated with platelets, resulting in intracellular calcium mobilisation, cytoskeletal rearrangement, degranulation, inside-out activation of αIIbβ3 and ultimately in platelet aggregation (Andrews, Lopez and Berndt, 1997; López et al., 1998; Bodnar et al., 1999; Gu et al., 1999; Berndt et al., 2001). GPIbα is also known to bind α-thrombin, thereby inducing thrombin dependant platelet aggregation and can bind to factor XI and XIIa which regulates the coagulation cascade (Berndt et al., 2001).
In addition, GPIb-IX-V is also thought to contribute to innate immune response. Interactions between GPIb-IX-V and neutrophil Mac-1 or P-selectin are suggested to facilitate the formation of platelet-leukocyte-endothelial multi-cellular complexes, localising immune response to the site of vascular insult (Cerletti, Evangelista and de Gaetano, 1999; Cerletti, de Gaetano and Lorenzet, 2010).

### 1.2.5 Platelets in haemostasis

Haemostasis is the process in which, following vessel injury, platelets form a clot which serves to limit the resulting haemorrhage (Rasche, 2001). This process is defined in two stages; primary haemostasis consists of the initial adhesion of platelets to the injured vessel and secondary haemostasis which is often referred to as the coagulation cascade. The original coagulation cascade proposed by Macfarlane (1964) suggested that coagulation was initiated by contact of the blood with foreign substances or damaged tissues. This contact then initiates a series of enzymatic steps in which coagulation factors, generally indicated by roman numerals, act firstly as a substrate and then as an activator or catalyst for the subsequent stages (Macfarlane, 1964). Since then, the majority of these 13 coagulation factors have been shown to circulate within the blood stream as zymogens, before being activated into serine proteases (Davie, Fujikawa and Kisiel, 1991). The exceptions are factors V and VIII which are glycoprotein cofactors as well as factor XIII which is a transglutaminase (Chaudhry and Babiker, 2018). Combined, these factors result in the coagulation cascade which concludes in the conversion of fibrinogen to fibrin and thrombus formation (Chaudhry and Babiker, 2018). The coagulation cascade can be separated into the three distinct extrinsic, intrinsic, and common activation pathways which are detailed in Figure 1.9 and summarised below.
1.2.5.1 The intrinsic pathway

The intrinsic pathway is the longer of the two pathways of secondary haemostasis. *In vitro* this pathway is initiated through platelet interaction with the exposed collagen within the damaged extracellular matrix of the vascular endothelium. This interaction initiates the activation of the serine kinase factor XII which becomes XIIa, which in turn acts as a catalyst activating factor XI to factor XIa. This activated XIa then results in the activation of factor X to Xa, giving rise to the subsequent stages of the coagulation cascade (Gailani and Renne, 2007).
1.2.5.2 The extrinsic pathway

The extrinsic pathway is the shorter of the two haemostatic pathways and was named so because of its dependence on the exogenous stimulation by tissue factor (TF) (Davie, Fujikawa and Kisiel, 1991). Following damage to the vessel wall, endothelial cells release tissue factor forming the activated TF-VIIa complex (Nemerson, 1988). This TF-VIIa complex is the key initiator of the protease coagulation cascade, resulting in the activation of both factor IX to IXa and X to Xa (MacKman, 2009). The extrinsic pathway also results in the production of low amounts of thrombin, which further amplifies to coagulation cascade by activating the glycoprotein cofactors V and VIII (Edgington et al., 1991).

1.2.5.3 The common pathway

The common pathway begins with the activation of factor X to Xa, which unifies both the intrinsic and extrinsic pathways. Activated factor X, factor V, tissue phospholipids, platelet phospholipids as well as calcium ions form the thrombinase complex which cleaves prothrombin (factor II) to thrombin (factor IIa) (Coughlin, 2000). Thrombin then cleaves fibrinogen into insoluble fibrin and activates factor XIII, which covalently crosslinks the fibrin polymers, forming the dense fibrin mesh and a definitive secondary haemostatic plug (Palta, Saroa and Palta, 2014).
1.2.6 Platelet adhesion, aggregation and plug formation

The complex series of events that result in normal haemostatic response not only require the enzymatic stages of the coagulation cascade but also rely on fundamental changes in platelet phenotype and cellular activity (Gailani and Renné, 2007). In platelets, primary adhesion or primary haemostasis refers to the attachment of platelets to the sub endothelium, while platelet to platelet ‘adhesion’ is termed aggregation to differentiate the two processes entirely (Clemetson and Polgár, 1997).

Upon the initial insult to the vessel wall, platelets are recruited to the injury site via interactions with the exposed subendothelial extracellular matrix (ECM), resulting in primary adhesion (Ruggeri, 2009). Following contact with the ECM, the platelet glycoprotein (GP) complex Ib-IX-V binds to von Willebrand factor associated with the exposed collagen fibres of the subendothelium. This initial tethering with GPIb-IX-V then leads platelets to undertake subsequent activation signalling and phenotypic cytoskeletal spreading/flattening (Ruggeri and Mendolicchio, 2007).

Once activated, platelets then initiate a further barrage of cellular events, resulting in secondary haemostasis and platelet plug stabilisation. Platelet aggregation is initiated through conformational changes within GPIIbIIIa, which increases the receptors’ binding affinity for fibrinogen, resulting in platelet-platelet aggregation (Clemetson and Polgár, 1997). The growing platelet aggregate is stabilised through a multitude of platelet to platelet interactions contained within the thrombus and remains adhered to the vessel wall through further interaction with the secondary collagen receptors GPIIaIIa, GPIV and GPVI (Holinstat, 2017).

In addition to this, thrombin, as well as acting as a platelet agonist, converts fibrinogen to fibrin, forming a dense, insoluble, fibrin mesh that intertwines adhered platelets and
passing red blood cells, resulting in a definitive platelet plug (Tomaiuolo, Brass and Stalker, 2017). Once formed the haemostatic plugs become firmly adhered to the site of vascular injury, preventing further haemorrhage whilst also resisting the sheer force of circulatory blood flow (Minors, 2007).

### 1.2.7 The roles of platelet within innate and adaptive immunity

As well as having a substantial involvement within the haemostasis, platelets also hold a primary role within the immune system, which at the very principle level rapidly accumulate to the epicentre of any vascular injury and provide an immediate barrier against infection (Gardiner and Andrews, 2013). Platelets have been shown to accumulate and attract other immune cells to sites of infection through active chemotaxis and the release of antimicrobial chemokines, such as platelet factor 4 and interleukin 8 (Lowenhaupt et al., 1982; Juselius and MacDonald, 2004; Skoglund et al., 2008; Ali, Wuescher and Worth, 2015). Further studies have demonstrated that platelets have both the ability to internalize pathogens (Yeaman, 1997, 2010a, 2010b) and to exhibit antimicrobial activity which is triggered by bacterial LPS stimulation (Zielinski et al., 2001; Lopes-Pires et al., 2012). Bacterial LPS has also been shown to upregulate the production of IL-6, cyclooxygenase-2 and prostaglandin E2 through stimulation of toll-like receptor 4, the MAP-kinase pathway and Nf-kB signalling (Scott and Owens, 2008). Periodontitis has also been implicated in bacterial-platelet interactions, with periodontal pathogens, specifically *Porphyromonas gingivalis* increasing the levels of P-selectin excretion, triggering platelet activation (Laky et al., 2011; Ganji et al., 2014), as well as causing an up regulation of CD40L expression through TLR-2 and 4 interactions (Assinger et al., 2012).
1.3 Introduction Part III: Periodontitis and cardiovascular disease

1.3.1 The systemic impact of periodontitis

Periodontitis and the associated chronic periodontal inflammation have historically been linked to other systemic diseases (Kerr, 1951, 1962). Both periodontal diseases and systemic diseases share a number of the similar risk factors such as smoking, age and obesity and often have inflammation as an underlying principle basis (Beck et al., 1998; Paquette, Brodala and Nichols, 2007). A highly studied example of the systemic impact of periodontitis is the development of endocarditis. Although the specific factors that lead to endocarditis are unknown, it is believed that the increased bacterial load of predominantly Staphylococcus, Streptococcus and Enterococcus species found within the bloodstream that leads to infected cardiac tissue could originate from the dysbiotic biofilm found within periodontitis (Carinci et al., 2018).

Further to this, recent research has shown that periodontal bacteria are found within post-mortem human brains (Poole et al., 2013), have been implicated in nerve cell damage (Watts, Crimmins and Gatz, 2008) and shown to exacerbate pathogenesis (Miklossy, 2011) within Alzheimer's disease. In addition, there have been further links between periodontal pathogens and rheumatoid arthritis (RA) with genomic material derived from P. gingivalis been discovered within samples of patient synovial fluid (Martinez-Martinez et al., 2009; Reichert et al., 2013), encouraging suggestions that these bacteria may provide a novel mechanism for the progression of RA (Wegner et al., 2010). Further relationships between periodontal pathogens and diabetes have also been suggested by groups such as Demmer et al., 2008, with further groups
suggesting that periodontitis could produce adverse effects for patients with diabetes (Borgnakke et al., 2013) but also that diabetes may increase the progression of periodontitis (Chávarry et al., 2009). Furthermore, several mechanisms linking periodontitis to the atherosclerotic process and atherosclerotic disease such as stroke and myocardial infarction have been put forward (Joshipura et al., 2003; Reyes et al., 2013; Scannapieco and Cantos, 2016).

Although clear evidence links oral infection and systemic disease, the bacterial interactions that take place are likely to be complex and involve more than a singular mechanism. Due to this fact several theorised mechanisms that have been proposed by which periodontal disease may impact further than the immediate oral environment and have a distal effect (Table 1.3).

<table>
<thead>
<tr>
<th>Table 1.3 Theoretical mechanisms proposed to how explain periodontal disease may have a systemic impact. Adapted from Reyes et al (2013).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oral bacterial invasion of epithelial and endothelial cells;</strong></td>
</tr>
<tr>
<td>• Protects bacteria from host defence to extend bacterial survival</td>
</tr>
<tr>
<td>• Stimulates innate immune response and exacerbates systemic inflammation</td>
</tr>
<tr>
<td><strong>Oral bacteria penetrate and enter the vascular system;</strong></td>
</tr>
<tr>
<td>• Able to migrate and establish infection within other organs and tissues (e.g. brain abscess)</td>
</tr>
<tr>
<td>• Exacerbate or stimulate inflammation in non-localised tissues</td>
</tr>
<tr>
<td>• Directly impact other organs or tissues (e.g. arteries, liver.).</td>
</tr>
<tr>
<td><strong>Biologically active molecules from chronic oral inflammation enter the vascular system and impact both ongoing inflammation or organ/tissue systems (e.g. LPS, cytokines)</strong></td>
</tr>
</tbody>
</table>
1.3.2 Periodontal infection and atherosclerotic vascular disease

Cardiovascular disease (CVD) and atherosclerosis are globally a leading cause of death accounting for ~30% of all deaths worldwide (WHO, 2018). Various cross-sectional studies have suggested that periodontitis is directly associated with atherosclerotic cardiovascular disease independently of traditional risk factors such as smoking and obesity (Bahekar et al., 2007; Friedewald et al., 2009; Kebschull, Demmer and Papapanou, 2010).

When considered broadly, periodontal disease and atherosclerotic vascular disease (AVD) have a multitude of common etiological and epidemiological factors including sharing several risk factor groups (smoking, diabetes, diet) and producing correlative biological markers (C-reactive protein, interleukin 1, endothelial growth factor, interleukin 8 etc.) (Aarabi et al., 2015). Periodontal infection has also been shown to cause an increase in AVD associated markers (Eberhard et al., 2013). Studies have found bacterial DNA through PCR analysis of endarterectomy samples (Nakano et al., 2006, 2007, 2008; Pucar et al., 2007; Gaetti-Jardim et al., 2009), with both genetic material and viable cells of both *P. gingivalis* and *T. forsythia* found in atherosclerotic legions (Marcelino et al., 2010). Further studies have shown that periodontal pathogens can directly influence endothelial cells *in vitro*, causing the up-regulation of inflammatory cytokines, such as IL-1β, IL-6 and IL-8, as well as causing up-regulation of adhesion molecules, monocyte chemotactic protein (MCP-1), intracellular adhesion molecule (ICAM-1), vascular adhesion molecule (VCAM-1), and endothelial-leukocyte adhesion molecule (E-selectin) (Takahashi et al., 2006; Ho et al., 2009). Interestingly, *P. gingivalis* and its fimbriae are able to induce cell adhesion molecule cleavage and endothelial apoptosis (Sheets et al., 2005, 2006). *In vitro* studies have demonstrated
that monocytes suffer very similar consequences to endothelial cells when exposed to periodontal pathogens (Hajishengallis et al., 2006). Further reports also highlight an increased adhesion of monocytes to P. gingivalis infected endothelial cells (Roth, Moser, et al., 2007), with both P. gingivalis and T. forsythia causing an increase in monocytic cytokine and adhesion molecule production (Pollreisz et al., 2010; Sahingur et al., 2010). P. gingivalis has been described to increase the low density lipoprotein (LDL) uptake of monocytes, promoting the formation of foam cells (Giacona et al., 2004). Although conclusive evidence is yet to be presented, three specific and distinct pathways have been proposed as to how periodontal pathogens may migrate into the vascular system; Firstly, bacteria conduct trans-cellular migration, moving through multiple epithelial cells until reaching vascular endothelial cells (Kebschull, Demmer and Papapanou, 2010). Secondly, the pathogen either invade or exploit phagocytosis of host immune cells, evading the intracellular digestion processes and elicit a 'Trojan horse' method of transportation and thirdly, external influence, such as dental work, chewing or brushing of the teeth and gums, directly ruptures the vascular system , bridging the gap to the periodontal infection (Reyes et al., 2013). It is therefore essential to understand the key stages of AVD progression and how periodontal pathogens may influence this process. The following three figures describe; (A) the potential role of periodontal pathogens in the initiation and development of endothelial dysfunction (Figure 1.10a), (B) how these pathogens may contribute to the formation of fatty streaks and atherosclerotic plaques (Figure 1.10b) and (C) the proposed pathways that model the maturation and rupture of atherosclerotic plaques (Figure 1.10c) (Kebschull, Demmer and Papapanou, 2010).
Figure 1.10 Schematic representation of the potential role of periodontal pathogens in atherosclerotic vascular disease progression. (A.) Vascular endothelial cells are exposed to and invaded by periodontal pathogens e.g. *P. gingivalis*, which proceed to multiply intracellularly, triggering activation of toll-like receptor 2 (TLR2) and an up regulation of pro-inflammatory cytokines, cell adhesion molecules and apoptosis. (B.) Further potential progression of atherosclerotic plaque formation, through foam cell formation due to exposure to periodontal pathogens. Apoptosis of low-density laden (LDL) macrophages cause accumulation of lipids. Periodontal pathogens also influence the induction of smooth muscle cell proliferation, extracellular matrix (ECM) build-up and the attraction of T-cells within the plaque. (C) Exposure to periodontal pathogens causes the up regulation of endothelial apoptosis or anti-endothelial auto antibodies. Pathogen assisted breakdown of the ECM matrix also occurs by plaque macrophages, T-cells and plasma cells leading to mature plaque formation and subsequent vessel rupture. (Kebschull, Demmer and Papapanou, 2010)
Another mechanism that resides at the heart of atherosclerotic biology is the substantial role of platelets. Platelets can be activated either indirectly, through the vascular endothelium and exposed surface markers or through direct interaction with bacterium or their biological products such as LPS (Jennings, 2009). Oral pathogenic bacteria have been described to interact with platelets through toll-like receptors (TLR's), causing activation and the release of activation markers such as P-selectin and ADP (Shiraki et al., 2004; Blair et al., 2009; Zhang et al., 2009). Both *T. forsythia* and *P. gingivalis* have been described to increase the activation of platelets, inducing the up regulation of P-selectin and platelet-monocyte complexes (Nicu et al., 2009; Papapanagiotou et al., 2009; Laky et al., 2011), with *P. gingivalis* also inducing the pro-thrombotic pathway (Roth et al., 2009). However, the mechanisms underlying these interactions and the resulting consequences remain to be elucidated.
1.4 Research rationale and Thesis aims

The periodontopathogens *Porphyromonas gingivalis* and *Tannerella forsythia* found within the chronic oral disease of periodontitis have been suggested to contribute to and exacerbate the progression of atherosclerotic vascular disease. It is hypothesised that through the destructive phenotypes of chronic periodontitis and the subsequent localised bleeding within the periodontium, opportunistic pathogens *P. gingivalis* and *T. forsythia* are able to gain access to the vasculature. During this process, these periodonto-pathogens are able to directly or indirectly interact with circulatory platelets, resulting in an activated platelet state and the formation of platelet aggregates. It is then this altered platelet state which exacerbates or contributes to the progression of atherosclerotic vascular disease.

In order to investigate the potentiated interactions between periodonto-pathogens and mammalian platelets:

- The use of the megakaryocytic-like cells, CHRF-288-11 was investigated as a putative *in vitro* platelet-like cell model.
- A multicolour flow cytometry panel was developed to study the effect of periodontal pathogen interactions on platelet marker expression.
- The direct interactions of pathogens with platelets were characterised.
- The possible induction of platelet aggregation by a range of *P. gingivalis* and *T. forsythia* strains at varying multiplicities of infection was investigated.
- The mechanisms by which periodonto-pathogen might induce platelet activation were determined.
Chapter 2: Materials and methods
2.1 Materials

All chemical reagents unless otherwise stated were purchased from Sigma-Aldrich or ThermoFisher Scientific. All manufacturers and suppliers are shown in Table 2.1.

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<td>Biolegend</td>
<td>London, UK</td>
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<td>BMG Labtech</td>
<td>Buckinghamshire, UK</td>
</tr>
<tr>
<td>G E Healthcare</td>
<td>Buckinghamshire, UK</td>
</tr>
<tr>
<td>Gibco, ThermoFisher Scientific</td>
<td>Leicestershire, UK</td>
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<td>Hawksley</td>
<td>Sussex, UK</td>
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<td>Invivogen</td>
<td>Toulouse, Fr</td>
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<td>Hampshire, UK</td>
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<td>Sigma-Aldrich</td>
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<td>Sysmex</td>
<td>Buckinghamshire, UK</td>
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<td>ThermoFisher Scientific</td>
<td>Leicestershire, UK</td>
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<tr>
<td>TOCRIS</td>
<td>Gloucestershire, UK</td>
</tr>
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</table>

*Table 2.1 Manufacturers and suppliers.*
2.2 Antibodies

All antibodies against *P. gingivalis* and *T. forsythia* were kindly donated by Dr Graham Stafford, School of Clinical Dentistry, University of Sheffield, Sheffield, UK. Details of antibodies used in this study are provided in the Table 2.2 below:

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Host Animal</th>
<th>Raised Against</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 1B5</td>
<td>Mouse</td>
<td><em>Porphyromonas gingivalis</em> LPS</td>
</tr>
<tr>
<td>TF S-Layer</td>
<td>Rabbit</td>
<td><em>Tannerella forsythia</em> S-layer</td>
</tr>
<tr>
<td>TF Whole Cell 1</td>
<td>Rabbit</td>
<td><em>Tannerella forsythia</em> ATCC 43037</td>
</tr>
<tr>
<td>PG Whole Cell 1</td>
<td>Rabbit</td>
<td><em>Porphyromonas gingivalis</em> NCTC 11834</td>
</tr>
<tr>
<td>PG Whole Cell 2</td>
<td>Rabbit</td>
<td><em>Porphyromonas gingivalis</em>, NCTC 11834</td>
</tr>
<tr>
<td>α nanH Sialidase</td>
<td>Rat</td>
<td><em>Tannerella forsythia</em> ATCC 43037, sialidase mutant (ΔnanH)</td>
</tr>
<tr>
<td>α nanS Sialate-exterase</td>
<td>Rat</td>
<td><em>Porphyromonas gingivalis</em> ATCC 381, sialate-O-acetylesterase mutant (ΔnanS)</td>
</tr>
</tbody>
</table>

Table 2.2 Bacterial specific antibodies used within this study.
Table 2.3 shows mammalian antibodies used throughout this body of work, with Table 2.4 detailing secondary antibodies used within this study.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Alternate Name</th>
<th>Host Species</th>
<th>Supplier and Catalogue Number</th>
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<tr>
<td>Integrin β1</td>
<td>CD29</td>
<td>Mouse</td>
<td>Abcam #ab30394</td>
</tr>
<tr>
<td>Integrin β3</td>
<td>CD61</td>
<td>Mouse</td>
<td>Abcam #ab34409</td>
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<tr>
<td>mToR</td>
<td>N/A</td>
<td>Rabbit</td>
<td>Cell Signalling #7C10</td>
</tr>
<tr>
<td>Phalloidin-X Texas Red</td>
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<td>N/A</td>
<td>ThermoFisher #T7471</td>
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<td>Zombie Green</td>
<td>N/A</td>
<td>N/A</td>
<td>Biolegend #423111</td>
</tr>
<tr>
<td>CD42b-PE</td>
<td>GP1b</td>
<td>Mouse</td>
<td>Biolegend #303906</td>
</tr>
<tr>
<td>CD61-PE/Cy7</td>
<td>Integrin β3, GPIIa</td>
<td>Mouse</td>
<td>Biolegend #336416</td>
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<tr>
<td>CD41-Pacific Blue</td>
<td>Integrin αIIb, GPIIb</td>
<td>Mouse</td>
<td>Biolegend #303714</td>
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<tr>
<td>CD62P-PE/Cy7</td>
<td>GMP-140, PADGEM</td>
<td>Mouse</td>
<td>Biolegend #304922</td>
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<tr>
<td>CD41/61-APC</td>
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<td>Biolegend #359808</td>
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<td>PE-IgG Control</td>
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<tr>
<td>Pacific Blue-IgG Control</td>
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<td>Biolegend #4000222</td>
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Table 2.3 Primary antibodies used throughout this study. Antibodies were optimised and used at assay dependant concentrations detailed within each relevant section of Chapter 2.

<table>
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<th>Specificity</th>
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<td>Goat</td>
<td>Invitrogen #A11008</td>
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<tr>
<td>Anti-Mouse Alexa Fluor 488</td>
<td>Goat</td>
<td>Abcam #ab150117</td>
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</tbody>
</table>

Table 2.4 Secondary antibodies used throughout this study.
2.3 Bacterial Strains and Mutants

All bacterial strains used were kindly donated by Dr G. Stafford and details of the strains are shown in Table 2.5.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>Relevant characteristic(s)</th>
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<tbody>
<tr>
<td><em>Tannerella forsythia</em></td>
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<td>ATCC Strain</td>
</tr>
<tr>
<td></td>
<td>43037 Δ<em>NanH</em></td>
<td><em>NanH</em> sialidase deficient mutant of ATCC 43037 (ery²) (Honma, Mishima and Sharma, 2011)</td>
</tr>
<tr>
<td></td>
<td>43037 Δ<em>WecC</em></td>
<td><em>WecC</em> isogenic mutant of ATCC 43037 (ery²) (Honma <em>et al</em>., 2007)</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>NCTC 11834</td>
<td>NCTC Strain</td>
</tr>
<tr>
<td></td>
<td>NCTC 11834 Δ<em>OmpA1</em></td>
<td><em>OmpA1</em> (PGN_0729) deletion mutant of ATCC 33277 (ery²) (Naylor <em>et al</em>., 2016)</td>
</tr>
<tr>
<td></td>
<td>NCTC 11834 Δ<em>OmpA2</em></td>
<td><em>OmpA2</em> (PGN_0728) deletion mutant of ATCC 33277 (ery²) (Naylor <em>et al</em>., 2016)</td>
</tr>
<tr>
<td></td>
<td>ATCC 381</td>
<td>ATCC Strain</td>
</tr>
<tr>
<td></td>
<td>ATCC 381 Δ<em>Sia0352</em></td>
<td>Sialidase 0352 deficient mutant of ATCC 381 (ery²)</td>
</tr>
<tr>
<td></td>
<td>ATCC W50</td>
<td>ATCC Strain</td>
</tr>
</tbody>
</table>

Table 2.5 Bacterial strains and mutants used within this study. Ery² - erythromycin resistance.
2.4 Bacterial Growth

*P. gingivalis* strains were maintained on fastidious anaerobic agar (FA) supplemented with 5% horse blood (Oxoid, ThermoFisher) while *T. forsythia* strains were maintained on FA agar supplemented with 5% horse blood, 0.17 mM N-acetylmuramic acid (NAM) and 25 μg/mL gentamycin.

For growth in liquid cultures, *P. gingivalis* was grown in brain heart infusion (BHI) (Oxoid, ThermoFisher) broth supplemented with 0.4 % yeast extract, 1 mg/mL vitamin K1, 1 mg/mL hemin and 250 μg/mL cysteine.

*T. forsythia* was grown as liquid cultures in tryptic soy broth (TSB) supplemented with 0.5% yeast extract, 5 μg/mL hemin, 0.5 μg/mL vitamin K, 0.17 mM NAM and 0.1% cysteine.

Erythromycin (10 μg/mL) was included as a selective antibiotic in the media after autoclaving where appropriate (see table 2.5) and bacteria grown on solid surface or as liquid cultures were incubated at 37 °C under anaerobic conditions (10% H₂, 80% N₂, 10% CO₂). Bacterial counts were determined using a bacterial counting chamber (Hawksley).

2.4.1 Long Term Bacterial Storage

For long term storage, bacterial cultures were kept at -80 °C. Routine bacterial cultures were maintained as in section 2.4 before being removed from supplemented FA plates, resuspended in ice-cold fresh growth medium containing 17% glycerol and immediately stored.
2.5 Megakaryocytic Cell Culture and Differentiation

2.5.1 Megakaryocyte Cell Line CHRF-288-11

The CHRF-288-11 cell line was kindly provided by Prof. Willem Ouwehand & Dr Cedric Ghevaert (Division of Transfusion Medicine, Department of Haematology, University of Cambridge, Cambridge, UK). The CHRF-288-11 cells were routinely cultured in RMPI-1640 medium containing L-glutamine (Gibco, ThermoFisher Scientific) supplemented with 1% non-essential amino acids and 10% foetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO₂.

2.5.2 Cell Passaging

CHRF-288-11 cells were grown until confluent (~1x10⁶ cells/mL) before passaging. Cells suspensions were transferred to a fresh tube before centrifugation (200 g, 5 min, room temperature (RT)). The supernatant was discarded and the cell pellet was resuspended in phosphate-buffered saline (PBS). Following further centrifugation (200 g, 5 min, RT), cells were resuspended in fresh RPMI at a density of 1x10⁵ cells/mL.

2.5.3 Cryopreservation of Cells

Cells were cryopreserved for long term storage. Cells at confluency (1x10⁶ cells/mL) were pelleted at 200 g for 5 min at RT before being resuspended and washed in PBS. Cells were then further centrifuged for 5 min (200 g, RT) and re-suspended in freezing media (complete RPMI-1640 containing 10% dimethyl sulfoxide (DMSO). Cells at a density of ~5x10⁵ cells/mL were then transferred into cryopreservation tubes before being placed into a Mr Frosty™ for 24 hours at -80 °C, after which the vials were transferred to liquid nitrogen for long term storage. When needed, cells were revived by quickly thawing in a water bath at 37 °C. Cells were then immediately transferred
into fresh culture media and cultured for 6h. After this time period, the cell media was replaced to remove the DMSO and normal cell culture protocol was then followed.

2.5.4 CHRF-288-11 Differentiation

For differentiation, CHRF-288-11 cells were seeded in routine culture medium (RPMI-1640 with L-glutamine (Gibco, ThermoFisher Scientific), 1% non-essential amino acids, 10% FBS) containing 50 ng/mL of Phorbol 12-myristate 13-acetate (PMA). Cells were then cultured for five days without a media change at 37 °C in a humidified incubator with 5% CO₂. For experiments requiring cells in suspension, differentiated CHRF-288-11 cells were washed twice with PBS and incubated in 1x trypsin-EDTA for two min at 37°C in a humidified incubator with 5% CO₂. Cell detachment was confirmed using light microscopy and an equal volume of complete RPMI-1640 was added to neutralise trypsin activity. After detachment cells were then processed in an assay dependant manner.

2.5.5 Cell Counting

Cells were counted using the Countess II FL automated cell counter (Invitrogen). Cell suspensions were removed and mixed with an equal volume of trypan blue before being transferred into a countess chamber slide. Cell counts were then generated automatically, with none viable cells being discounted by trypan blue exclusion.

2.6 Platelet Isolation

Blood was obtained from healthy volunteers within the Biomolecular Sciences Research Centre, Sheffield Hallam University, UK, with informed consent. The use of platelets in this study was approved by the Sheffield Hallam University Research Ethics Committee (Ethics application No. HWB-BIO-03), Sheffield Hallam University, Sheffield, UK.
2.6.1 Blood collection

After a tourniquet had been applied, the first sample was drawn into an EDTA-containing Vacutainer. The tourniquet was then removed and any subsequent samples were drawn into sodium citrate Vacutainers, allowing blood to flow by gravity. All tubes were inverted to ensure full distribution of the anticoagulant and then processed accordingly. Full blood count (FBC) was determined using the EDTA blood sample by a haematology analyser (XP-300, Sysmex).

2.6.2 Platelet rich plasma preparation

Each sample was centrifuged at 200 g for 20 min at room temperature with no brake applied. After centrifugation, the straw-coloured platelet rich plasma (PRP) layer was removed using a sterile Pasteur pipette and transferred to a fresh tube. The isolated PRP was analysed by the Sysmex XP-300 to determine platelet concentration and confirm that the sample is free of other blood contaminants.

2.6.3 Platelet poor plasma preparation

Previously isolated PRP was further centrifuged at 800 g for 20 min at room temperature with no brake in order to pellet platelets. After centrifugation, the supernatant was carefully removed and transferred to a fresh tube before being utilised to set '0% aggregation' in an experiment dependant manner.

2.6.4 Modified Tyrode’s Buffer

Modified Tyrode’s Buffer was made by adding; 134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na₂HPO₄, 1 mM MgCl₂, 10 mM HEPES, to dH₂O and adjusting the pH to 7.4. Prior to use, the buffer was supplemented with 5 mM glucose, 25 mM CaCl₂ and 3 mg/mL BSA.
2.7 Phenotypic Analysis Methods

2.7.1 Antibiotic Protection Assays

Antibiotic protection assays were carried out in a Class II safety cabinet that had previously been sterilised by UV light. CHRF-288-11 cells were seeded at a density of $1 \times 10^6$ cells/mL into 24 well plates in complete RPMI media and allowed to differentiate for five days (Section 2.5.4). Before starting the antibiotic protection assay, all buffers and cell culture media were pre-warmed to 37 °C. Three 'sacrificial' wells were washed three times with PBS, before 1x trypsin-EDTA was added to the plate and incubated for two min at 37 °C in a humidified incubator with 5% CO$_2$. The trypsin-EDTA was then neutralised with an equal volume of complete media, the cell suspensions were removed, counted and averaged to determine the relevant bacterial count for an MOI of 1:100. The remaining wells were washed twice with PBS to remove any undifferentiated cells before incubating with complete RPMI supplemented with 2% BSA for one hour at 37 °C in a humidified incubator with 5% CO$_2$. During this incubation, $P. gingivalis$ strains (wild-type or mutants) were removed from 72 hour blood agar plates, resuspended in complete RPMI before being counted and adjusted to equal an MOI of 1:100. Following blocking of non-specific sites, wells were washed three times with PBS before the addition of the bacterial suspension. In parallel, the bacterial suspension was added to three wells not seeded with mammalian cells to assess bacterial viability throughout the assay. Well seeded with CHRF-288-11 cells but not infected were also included as controls. Samples were incubated for 90 min at 37°C in a humidified incubator with 5% CO$_2$. Following bacterial incubation, to determine percentage invasion, cells were washed three times with PBS, before being incubated for a further 60 min with complete RPMI media supplemented with 200 µM
of metronidazole in order to assess in the invaded population. All wells containing mammalian cells were then washed a further three times with PBS before the addition of sterile water and each well was scraped for one minute for ensure complete cell lysis. Cell lysates were then serially diluted and spotted onto blood agar plates using the Miles-Misra method. Well containing only bacterial cells were only serially diluted before being plated the same way. Blood agar plates with then incubated anaerobically at 37 °C for up to five days before colony forming units (CFU's) were counted and recorded.

2.7.2 Immunofluorescent Microscopy

2.7.2.1 Phenotypic characterisation of CHRF-288-11 differentiation
Sterile 13mm coverslips were added to each well of a 24-well plate. The wells were then washed twice with PBS before being seeded at a density of $1 \times 10^6$ cells and allowed to differentiate for up to seven days (Section 2.5.4). Cells were also seeded at a density of $5 \times 10^5$ cells as an undifferentiated control. After the appropriate differentiation period, cell media was removed, each well was washed three times with PBS before cells were fixed for 10 min in 4% paraformaldehyde (PFA). After fixation, cells were washed three times in PBS, permeabilised with 0.5% Triton-X in PBS for 8 min and washed a further three times with PBS. Cells were then blocked with 3% BSA for one hour at room temperature, washed three times with PBS before being incubated with the high affinity F-actin probe Texas Red-X phalloidin at a concentration of 1:3500 in PBS with 3% BSA for a further hour at room temperature, protected from light. Each well was washed a further three times with PBS before the coverslips were removed and mounted with ProLong Gold anti-fade mountant with
DAPI (Invitrogen). Each slide was then left in the dark for 24 hours at room temperature before imaging with a fluorescent microscope (Olympus BX60).

2.7.2.2 Phenotypic evaluation of bacterial challenge on differentiated CHRF-288-11 cells

Sterile 13 mm coverslips were added to each well of a 24-well plate. The wells were then washed twice with PBS before being seeded at a density of $1 \times 10^6$ cells and allowed to differentiate for five days (Section 2.5.4) at 37°C in a humidified incubator with 5% CO$_2$. Cells were also seeded at a density of $5 \times 10^5$ cells as an undifferentiated control. *P. gingivalis* (wild-type or mutants) or *T. forsythia* strains were removed from 72 hour blood agar plates, resuspended in RPMI before being counted and adjusted to equal an MOI of 1:100. Cells were then challenged with bacterial suspensions for 90 min at 37°C in a humidified incubator with 5% CO$_2$ before fixation in 4% PFA for 10 min at room temperature. As above, cells were washed three times in PBS, permeabilised with 0.5% Triton-X in PBS for 8 min and washed a further three times with PBS. Cells were then blocked with 3% BSA for one hour at room temperature, washed three times with PBS before being incubated with primary antibodies (see Table 2.3) at a relevant concentration in 3% BSA. Cells were washed three times with PBS and before further incubation with the corresponding secondary antibodies (see Table 2.4) at the desired concentration in 3% BSA. Where applicable the high affinity F-actin probe Texas Red-X phalloidin (diluted 1:3500) was also included with the secondary antibodies. Each well was washed a further three times with PBS before the coverslips were removed and mounted with ProLong Gold anti-fade mountant with DAPI. Each slide was then left in the dark for 24 hours at room temperature to allow the mountant to cure before imaging with a fluorescent microscope (Olympus BX60).
2.7.2.3 Visualisation of platelets challenged with perio-pathogens

Isolated platelets (PRP) in sodium citrate Vacutainers were counted using the Sysmex XP-300 and resuspended at 1x10^6 platelets/mL of modified Tyrode's buffer. Where applicable, this modified Tyrode's suspension also included either 20µM ADP or *P. gingivalis* strains that had been removed from 72 hour blood agar plates, counted and resuspended at an MOI of 1:10. Each platelet suspension was incubated at room temperature for 20 min before fixation in 0.5% PFA for 30 min again at room temperature. Each sample was then cytospun onto separate slide at 800 g for 20 min at room temperature before being washed three times with PBS, permeabilised for 8 min with 0.5% Triton-X, washed a further three times with PBS and blocked with 3% BSA for an hour at room temperature. Each slide was then incubated with primary antibodies (anti-integrin β1, anti-integrin β3, anti-mToR; see Table 2.3) for 1 hour at room temperature at a relevant concentration in 3% BSA, before being washed three times with PBS and further incubation with the corresponding secondary antibodies (see Table 2.4) at the desired concentration in 3% BSA for an hour at room temperature. Where applicable the high affinity F-actin probe Texas Red-X phalloidin was also included with the secondary antibodies at a concentration of 1:3500. Each slide was then washed three times with PBS before mounting with ProLong Gold antifade mountant containing DAPI. Each slide was left in the dark for 24 hours at room temperature before imaging with a fluorescent microscope (Olympus BX60).
2.7.3 Electron microscopy

2.7.3.1 White's Saline

White's saline A was made by adding; 2.4 M of sodium chloride (NaCl), 0.1 M of potassium chloride (KCL), 0.046 M of magnesium sulphate (MgSO₄), 0.064 M of calcium nitrate tetrahydrate (Ca(NO₃)₂ 4H₂O) to 100 mL of distilled water.

White's saline B was made by adding; 0.131 M of sodium carbonate (NaHCO₃), 0.008 M of sodium phosphate dibasic heptahydrate (Na₂HPO₄ 7H₂O), 0.004 M of potassium phosphate monobasic (KH₂PO₄ anhydrous), 0.0003 M of phenol red to distilled water. The solution was adjusted to pH 7.4 before being further distilled water was added to a total volume of 100 mL.

2.7.3.2 Electron microscopy of platelet/perio-pathogen interactions

Isolated platelets (PRP) were counted using the Sysmex XP-300 and transferred into fresh Eppendorph tubes (4 x 10⁸ platelets/test). Platelets were then infected with P. gingivalis (at an MOI of 1:10) that had been removed from 72 hour blood agar plates, resuspended in modified Tyrode's buffer and counted. As a positive control platelets exposed to 20 µM of ADP were also included. Samples were incubated for 20 min at room temperature before the addition of glutaraldehyde in equal volumes of both White’s saline A and B to give a final concentration of 0.1% glutaraldehyde. Samples were fixed for 30 min before centrifugation at 800 g for 20 min and resuspended in 3% glutaraldehyde in an equal volume of White's saline A and B for a further 60 min.

For SEM analysis, samples were post fixed using 2% aqueous osmium tetroxide for two hour at room temperature before being dehydrated in a graded series of ethanol solutions and partially dried in a 50/50 mixture of 100% ethanol and hexamethyldisilazane (HEX). Samples were fully dried in 100% HEX before being
mounted onto a pin-stub using Leit-C sticky tabs, gold coated using an Edwards S150B sputter coater and examined using a Tescan Vega3 LMU scanning electron microscope. Scanning electron microscope was operated at a voltage of 12 kV.

For TEM analysis, samples were post fixed in 2% aqueous osmium tetroxide, dehydrated using a graded series of ethanol solutions, cleared in epoxypropane (EPP) and infiltrated in 50/50 araldite resin:EPP mixture overnight on a rotor at room temperature. This mixture was replaced with two changes, over 8 hours with fresh araldite resin mixture before being embedded and cured at 60 °C for 72 hours. Ultrathin sections, approximately 85 nm thick, were cut on a Leica UC 6 ultramicrotome onto 200 mesh copper grids, stained for 30 min with saturated aqueous Uranyl Acetate followed by Reynold’s Lead Citrate for 5 min. Sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80 Kv. Electron micrographs were recorded using a Gatan Orius 1000 digital camera and Gatan Digital Micrograph software.

Sample processing and acquisition was conducted by Chris Hill at the department of Electron Microscopy Services, University of Sheffield.

2.7.4 Flow cytometry

2.7.4.1 Relative expression of platelet markers by CHRF-288-11 cells

Cell suspension of either differentiated or undifferentiated cells were counted using the Countess II cell counter and adjusted to 1x10^6 cells/mL in complete RPMI. Where required cells were exposed to either ADP (20µM) or P. gingivalis (MOI 1:100, wild type or mutant strains) as previously described. Platelet/bacteria samples were then incubated for 90 min at 37 °C in a humidified incubator with 5% CO₂. Cells were
pelleted by centrifugation at 200 g for 5 min at room temperature before resuspending in PBS. The cells were then centrifuged again for 5 min at 200 g, room temperature before the supernatants were discarded and the pellet gently resuspended in the residual volume. The expression of each cell marker was determined by incubating with the relevant antibodies or IgG controls (see Table 2.3) at room temperature for 20 min before each sample was washed with PBS, centrifuged at 200 g for 5 min at room temperature and supernatants then discarded. Each sample was re-suspended in PBS and analysed on a Beckman Coulter Gallios flow cytometer.

2.7.4.2 Phenotypic analysis of platelet/perio-pathogen interactions

Isolated platelets (PRP) were counted using the Sysmex XP-300 and resuspended in modified Tyrode's buffer at a density of 1x10^6/100 µL. *P. gingivalis* strains (wild-type or mutants) were removed from 72 hour blood agar plates and resuspended in modified Tyrode's buffer at an MOI of 1:10. Platelets were exposed to the bacteria or ADP (20µM) for 20 min at room temperature before fixation with 0.5% PFA for 30 min. After fixation, each sample was resuspended in PBS before being centrifuged at 800 g for 20 min at room temperature. The supernatants were then discarded and the pellet gently resuspended in the residual volume. The expression of each cell marker was determined by incubating with the relevant antibodies or IgG controls (see Table 2.3) at room temperature for 20 min. Each sample was then diluted in PBS and analysed on a Beckman Coulter Gallios flow cytometer.
2.7.5 Enzyme linked immunosorbent assays (ELISAs)

2.7.5.1 Development of a cell-based ELISA

To investigate whether oral pathogens interacted with integrins β1 and β3, an in-house ELISA was developed. Cells were seeded at a density of 3×10⁴ cells/well and allowed to differentiate for five days in a tissue culture grade 96-well plate (37 °C, 5% CO₂). Following differentiation, media containing non-differentiated and non-viable cells were removed and discarded and cells were washed twice with PBS. Three of the wells were trypsinised to recover attached cells and the average number of differentiated cells per well was determined. The differentiated cells were subsequently challenged with bacteria (MOI 1:100 in complete media) and incubated for 90 min in a humidified incubator (37 °C, 5% CO₂) after which supernatants were removed and discarded. The cells were washed twice with PBS containing 0.1% Tween20 (PBST) and blocked for one hour with PBS containing 3% BSA. Following blocking, cells were washed as described above and fixed using 4% paraformaldehyde. Wells were then incubated with anti-integrin β1 (2 µg/mL; Abcam), anti-integrin β3 (2 µg/mL, Abcam), a polyclonal rabbit IgG anti-\textit{P. gingivalis} (1:5000) or a polyclonal rabbit IgG anti-\textit{T. forsythia} (1:5000) for a further hour, after which cells were washed before incubation with secondary antibodies; either the peroxidase anti mouse IgG (1:40,000) or peroxidase goat anti-rabbit IgG (1:20,000) for one hour. Wells were then washed three times with PBST and 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Fisher Scientific) was added. The reaction was allowed to develop before stopping with 2 M H₂SO₄. Plates were read spectrophotometrically at 450 nm with a Clariostar microplate reader (BMG Labtech).
2.7.5.2 Recombinant protein-based ELISA

2.7.5.2.1 Coating Buffer
Sodium bicarbonate coating buffer was made by adding 0.019 M NaCO₃ and 0.035 M NaHCO₃ to 1 L of dH₂O, pH 9.6. The pH of this buffer was not adjusted and if not correct was remade.

2.7.5.2.2 Detection of *P. gingivalis* binding to recombinant integrins
Wells of 96-well Maxisorp™ microplates were coated with either recombinant integrin β1 or β3 at 5 μg/mL in coating buffer, overnight at 4 °C. After washing once with wash buffer (PBS with 0.05% Tween-20, 0.5 μM CaCl₂), non-specific binding sites were blocked with blocking buffer (PBS with 3% BSA and 0.5 μM CaCl₂) for one hour under agitation before washing a further two times. Bacterial cells were removed from FA agar, resuspended in blocking buffer before being added to the pre-coated plates and incubated at room temperature for one hour under agitation. Untreated wells were also included as a control. Wells were then washed three times with wash buffer before incubating with a polyclonal rabbit IgG anti-*P. gingivalis* (1:3500) or a polyclonal rabbit IgG anti-*T. forsythia* (1:3500) antibodies for one hour at room temperature under agitation. After washing three times with wash buffer, wells were incubated with peroxidase goat anti-rabbit IgG (1:20,000) for one hour under agitation, washed three times with PBST and 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Fisher Scientific) was added. The reaction was allowed to develop before stopping with 2 M H₂SO₄. Plates were read spectrophotometrically at 450 nm with a Clariostar microplate reader (BMG Labtech).
2.8 Platelet Function Methodology

2.8.1 Platelet Aggregation

2.8.1.1 Agonist preparation

2.8.1.1.1 Live perio-pathogens
Initially bacterial strains (wild-type or mutants) of both *P. gingivalis* and *T. forsythia* were removed from 72 hour blood agar plates and resuspended in modified Tyrode's buffer. These suspensions were then counted and calculated to give a MOI of either 1:10 or 1:1

2.8.1.1.2 Heat killed perio-pathogens
Strains of *P. gingivalis* or *T. forsythia* were prepared as above (Section 2.9.1.1.1) before being boiled for 5 min at 100 °C. In order to confirm bacterial viability, each bacterial suspension was transferred to a fresh blood agar plate and cultured at 37 °C in an anaerobic environment for five days.

2.8.1.1.3 2.9.1.1.3 Bacterial supernatants
Wild-type strains of *P. gingivalis* were grown in liquid cultures until they reached an optical density (O.D.) of 0.8 at 600 nm. Each culture was centrifuged at 13,000 g for 5 min at room temperature before the supernatants were removed and passed through a 0.2 µm filter. 20 µL of this suspension was then added to platelet suspensions during aggregation determination.

2.8.1.1.4 *P. gingivalis* lipopolysaccharide
Commercial lipopolysaccharide (LPS; Invivogen) purified from *P. gingivalis* NCTC 11834 was used in aggregation investigations at concentrations of 10, 20 and 40 µg/mL.
2.8.1.2 Platelet inhibitors

Before measuring aggregation, 250 µL of PRP was transferred into individual cuvettes and platelets were incubated with selective platelet inhibitors (shown in Table 2.6) for 15 min at room temperature. Each cuvette was then analysed routinely as described in 2.9.1.3.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Supplier</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
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<td>Invivogen</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Invivogen</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Integrin α2β1</td>
<td>TOCRIS</td>
<td>20 µM</td>
</tr>
<tr>
<td>Integrin β3</td>
<td>ABBIOTEC</td>
<td>11.2 µg/mL</td>
</tr>
</tbody>
</table>

Table 2.6 Platelet inhibitors.

2.8.1.3 Light transmission aggregometry

Prior to the measurement of aggregatory responses, each channel of the AggRAM light transmission aggregometer (Helena Biosciences), was blanked with PPP of the same donor. To measure aggregation, PRP was transferred into individual cuvettes containing a siliconised magnetic stirrer and incubated for one minute at 37 °C. Where applicable each agonist was then added to each cuvette and the aggregatory response was recorded for 10 min at 37 °C with continuous stirring at 650 nm. As a positive control measure and to determine 100% aggregation, PRP treated with 20 µM ADP was also included. The raw data files were exported and further processed using Microsoft Excel to determine initiation of aggregation (AG0) and time taken to reach 50% aggregation (AG50).
2.8.2 Platelet Calcium Mobilisation

Isolated PRP was initially counted using the Sysmex XP-300 before incubating with 5µM FURA-2/AM for 45 min at room temperature. Platelets were then pelleted at 800 g for 20 min at room temperature before being resuspended in modified Tyrode's buffer at a concentration of 4x10^8 platelets/mL. Wild-type or mutant strains of *P. gingivalis* were removed from 72 hour blood agar plates, resuspended in modified Tyrode's buffer and counted to determine an MOI of 1:10. The preloaded platelet suspension was transferred to each well of a 96-well plate, before recording the excitation of fluorescent intensities at both 340 and 380 nm simultaneously using a Clariostar microplate reader (BMG Labtech). The fluorescent emission of each test was recorded at 510 nm for 210 seconds with the initial 20 seconds being used as a baseline before automatically injecting platelet agonists or a buffer control. Intracellular calcium levels are then determined using the following equation;

\[
Relative \, fluorescence \, of \, intracellular \, calcium = \frac{Emission \, at \, 340nm}{Emission \, at \, 380nm}
\]
2.8.3 Platelet Granule Release Quantitation by Enzyme-linked Immunosorbent Assay (ELISA)

PRP was initially counted using the Sysmex XP-300 before platelets were pelleted at 800 g for 20 min at room temperature before resuspending at a density of 2x10^8/mL in modified Tyrode's buffer. Strains of *P. gingivalis* (wild-type or mutants) were removed from 72 hour blood agar plates, resuspended in modified Tyrode's buffer, counted and adjust to an MOI of 1:10. 2x10^8 platelets were then incubated with bacteria or ADP (20 µM) for 20 min at room temperature before fixing in 0.5% PFA for 30 min, again at room temperature. Samples were centrifuged at 13,000 g for 5 min at 4 °C before the supernatants were removed and processed following the manufacturer's instructions of one of the following ELISA kits; human platelet factor 4 (PF4) (Abcam), interleukin 1β (IL-1β) (Bio-Techne LTD) or Serotonin (Abcam). ELISA plates were read using and the data recorded using a Clariostar microplate reader (BMG Labtech).

2.9 Statistical Analysis

Statistical analysis was conducted using the StatsDirect3 software (StatsDirect Ltd, Cheshire, UK). Data normality was determined by a D'Agostino & Pearson normality test before subsequent statistical analysis.

Parametric data was analysed using paired t-test. Non-parametric paired data was analysed using the Friedman test whereas non-paired non-parametric data was evaluated using the Kruskal-Wallis Test (Conover-Iman). The threshold for statistical significance was set at p<0.05; * = P≤ 0.05, ** = P≤ 0.01 and *** = P≤ 0.001. All experiments consisted of at least three biological replicates.
Chapter 3: Characterisation of an *in vitro* cellular model to study platelet-periodontal pathogen interactions
3.1 Introduction

The development and application of megakaryocytic cell lines, derived from patients with leukaemia, allow a unique opportunity to study the maturation and differentiation of megakaryocytes and their associated platelet-like phenotypes (Saito, 1997). The overall aim of this thesis is to investigate the effect of periodontal pathogens on platelet aggregation, activation and function. However, as experimental work on platelets requires fresh blood which needs to be immediately used due to an inability to effectively store samples (Bausset et al., 2012), the megakaryocytic cell line, CHRF-288-11 was recruited as a cellular model to study platelet-pathogen interactions.

The CHRF-288-11 cell line is a megakaryocytic cell line, which was initially isolated from a biopsy of a metastatic tumour in a 17 month old infant with acute megakaryoblastic leukaemia (Fugman et al., 1990). Further characterisation of this cell line indicated that CHRF-288-11 cells can be used as an effective megakaryocyte/platelet model (Lev-Lehman et al., 1997), which can be matured towards platelet production (Deutsch et al., 2008) as the cells express platelet derived growth factors (Yang et al., 1997) as well as expressing platelet markers including integrins which are involved in platelet adhesion, aggregation and activation (Fugman et al., 1990; Conran and Hemming, 1998; Nurhayati, Ojima and Taya, 2015).
3.2 Aims

The overall aim of this chapter is to validate a differentiated megakaryocytic cell line for the study of platelet-periodonto-pathogen interactions. This will be achieved by:

- Investigating the use of the CHRF-288-11 cells as an *in vitro* platelet-like cell line by determining expression of platelet specific markers
- Investigating CHRF-288-11 cells as an *in vitro* platelet-like model to study platelet pathogen interactions
3.3 Results

3.3.1 Optimisation of CHRF-228-11 differentiation

Differentiation and maturation of CHRF-288-11 (CHRF) cells following exposure to phorbol 12-myristate 13-acetate (PMA) was investigated by immunofluorescence microscopy. PMA at both concentrations (10 and 50 ng/ml) successfully induced CHRF differentiation over the period investigated.

Successful differentiation was determined through cellular adherence, DAPI staining for nucleic morphology and an F-actin probe for cytoskeletal phenotyping. Representative micrographs are shown for both 10 ng/ml (Figure 3.1) and 50 ng/ml (Figure 3.2) PMA treatments. Both 10 and 50 ng/ml of PMA treatment induced differentiation from the day one time point as characterised by development of large multi-lobed nuclei and the formation of pseudopodial structures that protrude from the main cellular body when compared to the control cells. No changes were observed with cells treated with DMSO vehicle control, with consistent low cell numbers observed across all investigated time points. The observed phenotypic cellular differentiation was maintained throughout the day three, five and seven time points within both PMA treatments, with both treatments resulting in highly heterogeneous cellular populations with varying pseudopodia and no distinctive cytoskeletal structure. Cellular viability was also confirmed by trypan blue exclusion and remained at ~98% throughout all PMA treatments (data not shown). It was determined that 50ng/ml for a period of five days yielded the most consistent differentiated cell densities and was therefore selected for future investigations utilising this cellular model.
Figure 3.1 CHRF-288-11 differentiation with 10 ng/ml of PMA. CHRF-288-11 cells were differentiated onto glass coverslips before being stained with a high affinity F-actin probe and DAPI. DAPI staining revealed the formation of large multi-lobed nuclei within 24 hours of PMA treatment. F-actin positivity showed the development of large pseudopodia that protruded from the cell body and became more defined over a seven day time course. DMSO was included as a vehicle control and is shown at the 7 day time point. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.2 CHRF-288-11 differentiation using 50 ng/ml of PMA. CHRF cells were differentiated onto glass coverslips before being treated with an F-actin probe and the nucleic stain DAPI. Staining revealed the formation of large multi-lobed nuclei and large pseudopodial structures over the seven days of treatment. DMSO is included as a vehicle control and is shown at the 7 day time point. Images are representative of at least three independent observations. Scale bars= 50 μm.
To further characterise the differentiated CHRF cells, expression of megakaryocytic/platelet surface protein and activation markers was determined by flow cytometry. A general increase in expression of CD41 and CD41/61 was observed up to five days of culture after which a decrease was observed with both PMA treatments (10 and 50 ng/ml). Over the seven day time course CHRF-288-11 cells were found not to express CD42b, throughout both the 10 and 50 ng/ml treatments, whilst the expression of CD62P increased almost two-fold compared to the DMSO controls (Figure 3.3). No significant differences were observed between 10 and 50 ng/ml PMA treatments at any of the time points investigated. The decrease in overall expression at day seven was attributed to cell detachment.
Figure 3.3 Analysis of megakaryocytic protein expression following CHRF-288-11 differentiation. CHRF-288-11 cells were treated with either 10 or 50 ng/ml of PMA and differentiation was monitored over a period of seven days. Cells were analysed by flow cytometry to determine the relative expression of cell surface proteins and platelet activation markers: CD41 (A), CD41/61 (B), CD62P (C) and CD42b (D). Expression of CD41, CD41/61 and CD62P increased with time in both PMA treatments when compared to the DMSO vehicle control up to day five before decreasing. CHRF cells did not express CD42b across all treatments and time points. No changes were observed in the DMSO vehicle control across all time points, with the day seven time point being included as a representative control. Data is expressed as the mean fluorescent index (MFI) ±SEM, n=3.
3.3.2 Periodontal pathogen invasion and adhesion of CHRF-288-11 cells

Following optimisation of CHRF differentiation, the ability of periodontal pathogens to adhere to and invade the differentiated CHRF-288-11 cells were investigated. Initially, undifferentiated and differentiated cells were treated with either *P. gingivalis* NCTC 11834 or *T. forsythia* ATCC 43037 (MOI of 1:10 or 1:100) and probed with pathogen specific antibodies, a high affinity actin probe and DAPI as a nucleic stain.

It was observed that, *P. gingivalis* associated with and invaded the undifferentiated CHRF-288-11 cells (Figure 3.4), with association and invasion increasing following CHRF-288-11 differentiation independent of MOI (Figure 3.5). Similarly *T. forsythia* was shown to interact with undifferentiated CHRF-288-11 cells (Figure 3.6) with cellular association and invasion increasing following CHRF differentiation (Figure 3.7) at both MOIs investigated. Within both undifferentiated and differentiated cells, *P. gingivalis* and *T. forsythia* were shown to localise within the cell membrane and the perinucleic space.
Figure 3.4 Invasion of CHRF-288-11 cells by *P. gingivalis* NCTC 11834. Undifferentiated CHRF-288-11 cells were challenged with *P. gingivalis* NCTC 11834 (MOI of 1:10 and 1:100) for 90 minutes at 37 °C, before being fixed and mounted onto glass microscope slides. Cells were then permeabilised and probed with a pathogen specific antibody, PGWC1 (green), an F-Actin probe (red) and the nucleic stain DAPI (blue). *P. gingivalis* NCTC 11834 localised with and invade CHRF-288-11 cells (white arrows). Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.5 *P. gingivalis* invades differentiated CHRF-288-11 cells. CHRF cells were differentiated for five days before being challenged with *P. gingivalis* NCTC 11834 (MOI 1:10 or 1:100) and fixed. Cells were then permeabilised and stained with a *P. gingivalis* specific antibody (PGWC1; green), an F-actin probe (red) and mounted with DAPI (blue). A high level of invasion was observed with differentiated CHRF cells, with *P. gingivalis* localising within the membrane and nucleic regions of the cells. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.6 CHRF-288-11 cellular invasion by *T. forsythia* ATCC 43037. Undifferentiated CHRF-288-11 cells were exposed at an MOI of 1:10 or 1:100 to *T. forsythia* ATCC 43037 before being fixed and permeabilised. Cells were then stained with a pathogen specific antibody, TFWC (green), an F-actin probe (red) and DAPI (blue). These images demonstrate that *T. forsythia* invades and adheres to undifferentiated CHRF-288-11 cells (white arrows). Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.7 Invasion of differentiated CHRF-288-11 cells by T. forsythia. Cells were challenged with T. forsythia at an MOI of either 1:10 or 1:100 before fixation. Cells were then permeabilised and probed with a pathogen specific antibody, TFWC (green), a high affinity F-actin probe (red) and DAPI (blue). A high level of cellular invasion was observed with bacterial cells localising within the cellular membrane and around the nuclei (white arrows). Images are representative of at least three independent observations. Scale bars= 50 μm.
To quantitate cellular invasion, an antibiotic protection assays were performed on differentiated CHRF-288-11 cells (Figure 3.8). Differentiated CHRF-288-11 cells were challenged with wild-type strains of *P. gingivalis* (NCTC 11834, ATCC W50) or *T. forsythia* (ATCC 43037). To determine the role of the *P. gingivalis* outer-membrane protein (OMPA), cells were also challenged with deficient mutants lacking the two major subunits of the OMPA protein (NCTC 11834 ΔompA1, NCTC 11834 ΔompA2).

All of the wild-type strains and mutants tested successfully adhered to and invaded the megakaryocytic-like cells. Although some variation in both cellular adhesion and invasion were observed, no significant differences were calculated between any of the variables tested.
Figure 3.8 Bacterial adhesion and invasion of CHRF-288-11 cell by periodontal pathogens. Differentiated CHRF-288-11 cells were challenged with *P. gingivalis* (NCTC 11834, NCTC 11834 Δ*omp*A1, NCTC 11834 Δ*omp*A2, ATCC W50) or *T. forsythia* (ATCC 43037) for 90 minutes (37 °C, 5% CO₂). Invasion was defined as the percentage of bacterial inoculum protected from metronidazole, whereas total associated is the total percentage of bacterial cells recovered. The adhered population is then determined by subtracting the ‘invaded’ from ‘total associated’. All *P. gingivalis* and *T. forsythia* strains tested were shown to adhere to and invade differentiated CHRF-288-11 cells. Data is presented as a percentage recovery relative to the total inoculum. ±SEM, n=3.
3.3.3 Analysis of CHRF-288-11 interactions with periodontal pathogens by immunofluorescent microscopy

Following the initial immunofluorescent characterisation of periodontal-pathogen invasion of CHRF cells, further investigation focussed on exploring the impact of pathogen invasion on CHRF protein expression. The effects of *P. gingivalis* NCTC 11834 and *T. forsythia* ATCC 43037 were determined in relation to the expression of two proteins of interest; the Mammalian Target of Rapamycin (mToR) and integrin β3.

### 3.3.3.1 Analysis of CHRF-288-11 mToR expression following incubation with periodontal pathogens

Undifferentiated CHRF-288-11 cells expressed mToR and the rate of bacterial association increased with increasing MOI with both *P. gingivalis* NCTC 11834 (Figure 3.9) and *T. forsythia* 43037 (Figure 3.10). However, no variances in protein expression were observed in either bacterial treatment when compared to the untreated control.

In contrast, while differentiated CHRF-288-11 cells also showed/demonstrated increased bacterial association with increasing MOI, the overall expression of mToR decreased following exposure to both *P. gingivalis* NCTC 11834 (Figure 3.11) but not with *T. forsythia* 43037 (Figure 3.12).
Figure 3.9 The effect of *P. gingivalis* invasion on mToR expression in CHRF-288-11 cells. CHRF cells were challenged with *P. gingivalis* NCTC 11834 for 90 minutes before being fixed and permeabilised. Cells were then probed for mToR expression (red), a pathogen specific antibody (PGWC1, green) and DAPI. Images highlight the CHRF-288-11 cells express mToR but its relative expression is not effected through bacterial association at an increasing MOI. Images are representative of at least three independent observations. Scale bars= 50μm.
Figure 3.10 Determination of mToR expression following exposure to *T. forsythia* in CHRF-288-11 cells. Cells were challenged with *T. forsythia* ATCC 43037 for 90 minutes before fixation. Cells were then permeabilised and stained for mToR (red), DAPI (blue) and *T. forsythia* (green). Despite bacterial association increasing with an increasing MOI, no changes in mToR expression were observed across any treatments. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.11 *P. gingivalis* decreases mToR expression in differentiated CHRF-288-11 cells. CHRF-288-11 cells were differentiated for five days before being challenged with *P. gingivalis* at an MOI of either 1:10 or 1:100. Cells were then fixed, permeabilised and probed for DAPI (blue), mToR (red) and *P. gingivalis* (PGWC1, green). Large lobed nuclei confirm cellular differentiation, with bacterial association also increasing with an increasing MOI. Bacterial treatment resulted in no change within expression of mToR at both an MOI of 1:10 and 1:100. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.12 Effect of *T. forsythia* invasion on mToR expression in differentiated CHRF-288-11 cells. Cells were differentiated for five days before being exposed to *T. forsythia* ATCC 43037 for 90 minutes and fixed. Cells were then permeabilised before being stained for mToR expression (red), nucleic phenotyping (DAPI, blue) and bacterial cells (TFWC, green). Bacterial treatment with *T. forsythia* resulted in no change within mToR staining despite an increased bacterial association at both an MOI of 1:10 and 1:100. Images are representative of at least three independent observations. Scale bars= 50 μm.
3.3.3.2 Analysis of CHRF-288-11 integrin β3 expression following incubation with periodontal pathogens

Undifferentiated and differentiated CHRF-288-11 cells expressed integrin β3, uniformly diffused across the cell membrane (Figure 3.13-3.16). Exposure to bacterial cells at an increasing MOI resulted in an increased bacterial association and invasion by both *P. gingivalis* NCTC 11834 and *T. forsythia* 43037. Within both undifferentiated (Figure 3.13 and 3.14) and differentiated (Figure 3.15 and 3.16) cells, exposure to either pathogen resulted in an increased expression of integrin β3 although no localisation between either pathogen or integrin β3 could be determined.
Figure 3.13 The effect of *P. gingivalis* invasion on integrin β3 expression in CHRF-288-11 cells. CHRF cells were challenged with *P. gingivalis* for 90 minutes before cells were fixed and permeabilised. Cells were then treated with anti-β3 (red) and pathogen specific (PGWC1, green) antibodies before being mounted with DAPI (blue). Incubation with *P. gingivalis* increases staining of integrin β3 most notably at an MOI of 1:100. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.14 Relative integrin β3 expression following exposure to *T. forsythia* ATCC 43037. CHRF cells were treated with *T. forsythia* ATCC 43037 for 90 minutes before associated bacterial cells were removed. Cells were then fixed, permeabilised and probed for integrin β3 (red), *T. forsythia* ATCC 43037 (TFWC, green) and mounted with DAPI (blue). Bacterial challenge with *T. forsythia* ATCC 43037 increases integrin beta 3 expression at an MOI of 1:10 and 1:100. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.15 The effect of *P. gingivalis* invasion on integrin β3 expression by differentiated CHRF-288-11 cells. CHRF-288-11 cells were differentiated for five days before being challenged with *P. gingivalis* NCTC 11834 at an MOI of with 1:10 or 1:100 for 90 minutes at 37 °C. Cells were then fixed, permeabilised and probed for *P. gingivalis* NCTC 11834 (PGWC1, green), integrin β3 expression (red) and DAPI (blue). Incubation with *P. gingivalis* NCTC 11834 increased β3 expression at both an MOI of 1:10 and 1:100 when compared with the untreated controls. Images are representative of at least three independent observations. Scale bars= 50 μm.
Figure 3.16 *T. forsythia* increases β3 expression in differentiated CHRF-288-11 cells. CHRF-288-11 cells were differentiated before being exposed to *T. forsythia* ATCC 43037 at an MOI of either 1:10 or 1:100. Cells were then fixed and permeabilised before being stained for β1 (red), *T. forsythia* ATCC 43037 (TFWC, green) and DAPI (blue). Following exposure to *T. forsythia* ATCC 43037, integrin β1 staining increased at both an MOI of 1:10 and 1:100. Images are representative of at least three independent observations. Scale bars= 50 μm.
3.3.4 Flow cytometric analysis of megakaryocytic/platelet markers following *P. gingivalis* challenge

Having demonstrated that *P. gingivalis* invade differentiated megakaryocytic-like cells, impacting on integrin β1 and mToR expression, further investigations were undertaken to determine the effect of periopathogens on surface megakaryocyte/platelet markers. Using flow cytometry, *P. gingivalis* association with differentiated CHRF cells and the relative expression of CD41, CD61, CD41/61 and CD62P were quantified (Figure 3.17).

Using this approach, sub-populations of cells associated with *P. gingivalis* (wild-type or OMPA deficient mutants) could not be identified as no significant increase in PGWC1 fluorescence was detected when bacterial treated samples were compared to untreated controls (Figure 3.17a). Similarly, no significant change in integrin expression was detected with either CD41 (Figure 3.17b), CD61 (Figure 3.17c), CD41/61 (Figure 3.17d) or CD62P (Figure 3.17e) following bacterial treatment with wildtype of mutant *P. gingivalis*. 
Figure 3.17 Quantitation of megakaryocytic surface marker expression following challenge with *P. gingivalis* using flow cytometry. Differentiated CHRF-288-11 cells were challenged with either wild-type *P. gingivalis* (NCTC 11834, ATCC W50) or the OMPA deficient mutants NCTC 11834 ΔompA1 and NCTC 11834 ΔompA2 before being treated with antibodies to detect the expression of platelet surface antigens and pathogen presence (PGWC1). No significant changes in detection of bacterial association (A) or the expression of CD41 (B), CD61 (C), CD41/61 (D) and CD62P (E) following bacterial challenge. Data is expressed as ±SEM, n=3.
3.3.5 Development of a cell-based ELISA for immunophenotyping of CHRF-288-11 cells following periodontal pathogen challenge

To further quantify periodontal pathogen invasion of CHRF-288-11 cell and the effect of pathogen invasion on surface protein expression, a cell-based ELISA was developed. Cells were differentiated onto a solid surface and probed for bacterial binding using pathogen specific antibodies (PGWC1, TFWC) or the relative expression of CHRF integrins β1 and β3.

Following bacterial challenge at an MOI of 1:100, significant binding of *P. gingivalis* NCTC 11834 and ATCC W50 was observed ($p<0.001$; Figure 3.18a), as well as a significant increase in expression of integrin β1 ($p<0.001$, Figure 3.18b). *P. gingivalis* NCTC 11834 was also found to induce a significant increase of β3 expression ($p<0.001$; Figure 3.18c). A slight increase in integrin β3 expression was also observed following exposure to ATCC W50, although this was determined as not statistically significant (Figure 3.18c). *T. forsythia* ATCC 43037 did not associate with differentiated CHRF-288-11 cells (Figure 3.18a) or induce any change in either integrin β1 (Figure 3.18b) or β3 (Figure 3.18c) expression.
Figure 3.18 Analysis of bacterial association and integrin expression by a cell-based ELISA. CHRF-288-11 were differentiated into well-plates before being challenged with periodontal pathogens (MOI 1:100), fixed and probed with pathogen specific antibodies (PGWC1 or TFWC) (A) or for expression of integrin β1 (B) and integrin β3 (C). Significant binding of \textit{P. gingivalis} NCTC 11834 and ATCC W50 was detected at an MOI of 1:100 (A). A significant increase in expression of integrin β1 was detected following treatment with \textit{P. gingivalis} NCTC 11834 and ATCC W50 (B), with \textit{P. gingivalis} NCTC 11834 also stimulating a significant increase in β3 expression (C). Data is expressed as ±SEM, \( p=*** <0.001, n=3 \).
3.3.5.1 Is *P. gingivalis* association and interaction with CHRF-288-11 dependent on the multiplicity of infection?

Investigations to determine whether the observed association to CHRF-288-11 cells by *P. gingivalis* was MOI dependant. Differentiated CHRF-288-11 cells were challenged with *P. gingivalis* at an increasing MOI before cells were fixed and probed with pathogen specific antibodies (PGWC1). *P. gingivalis* NCTC 11834 (Figure 3.19a) and ATCC W50 (Figure 3.19b) were both shown to increase in bacterial association relative to an increasing MOI.
Figure 3.19 Specific periodontal pathogen interaction with differentiated CHRF-288-11 cells. Differentiated CHRF-288-11 cells were challenged with bacterial cells at an increasing MOI before fixation and detection with pathogen specific antibodies. The data demonstrates specific, pathogen binding in a direct relationship with increasing MOI following treatment with *P. gingivalis* NCTC 11834 (A), *P. gingivalis* ATCC W50 (B). Data is expressed as ±SEM, n=3.
After confirming that periodontal pathogen interaction was specific and MOI dependant, the relationship between integrin expression and bacterial invasion was investigated. As above, differentiated CHRF-288-11 cells were challenged with a titrated MOI of periodontal pathogens before fixation and the relative expression of integrin β1 and β3 was detected (Figure 3.20). These results show that *P. gingivalis* NCTC 11834 stimulates an increase in expression of integrin β1 (Figure 3.20a) and integrin β3 (Figure 3.20b) at all the MOI's tested with the observed increase in expression appearing to be independent of MOI and of direct consequence, solely to bacterial treatment. Similar findings were also observed when cells were treated with *P. gingivalis* ATCC W50 (Figure 3.20c-d).
Figure 3.20 The effect of MOI on periodontal pathogen induced integrin expression in differentiated CHRF-288-11 cells. Differentiated CHRF cells were challenged with periodontal pathogens at an increasing MOI, before being fixed and probed for expression of integrin β1 and β3. Both *P. gingivalis* NCTC 11834 and ATCC W50 induced an increase in expression of integrin β1 (A and C) and β3 (B and D). Data presented is a representative figure of three independent observations.
3.4 Discussion

The overall aim of this study is to determine whether an in vitro megakaryocytic cell line can be used to study platelet-pathogen interactions. Due to the ability of platelets to become readily activated and the lack of reliable storage options, platelet studies require fresh platelets and as such, also rely on regular blood donations (Bausset et al., 2012). The availability of a platelet-like cell line will therefore help minimise the reliance on blood donations, an invasive procedure, whilst enabling studies to be conducted in vitro, prior to validation using fresh platelets isolated from donors.

Earlier studies have proposed the use of megakaryocytic-like cell lines such as DAMI (Greenberg et al., 1988) and UT-7 (Komatsu et al., 1991) within platelet research. However, as with many cell lines, megakaryocytic-like cells carry many caveats including misidentification, contamination as well as genomic and phenotypic instability (Geraghty et al., 2014). As an example, UT-7 cells have since been shown to express markers of different cell lineages including those associated with erythroid and myeloid phenotypes (Saito, 1997). Further to this, although the DAMI cell line seemed promising as an in vitro platelet model, since its initial characterisation it is reported to either be contaminated with or share genetic markers with the HEL erythroleukemia cell line (MacLeod et al., 1997). For these reasons, initial investigations focussed on characterising the CHRF-288-11 cell line and exploring its suitability as a platelet-like model.

Optimisations of the CHRF-288-11 cells confirmed that treatment with PMA (50 ng/ml) over a five day period yielded consistent CHRF differentiation. This was assessed by the characteristic changes in phenotype and illustrated by cell enlargement and the
formation of polyploidial, multi-lobed nuclei in line with previous reports by Fugman and colleagues (1990).

Further analysis of differentiated CHRF cells via flow cytometry confirmed the expression of several markers attributed to the megakaryocytic cell lineage and platelet production. In agreement with findings by Conran and Hemming (1998), CHRF cells were found to express both CD41 (αIIb, GPIIb) and CD61 (β3, GPIIIa), which form the heterodimer complex of CD41/61 that is expressed by both megakaryocytes (Duperray et al., 1987) and platelets (Wagner et al., 1996b). The megakaryocytic-like cells were also shown to express the activated epitope of CD41/61, which acts as a fibrinogen receptor during platelet activation (Shattil et al., 1985).

Additionally, an increase in CD62P expression, a protein normally contained within platelet alpha granules (Stenberg et al., 1985), was observed following PMA treatment on the cell surface of the differentiated megakaryocytic cells. Surprisingly, it was also found that CHRF-288-11 cells did not express CD42b (GPIbα) within either undifferentiated or differentiated cells. Although GPIbα is highly expressed on the surface of platelets (Li and Emsley, 2013), it is also expressed by megakaryocytes, with an increase in GPIbα expression being reported as a marker for both maturation and differentiation (Lepage et al., 2000).

Following the initial characterisation of the CHRF-288-11 cells and their associated platelet-like phenotype, further investigations explored whether this cell type could interact with oral pathogens. Despite the role of platelets within the immune system being widely published (Klinger and Jelkmann, 2002; Yeaman, 2010b; Gardiner and Andrews, 2013; Ali, Wuescher and Worth, 2015; Koupenova and Freedman, 2015), research surrounding bacterial interactions with megakaryocytes is highly limited.
Megakaryocytes are derived through haematopoietic stem cell differentiation and as a cell type are extremely rare, accounting for approximately 0.1% of nucleated cells within the bone marrow, which would suggest that interactions with bacterial cells are highly improbable. However, as well as residing within the bone marrow, megakaryocytes are also routinely found within circulating blood (Kallinikos-Maniatis, 1969) and have been shown to migrate to, terminally differentiate and produce platelets within the lung tissue (Howell and Donahue, 1937; Lefrançais et al., 2017) suggesting that although improbable, interactions with bacterial cells are not altogether impossible. It has also been previously reported that megakaryocytes express toll-like receptors, suggesting that they could hypothetically interact with bacterial cells (Beaulieu and Freedman, 2010), with a further study showing that isolated megakaryocytes can interact with heat-inactivated Escherichia coli, stimulating the translocation of complement protein C (Arbesu et al., 2016).

In contrast, it is well-established that the periodontal pathogens P. gingivalis and T. forsythia ATCC 43037 interact and invade a range of cell types including epithelial cells (Nakajima et al., 2006; Stathopoulou et al., 2010; Suwannakul et al., 2010; Mishima and Sharma, 2011; Stafford et al., 2013), osteoblasts (Zhang et al., 2010), trophoblasts (Inaba et al., 2009) as well as cells of the hematopoietic stem cell lineage such as monocytes (Pollreisz et al., 2010; Bloch et al., 2018). Periodontal pathogens have also been associated with increasing risk of cardiovascular disease (Kebschull, Demmer and Papapanou, 2010) while P. gingivalis has been shown to induce platelet aggregation (Curtis et al., 1993a). However, the mechanisms of these interactions are not fully understood.
Using the CHRF-288-11 cells as a platelet-like cell line, the interaction of both *P. gingivalis* and *T. forsythia* were investigated. Increased association of pathogens with differentiated CHRF cells were observed by immunofluorescence microscopy and further confirmed by ELISA. *P. gingivalis* and *T. forsythia* invaded the differentiated cells in an antibiotic-protection assay and were shown to localise within peri-nuclei regions. This observed cellular interaction with CHRF-288-11 cells agrees with previous findings that demonstrate that both *P. gingivalis* (Belton *et al*., 1999) and *T. forsythia* (Inagaki *et al*., 2006) are able to localise intracellularly with cellular invasion occurring in similar levels to that observed within host epithelium.

As well as characterising interactions with wild-type *P. gingivalis*, the role of the major outer membrane protein subunits OMPA1 and OMPA2 within CHRF-288-11 interactions were also explored. No significant differences were observed between either the Δ*omp*A1 or the Δ*omp*A2 deficient mutants when compared to the parent strain within CHRF-288-11 association as determined by antibiotic protection assays and flow cytometry. It has previously been shown that OMPA, specifically the OMPA2 subunit is fundamental for interactions between *P. gingivalis* and epithelial cells *in vitro* (Naylor *et al*., 2016). However the data produced within this chapter of work suggests that *P. gingivalis* interactions with CHRF-288-11 cells occur independently of either OMPA subunit, suggesting that both of the OMPA subunits can facilitate bacterial association with CHRF-288-11 cells independently. Further investigations therefore targeted understanding how *P. gingivalis* interacts with megakaryocytic-like cells.

As pathogens such as *Escherichia coli* (Arbesu *et al*., 2016) are known to activate platelets and alter the expression levels of surface platelet markers, the effect of *P.
*gingivalis* NCTC 11834 and ATCC W50 on the expression of specific cellular markers expressed by both CHRF-288-11 cells and mammalian platelets was investigated.

The mammalian target of rapamycin (mToR), a serine kinase that integrates with several key process including cell proliferation, protein synthesis and autophagy (Hay and Sonenberg, 2004) has been identified within previous research by Stafford and colleagues (2013) as a target for *P. gingivalis* host cell manipulation. Within this chapter of work, immunofluorescent analysis revealed that incubation with *P. gingivalis* resulted in a decreased expression of mToR within differentiated CHRF-288-11 cells in an MOI dependant relationship, whereas incubation with *T. forsythia* did not. In agreement with these findings, within epithelial cells, gingipains derived from *P. gingivalis* have been shown to degrade mToR and manipulate cell signalling pathways (Stafford *et al.*, 2013) and could suggest a similar mechanism in which *P. gingivalis* interacts with megakaryocytic-like cells.

Further to this, *P. gingivalis* is known to interact with host cells through multiple pathways including targeting and manipulating integrin expression, such as integrin β1 (Yilmaz, Watanabe and Lamont, 2002; Li *et al.*, 2013) and β3 (Li *et al.*, 2013; Boisvert, Lorand and Duncan, 2014). Within this chapter of work, it was investigated as to whether *P. gingivalis* could affect integrin β1 and β3 expression within the CHRF-288-11 cell line. Immunofluorescent images highlighted an overall increase in CHRF-288-11 expression of integrin β3 following bacterial challenge with both *P. gingivalis* and *T. forsythia*. It was also found that CHRF-288-11 integrin β3 expressions was uniformly diffused throughout the cell membranes and no distinct localisation between integrin expression and the presence of either *P. gingivalis* or *T. forsythia*. This initial data was also further confirmed through the application of a cell based ELISA, which
demonstrated an increase in expression in both integrin β1 and β3 following exposure to both *P. gingivalis* NCTC 11834 and ATCC W50 that was not dependent on MOI. Surprisingly however, further application of this developed ELISA revealed that *T. forsythia* did not bind to either recombinant β1 or β3, suggesting that other external factors may be needed for *T. forsythia* to associate with CHRF-288-11 cells for example through binding of lectin-like receptors or interactions with host toll-like receptors through expressed leucine-rich repeat motifs (Sharma, 2010).

Although the development of this protocol is novel within the study of megakaryocytic-like cells, it has previously reported that several bacterial species including *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli* can induce and amplify platelet activation through interactions with the integrin complex αIIbβ3 (Arman et al., 2014; Watson et al., 2016), with *P. gingivalis* also being shown to bind to both integrin αvβ3 and α5β1 when overexpressed with a Chinese hamster ovary cell lines. However research surrounding the interactions between either *P. gingivalis* or *T. forsythia* and cells of megakaryocytic lineage is to date extremely limited. Within this study, a more in-depth analysis of integrin expression by flow cytometry following exposure to periodontal pathogens was attempted but was deemed unsuccessful and did not generate any observable differences within any of the makers tested. It was originally hypothesised that sub-populations of invaded CHRF-288-11 cells could be identified through the application of pathogen specific antibodies but due to experimental and equipment limitations this could not be achieved.

The application of the CHRF-288-11 cell line within this study has provided an unlimited and rapid screening methodology of bacterial interactions with several markers specific to mature mammalian platelets. However it must be noted that as
with many megakaryocytic cell lines, the CHRF-288-11 cells are of leukemic origin (Fugman et al., 1990) and despite the CHRF-288-11 cells actively expressing multiple phenotypes associated with both megakaryocytes and platelets, it is important to confirm any finding within a highly purified platelet population. Further to this, recent developments within megakaryocyte culture techniques are detailing the routine culture of primary megakaryocytes and the production of large volumes of platelets in vitro (Moreau et al., 2016; Strassel, Gachet and Lanza, 2018), meaning that the use of megakaryoblast cell lines as well as the need for regular blood acquisition could soon be abolished entirely within the field of platelet research.
3.5 Summary

In conclusion, this study has highlighted that the CHRF-288-11 megakaryocytic-like cells can be utilised through a series of methodologies to study the pathogenic effects of periodontal pathogens on platelets and the megakaryocytic cells in vitro. This developed methodology could be applied to understand how these anaerobic bacteria can stimulate host cell responses and how platelets behave following infection.

Although multiple previous have studies have shown that periodontal pathogens can infiltrate and interact various cell types (Lamont et al., 1995; Deshpande, Khan and Genco, 1998; Simin F Nakhjiri et al., 2001; Yilmaz, Watanabe and Lamont, 2002; Yilmaz et al., 2008; Kirschbaum et al., 2010a; Suwannakul et al., 2010; Honma, Mishima and Sharma, 2011; Stafford et al., 2013; Naylor et al., 2016), as well as alter host cellular functions (Darveau et al., 1998; Tervahartiala et al., 2000; Kiili et al., 2002; Yilmaz et al., 2003, 2008; Nakajima et al., 2006; Posch et al., 2011; Settem et al., 2013; Stafford et al., 2013; Ksiazek, Mizgalska, Enghild, et al., 2015; Lee et al., 2015; Naylor et al., 2016) very little work has focussed on how these bacterial species may impact platelets or result in atherosclerotic phenotypes (Yun et al., 2005; Nicu et al., 2009; Papapanagiotou et al., 2009; Arman et al., 2014). More specifically, no current work has explored; how T. forsythia may affect any haematopoietic cell lineages, how periodontal pathogens could affect platelet function or how platelet/periopathogen interactions and the resulting consequences occur at the cellular level. Further work is needed to expand these initial studies to fully characterise the effects of P. gingivalis and T. forsythia on CHRF-288-11 cells as well as moving on to validating these finding in platelets, in order to answer these questions.
Chapter 4: Can periodontal pathogens interact with and induce platelet aggregation?
4.1 Introduction

The interactions between prokaryotes and mammalian platelets have been well documented with detailed studies showing that a broad spectrum of bacterial species can not only bind to platelets, but also induce platelet aggregation and activation (Clawson and White, 1971a, 1971b; Clawson, 1973; Clawson, Rao and White, 1975; Kerrigan, 2015). Examples of such interactions include the induction of aggregation and degranulation of platelets by *Staphylococcus aureus* (Hawiger *et al.*, 1979) and the indirect interaction between *Streptococcus pyogenes* and platelets that is mediated by the availability of fibrinogen and von Willebrand factor (vWF) (Kurpiewski *et al.*, 1983; Johnson and Bowie, 1992). Further to this several orally derived bacterial species, including *Streptococcus spp.*, *Staphylococcus spp.*, *Pseudomonas aeruginosa* and *Porphyromonas gingivalis*, have been implicated in cardiovascular disease (CVD) (McNicol and Israels, 2010; McNicol, 2015). It is thought that bacteria are able to gain access to the circulation, penetrate vascular walls causing secondary infections that ultimately lead to the formation of atherosclerotic plaques which then rupture and lead to platelet clotting and atherothrombosis (Kaplan and Jackson, 2011). Several studies have also suggested that bacterial-platelet interactions are crucial to the pathogenesis of some cardiovascular pathologies, as reviewed by McNicol and Israels (2010).

Blood platelets undoubtedly play a predominant role within haemostasis, but their emerging role within antimicrobial host defence and in modulation of inflammatory responses could hold a crucial link that underpins the mechanism behind thrombotic and inflammatory events (Von Hundelshausen and Weber, 2007). In the context of periodontal pathogens, oral pathogenic bacteria have been reported to interact with
platelets through toll-like receptors (TLR’s), causing platelet activation and the release of activation markers such as P-selectin and ADP (Shiraki et al., 2004; Blair et al., 2009; Zhang et al., 2009) as well as inducing the pro-thrombotic pathway (Roth et al., 2009). However the exact mechanisms by which these interactions occur are not fully elucidated.

The sequence of events that follows platelet-bacterial association can be defined as; contact, shape change, early aggregation and irreversible aggregation (Clawson and White, 1971a). The use of light transmission aggregometry was initially developed in the 1960’s (Born and Cross, 1963; Vigdahl, Marquis and Tavormina, 1969) and is still recognised as the gold-standard approach in platelet function testing in relation to cardiovascular diseases (Michelson, 2004). This chapter expands on the initial findings that *P. gingivalis* can induce platelet aggregation (Naito et al., 2006; Naito, 2007; Li et al., 2008) and investigates how viability and virulence factors of both *P. gingivalis* and *T. forsythia* could act as contributing factors to platelet/periopathogen interactions.

### 4.2 Aims

The studies described in this chapter aim to determine whether the periodontal pathogens *P. gingivalis* and *T. forsythia* induce platelet aggregation. This was investigated by determining:

- The possible induction of platelet aggregation by a range of *P. gingivalis* and *T. forsythia* strains at varying multiplicities of infection.
- The contribution of virulence associated bacterial proteins on the induction of platelet aggregation.
- Whether heat-killed bacteria can induce platelet aggregation.
4.3 Results

4.3.1 Platelet aggregation with wildtype *P. gingivalis* and *T. forsythia*

Light transmission aggregometry was initially used to screen wildtype (WT) *P. gingivalis* strains NCTC 11834, ATCC W50, ATCC 381 and *T. forsythia* ATCC 43037 to determine whether they induced platelet activation at varying multiplicity of infection (MOI). Isolated platelet rich plasma (PRP) was exposed to wildtype *P. gingivalis* at an MOI of 1:1 and 1:10. The rate of platelet aggregation was recorded over a period of 10 minutes at 37 °C and compared to untreated PRP or PRP treated with ADP (20 μM), as a negative and positive control respectively. A representative aggregation plot is shown in Figure 4.1a. As expected ADP induced platelet aggregation (red), while no aggregation was observed with untreated PRP (blue). *P. gingivalis* NCTC 11834 was also observed to induce platelet aggregation which reached a peak at 8 minutes when compared to ADP with maximum aggregation reached within 4 minutes.

Interestingly, aggregation was observed to be strain dependent with significant aggregation induced by *P. gingivalis* NCTC 11834 at an MOI of both 1:1 (*p*<0.05) and 1:10 (*p*<0.001), and by *P. gingivalis* ATCC 381 at an MOI of 1:10 (*p* <0.001; Figure 4.1b). With *P. gingivalis* ATCC W50, no aggregation was observed at either MOI within the 10 minute period (Figure 4.1b). In contrast to *P. gingivalis*, aggregation was not induced by wildtype *T. forsythia* at either MOI under the same experimental conditions (Figure 4.1c).
Figure 4.1 Induction of platelet aggregation by wildtype (WT) periodontal pathogens. (A) Example aggregation plot. An example trace of light transmission aggregometry recorded over 10 minutes at 37°C with *P. gingivalis* (green). Aggregation induced by ADP (20 μM, red) represents 100% aggregation and was included as a positive control. Untreated PRP (blue) was included as a negative control and demonstrates 0% aggregation. (B) Platelet aggregation by WT *P. gingivalis*. WT *P. gingivalis* NCTC 11834 and ATCC 381 triggered platelet aggregation at MOI of 1:1 and 1:10. Aggregation was not observed with ATCC W50 at either MOI investigated over the 10 minute period. (C) Platelet aggregation by WT *T. forsythia*. Platelet aggregation was not observed in the presence of WT *T. forsythia* at an MOI of either 1:1 or 1:10 ADP (20 μM) and untreated PRP are included as positive and negative control respectively. Data is expressed as ±SEM, $p= *<0.05$, $**<0.001$. n=4.
4.3.2 Investigation of the effect of bacterial virulence on platelet aggregation in induced by *P. gingivalis*

After the initial findings that wildtype *P. gingivalis* induces platelet aggregation in a strain dependant manner, further investigations were conducted to explore whether factors associated with bacterial virulence and/or interaction with cells could affect either the total percentage or the rate of aggregation. The ability of the *P. gingivalis* mutants *P. gingivalis* NCTC 11834 ΔompA1, *P. gingivalis* NCTC 11834 ΔompA2 and *P. gingivalis* ATCC 381 ΔSia0352 (described in Section 3.4) on platelet aggregation was determined. All mutants investigated, induced aggregation (*p*<0.001) at MOI 1:10, which was comparable to the parental strain (Fig 4.2).

The rate of aggregation was determined by calculating the time taken to reach 50% aggregation (AG50). Interestingly, AG50 values were found to vary when wildtype and mutants were compared (Figure 4.2). Both PG mutants deficient in the outer-membrane proteins OMPA1 (*P. gingivalis* NCTC 11834 ΔompA1) and OMPA2, (*P. gingivalis* NCTC 11834 ΔompA2) significantly induce platelet aggregation when compared to the parent wildtype strain (*p*<0.05, 0.01). The ΔompA2 mutant also exhibited a faster rate of aggregation when compared to the ΔompA1 mutant. This finding was also observed with *P. gingivalis* ATCC 381 ΔSia0352, which induced a significantly faster rate of platelet aggregation when compared to the wildtype strain (*p*<0.01; Fig. 4.2).
Figure 4.2 The effect of virulence associated proteins on platelet aggregation. (A) Induction of aggregation by mutants of *P. gingivalis*. Following exposure to mutants or WT strains of *P. gingivalis*, significant levels of aggregation were observed when compared to untreated PRP samples. (B) Time taken for platelet aggregation to reach 50% (AG50) of platelet aggregation when stimulated by WT *P. gingivalis* and mutants. Both mutants deficient in the outer membrane proteins (OMPA1 and OMPA2) and the Sialidase deficient strain (ΔSia0352) significantly induced a faster rate of aggregation when compared to the relevant WT strains. The ΔompA2 mutant was also observed to induce aggregation a faster rate when compared to the ΔompA1 mutant. ADP (20 μM) was included as a positive control for platelet aggregation. Data is expressed as ±SEM, *p*≤0.05, **p**≤0.01, ***p**≤0.001, n=4.
4.3.3 Investigation of aggregation induced by *T. forsythia* mutants

After previous experiments (Section 4.3.2) revealed that bacterial virulence associated factors could affect the rate of platelet aggregation it was hypothesised that the virulence factors associated with *T. forsythia* could be preventing an aggregatory response. In order to investigate this both the *T. forsythia* ATCC 43037 ΔnanH deficient mutant and the *T. forsythia* ATCC 43037 ΔwecC isogenic mutant (described in Section 3.4) were investigated in parallel with the wildtype strain. Contrary to the hypothesis, none of the bacterial stimulants caused platelet aggregation when compared to untreated PRP and PRP treated with ADP (20 μM) as a negative and positive control respectively (Figure 4.3).

![Figure 4.3 Platelet aggregation by *T. forsythia* WT strain and mutants.](image)

Platelets treated with *T. forsythia* ATCC 43037 or one of the two mutants (*T. forsythia* ATCC 43037 ΔnanH or *T. forsythia* ATCC 43037 ΔwecC) did not induce platelet aggregation at an MOI of 1:10. ADP (20 μM) was included as a positive control and all samples were directly compared to untreated PRP. Data is expressed as ±SEM, *p=***<0.001*. n=4.
4.3.4 Investigations into the effect of bacterial viability on platelet aggregation

After it had been demonstrated that platelet aggregation can be induced by viable periodontal pathogens, investigations were conducted to elucidate whether the pathogens need to be viable to elicit this effect. *P. gingivalis* NCTC 11834, ATCC W50 and *T. forsythia* ATCC 43037 were heat treated and the effect of the resulting heat-killed bacteria on platelet aggregation was determined. It was observed that both viable and heat killed *P. gingivalis* induced significant aggregation (*p* <0.01, 0.001), however when compared to the wildtype, the heat killed bacteria showed a significant decrease in percentage aggregation (*p*<0.001; Figure 4.4a). In contrast to this, although some aggregatory response was observed with wild type *P. gingivalis* ATCC W50, no significant aggregation was recorded with *T. forsythia* ATCC 43037 or *P. gingivalis* ATCC W50 in either treatment (Figure 4.4a)

Earlier findings demonstrated that *P. gingivalis* lacking the outer membrane proteins OMPA1 and OMPA2 showed a significant increase in the rate of platelet aggregation. To further understand these findings, investigations were designed to explore whether bacterial viability could impact the aggregatory response as seen with wildtype *P. gingivalis* (Figure 4.4a). All bacterial treatments successfully resulted in stimulating platelet aggregation, however only the viable treatments resulted in significant readings (Figure 4.4b). Total percentage aggregation does not account for the time taken for aggregation to initiate or the rate of the recorded aggregatory response. To overcome this factor the data was further processed to analyse the time take for each sample to reach AG50. The analysis highlights that all bacterial treatments induce platelet aggregation (Figure 4.4b) but also that with heat killed, non-viable bacteria
there was a significant decrease in the rate of aggregation (Figure 4.4c) suggesting that interactions with viable bacteria are needed to induce a platelet aggregatory response.
Figure 4.4 The effect of bacterial viability on platelet aggregation. (A) Aggregation by periodontal pathogens. Both wild type (WT) and heat killed (HK) *P. gingivalis* NCTC 11834 induced a significant level of platelet aggregation. In contrast, both WT and HK *P. gingivalis* ATCC W50 and *T. forsythia* ATCC 43037 did not induce significant aggregation. Interestingly, the HK treated *P. gingivalis* NCTC 11834 bacterium produced a significant reduction in aggregatory response when compared to the WT. (B) Induction of aggregation by viable and heat killed *P. gingivalis* deficient in the outer membrane proteins. Significant levels of aggregation was observed by *P. gingivalis* NCTC 11834 and mutants (*P. gingivalis* NCTC 11834 ΔompA1 and *P. gingivalis* NCTC 11834 ΔompA2) when compared to untreated PRP. All HK treatments also induced some level of aggregation but none of these findings were statistically significant. (C) AG50 of aggregation triggered by both viable and HK *P. gingivalis*. Platelets were exposed to both viable and HK bacteria before aggregation times were recorded against A significant decrease in the time taken to reach 50% aggregation was observed in the WT *P. gingivalis* NCTC 11834 and both mutant strains (PG NCTC 11834 ΔompA1 and PG NCTC 11834 ΔompA2). Aggregation was measured following exposure to pathogens (WT and HK) at an MOI of 1:10 for 10 minutes at 37 °C. Data is expressed as ±SEM, p= **≤0.01, ***≤0.001. n=4.
4.4 Discussion

Understanding the relationship by which periodontal pathogens and platelets interact is crucial to explicate the postulated impact of oral pathogens on cardiovascular disease. This chapter investigated the effect of periodontal pathogens on the induction of platelet aggregation. The data presented here corroborate previous findings suggesting that *P. gingivalis* NCTC 11834 interacts with platelets and induces aggregation at increasing MOI (Naito *et al.*, 2006; Naito, 2007; Li *et al.*, 2008).

This study demonstrates that *P. gingivalis* induced aggregation is strain specific as aggregation was observed with both *P. gingivalis* NCTC 11834 and *P. gingivalis* ATCC 381 strains but not with ATCC W50. This is in contrast to the findings of Klarström Engström and co-workers (2015) who reported that ATCC W50 caused approximately 50% aggregation when compared to NCTC 11834. The two studies however are not strictly comparable due to substantial differences in the methodology used. Klarström Engström *et al* (2015) conducted aggregation experiments in a modified Krebs Ringer glucose buffer, which replaces chelated cations that are lost within the platelet isolation process, over a longer time frame (15 minutes) and in the absence of relevant positive controls. In this work, all assays were conducted using isolated platelet rich plasma in citrate buffer and aggregation was monitored over 10 minutes. Throughout the optimisation of initial aggregation experiments with wildtype *P. gingivalis* strains within this body of work, it was noted that after three passages of bacterial culture all experimental conditions began to induce platelet aggregation. It is well documented that bacterial species are able adapt their behaviour and phenotype according to environmental conditions (Friedman *et al.*, 2015) and for this reason all experiments
reported within this thesis were done with bacteria that had undergone no more than three passages.

Strain-specific induction of platelet aggregation has been previously reported with other bacterial species including *Helicobacter pylori* which is associated with peptic stomach ulcers and *Streptococcus pneumoniae*, one of the underlying causes of bacterial pneumonia (Corcoran *et al*., 2007; Keane *et al*., 2010), thereby suggesting that strain-specific factors may affect the pathogenicity of different bacterial isolates. This is further illustrated by the fact that strain variances are known to impact on the invasive and virulent characteristics of *P. gingivalis* (Jandik *et al*., 2008; Suwannakul *et al*., 2010; Baek *et al*., 2015). It was previously reported that both *P. gingivalis* NCTC 11834 and *P. gingivalis* ATCC 381 are highly invasive when compared to *P. gingivalis* ATCC W50 in human umbilical vein endothelial cells (HUVEC) and KB epithelial cell lines when characterised via antibiotic protection assays (Dorn *et al*., 2000).

Other work has also begun to elucidate the underlying genetics which may explain the observed variances across bacterial isolates. Igboin and colleagues (2009) demonstrated that, when compared by heteroduplex and ribosomal intergenic spacer region (ISR) sequencing, *P. gingivalis* strains NCTC 11834 and 381 were genetically highly related but are both highly divergent when similarly compared with *P. gingivalis* ATCC W50. These findings by Igboin *et al*., (2009) not only explain the observations of this study that NCTC 11834 and 381 but not ATCC W50 caused platelet aggregation, but may also provide an insight into the underlying genetic variances that contribute to *P. gingivalis* phenotypes and virulence.

As well as strain specific variations, it was also hypothesised that periodontal-pathogen mediated platelet aggregation could be dependent on specific bacterial membrane
proteins and their involvement in mammalian cellular interactions. Therefore the role of the outer-membrane proteins OMPA1 and OMPA2 was investigated.

Previous studies by Suwannakul and colleagues (2010) identified the OMPA protein as being important in host interactions. Further studies demonstrated that two major subunits of the outer membrane proteins namely OMPA1 and OMPA2 were important in biofilm formation as well as epithelial cell interactions (Naylor et al., 2016). In this study, the deletion of either subunit OMPA1 and OMPA2 did not impact on the overall percentage of aggregation although the data suggest a possible role in triggering the onset of an aggregatory response. When compared to the parent strain, both OMPA1 and OMPA2 mutants were shown to significantly decrease the time taken to initiate platelet aggregation \( (p<0.05\) and \( <0.001\) for the OMPA1 and OMPA2 mutants, respectively). OMPA2 was also observed to induce aggregation at a faster rate than the OMPA1 mutant, but this difference was not statistically significant at the 0.05 level.

In addition to the OMPA proteins, the \textit{P. gingivalis} sialidase enzyme 0352 has also been implicated in cellular interactions and bacterial pathogenesis. Work by Aruni et al. (2011) identified that 0352 sialidase facilitates bacterial-host interactions within the periodontal pocket by allowing the exposure of potential binding receptors on glycoproteins and glycolipids. Further to this, sialidase activity has also been directly associated with the expression of virulence factors in \textit{P. gingivalis} including LPS, capsule, gingipain activity, fimbriae as well as circumventing host response (Aruni et al., 2011; Li et al., 2017; Yang et al., 2018). When the \textit{P. gingivalis} ATCC 381\(\Delta\)Sia0352 was screened in platelet aggregation, no differences were observed, with both the mutant and parent strain inducing a significant level of percentage aggregation \( (p<0.001)\). However, similarly to the findings with the OMP deficient mutants, \textit{P.}
*P. gingivalis* ATCC 381 ΔSia0352 caused a significantly faster onset of platelet aggregation when compared to the wildtype *P. gingivalis* ATCC 381 (*p*<0.001).

The *P. gingivalis* mutants screened throughout these aggregation studies were selected on the basis of their involvement in cellular interactions. Despite the stark biological differences between the OMPA and the ΔSia0352 mutants, the data suggests that disrupting bacterial/cellular interactions, results in a faster rate of aggregation more akin to that of a naturally occurring stimulant such as ADP. The process of delaying the aggregatory response that was observed with wildtype strains of *P. gingivalis* (NCTC 11834 and ATCC 381) could reflect a bacterial counterstrategy against the antimicrobial properties of platelets. Similar findings by Svensson and colleagues (2014) suggested that *Streptococcus pyogenes* not only initiates platelet aggregation, but exploits the fact that the formed aggregate is not significantly bactericidal allowing the bacterium to evade further host response, before disaggregating the platelets and escaping. *P. gingivalis* is well documented to supress and evade host response through a number of virulence factors including supressing complement activation though the secretion of LPS and gingipains, which promote bacterial survival (Hajishengallis, 2011). Taken together these observations suggest a possible mechanism in which *P. gingivalis* is able to hijack platelet aggregation and might be able to contribute to systemic pathogenesis.

In contrast to *P. gingivalis*, the systemic impact of *T. forsythia* is less characterised. To date limited studies have investigated interactions between *T. forsythia* and platelets. Under the same experimental conditions as *P. gingivalis*, wild type *T. forsythia* did not induce platelet aggregation. In an attempt to determine whether *T. forsythia* does not induce platelet aggregation or whether it actively evades platelet response, two
virulence factors of interest were selected. Firstly, the NanH sialidase is a virulence associated enzyme present in the outer membrane of *T. forsythia* that cleaves sialic acids on host glycoproteins, destroying their integrity and promoting cellular adhesion and invasion (Honma, Mishima and Sharma, 2011). Secondly, further work by Honma *et al.* (2007) identified that the *wecC* gene present in *T. forsythia* is responsible for regulating biofilm formation. Disruption of the *wecC* gene promotes exopolysaccharide synthesis, promotes biofilm formation and an overall increase in bacterial virulence (Honma *et al*., 2007). These two virulence associated characteristics of *wecC* and *nanH* were then screened in further aggregation experiments using the *T. forsythia* ATCC 43037Δ*nanH* deficient mutant and *T. forsythia* ATCC 43037Δ*wecc* isogenic mutant. Neither the parental strain nor the mutants resulted in an aggregatory response, suggesting that under these conditions, *T. forsythia* ATCC 43037 does not induce platelet aggregation. However, the lack of an aggregatory response does not indicate that *T. forsythia* cannot interact with platelets. Previous work has also identified that other bacterial species such as *Staphylococcus aureus* (Loughman *et al*., 2005) and *Streptococcus gordonii* (Kerrigan *et al*., 2007) do not interact directly with platelets but interact with them indirectly via bridging molecules. This indirect interaction process results in an increased lag time of up to 18 minutes to induce platelet aggregation (Loughman *et al*., 2005; Kerrigan *et al*., 2007) and could explain why no aggregation was recorded following stimulation by wild-type *T. forsythia* within this thesis during the 10 minute time period. It is also well established that bacterial species induce an 'all or nothing' response in relation to platelet aggregation (Kerrigan, 2015) and thus further experiments are needed to fully characterise the potential interactions between platelets and *T. forsythia*. 

123
In order to expand on initial aggregatory finding, the effects of bacterial viability were also explored within this chapter. The initial experiments screened both *P. gingivalis* (NCTC 11834 and ATCC W50) and *T. forsythia* (ATCC 43037) and found that both *P. gingivalis* ATCC W50 and *T. forsythia* ATCC 43037 did not induce significant levels of platelet aggregation with either the viable or heat-killed treatments. In contrast, *P. gingivalis* NCTC 11834 significantly induced platelet aggregation with both the viable and HK treatments (*p*<0.001), however comparatively the overall level of aggregation induced by the heat-killed *P. gingivalis* NCTC 11834 was significantly less in relation to the viable wildtype strain (*p*<0.05).

To probe this further, wild type *P. gingivalis* NCTC 11834 was comparatively screened by aggregation against the two OMPA deficient mutants OMPA1 and OMPA2. As previously mentioned, viable wild type NCTC11834 and both OMPA mutants were able to induce significant levels of aggregation (*p*<0.001). However in some cases, heat killed bacteria were observed to induce late-onset aggregation which is thought to reflect the variability in platelet response but this was not statistically significant. These finding were also compared to the rate at which aggregation occurred, with all the heat killed treatments (*P. gingivalis* NCTC 11834, OMPA1 and OMPA2) resulting in significantly longer times to reach AG50 when compared to the relevant viable treatments (*p*<0.01, 0.001). Taken together these findings suggest that while viability seems essential for a faster onset of platelet aggregation, it may be possible for non-viable bacterium to interact with platelets but at a slower rate. Similar findings were reported by Klarström Engström and colleagues (2015) who demonstrated that *P. gingivalis* NCTC 11834 induces platelet aggregation at an MOI of 1:1, whereas heat killing the bacterium completely eradicated any aggregatory response. The authors concluded that the heat killed treatment lacked gingipain proteolytic activity and the
ability to induce cytosolic calcium changes thereby preventing platelet activation and aggregation (Klarström Engström et al., 2015). However, the observed reduction in platelet aggregation could also be explained by the experimental approach used, as although heat-killing bacterium is a standard laboratory procedure, the process does denature key proteins involved in bacterial virulence, such as gingipains (Belton et al., 1999; Nakhjiri et al., 2001; Ankersmit, et al., 2007) and therefore it can be argued that these findings highlight the combined effect of denaturation and of bacteria cell viability.

4.5 Summary

In conclusion, this study demonstrates that viable *P. gingivalis* induces platelet aggregation in a strain dependant manner. Additionally, the data presented show that bacterial virulence associated with periopathogen/platelet interactions may reduce or dampen the aggregatory response. Within this study, neither wild-type *T. forsythia* nor any of the screened *T. forsythia* mutants were able to induce platelet aggregation. This suggests that *T. forsythia* cannot interact with platelets in the context that was observed with *P. gingivalis*. 
Chapter 5: Development and characterisation of a multicolour flow cytometry panel
5.1 Introduction

Platelets are small, anucleate discoid shaped blood cells, which play a fundamental role in both haemostasis and immune response (Sonmez and Sonmez, 2017). Normally, platelets circulate around the body in an unstimulated and resting form but, upon activation, they express a range of activation-specific markers (Jennings, 2009). This method was designed to identify and develop a panel for the simultaneous analysis of both platelet-surface and platelet-activation markers after treatment by an agonist or other stimulus following previously published guidelines on platelet analysis (Schmitz et al., 1998). Despite flow cytometry being routinely applied to the clinical analysis of whole blood to monitor platelet function in diseased states and being a well-established tool within platelet research (Michelson et al., 2000), very few comprehensive methodologies exist for quantitative analysis of platelet function that is relevant to their in vivo activity.

As platelets can be easily activated during sample processing (Wallén et al., 1997) and storage (Rinder et al., 1991; Vučetić et al., 2018) in the absence of inhibitors, a standardised single-step analysis panel was developed and optimised using freshly acquired platelets from healthy donors. This would enable the capture of key platelet surface markers, as well as those altered during activation, thereby reducing experimental variability and the need for large sample volume. This five-colour flow cytometry panel was optimised to allow the differential expression of platelet surface receptors to be quantified in both unstimulated and stimulated platelet populations, following treatment with adenosine diphosphate (ADP) or bacterial stimulation.
The panel was specifically designed to include commercially available antibodies in a single-step staining process to minimise the risk of spontaneous platelet activation during sample handling and preparation.
5.2 Aims

The aim of this chapter was to enable the quantification of a range of platelet surface markers following the addition of an agonist or bacterial stimulus. To minimise spontaneous activation of platelets and maximise the use of individual samples, a single-step protocol was developed to allow simultaneous measurement of key platelet markers. This chapter focusses on;

- The development of a multicolour flow cytometry panel to study marker expression in stimulated and unstimulated platelet populations following treatment with ADP
- The adaptation of an optimised multicolour flow cytometry panel to study the effect of periodontal pathogen interactions on platelet marker expression

The methodology developed within this chapter has been written up as an original paper and submitted to the journal Cytometry Part A.
5.3 Method development and gating strategy

This workflow has been optimised and validated for the analysis of freshly acquired platelets isolated from whole blood and from a range of healthy donors to ensure reproducibility.

5.3.1 Panel selection

To identify the platelet population, CD41 (αIIb, GPIIb) and CD61 (β3, GPIIIa) were selected as markers. CD41 is expressed exclusively on select early haematopoietic progenitors, megakaryocytes and platelets (Wiles and Keller, 1991; Mitjavila-Garcia et al., 2002), whilst CD61 is expressed on platelets, osteoclasts, fibroblasts, macrophages, and some tumour cells (Hynes, 1987; Savill et al., 1990; Engleman et al., 1997; Sloan et al., 2006). To determine levels of platelet activation following treatment, three markers and corresponding antibodies were selected. The antibody, against an activated epitope of the heterodimer CD41/CD61 (αIIbβ3, GPIIbIIIa) was included in the panel (Gardiner et al., 2007) as well as CD62P (P-selectin, GMP-140), a Type I transmembrane glycoprotein expressed on activated platelets (Ault et al., 1989). Finally, CD42b (GPIbα) was included within the panel because its shedding can be related to both platelet activation in treated samples and platelet viability in untreated samples (Bergmeier et al., 2003, 2004; Gardiner et al., 2007). The panel also includes IgG isotype controls although the specific focus of this work is on the comparison of unstimulated and stimulated platelets.
5.3.2 Flow cytometer instrument configuration

The panel was optimised for use on the Gallios flow cytometer (Beckman Coulter; Table 5.1). The Gallios flow cytometer was selected due to its three laser, 10 colour configuration and its routine application in both research and diagnostics. The Gallios flow cytometer has previously been applied to the phenotypic study of various haematopoietic cells including dendritic cells (Ferreira et al., 2013), monocytes (Morandi, Airoldi and Pistoia, 2014), B cells (Griffin and Rothstein, 2012), and platelets (Lacroix et al., 2010; Maugeri et al., 2012; Tynngård et al., 2015).
<table>
<thead>
<tr>
<th>Laser (nm)</th>
<th>Laser Power (mW)</th>
<th>Laser Type</th>
<th>Detector</th>
<th>Spectral Range for Detector (nm)</th>
<th>Dichroic Filter (nm)</th>
<th>Band Pass (nm)</th>
<th>Fluorochrome</th>
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<tr>
<td>488</td>
<td>22</td>
<td>Solid State Diode</td>
<td>FL-1</td>
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<td>550</td>
<td>525/40</td>
<td>Alexa Fluor 488</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>FL-2</td>
<td>560-590</td>
<td>595</td>
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<td>40</td>
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<td>FL-9</td>
<td>425-475</td>
<td>480</td>
<td>450/50</td>
<td>Pacific Blue</td>
</tr>
</tbody>
</table>

Table 5.1 Flow cytometer instrument configuration. This panel was optimised for a Gallios flow cytometer (Beckman Coulter), with a 10 colour, three laser configuration (Blue/Red/Violet). APC-allophycocyanin, Cy-cyanin, PE- R-phycoerythrin.
To assess suitability of conjugates in a multi-colour panel (Table 5.2) compensation matrix was generated using FlowJo to determine spill-over of signals (Table 5.3). FlowJo FACS data analysis software is a leading analysis platform that computes and applies compensation corrections and enables visualization of all flow cytometry data (Herzenberg et al., 2006).

Fluorescent intensities from single stain treatments were compared to those obtained through full panel analysis to account for potential steric hindrance and overlapping epitopes of antibodies. This single-stain approach allows both sample processing and analysis within four hours of blood sampling, which is critical for limiting spontaneous platelet activation and retaining in vivo platelet characteristics.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Antigen Reference</th>
<th>Stock Conc. (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD61</td>
<td>PE/Cy7</td>
<td>VI-PL2</td>
<td>Zola et al., (2007)</td>
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</tr>
<tr>
<td>CD42b</td>
<td>PE</td>
<td>HIP1</td>
<td>Clemetson et al., (1982); Fox, Aggerbeck and Berndt, (1988); Kuijpers et al., (1992)</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 5.2 Commercial reagents used. Samples were fixed with 0.5% paraformaldehyde and then incubated with listed antibodies for 20 minutes at room temperature in the dark prior to analysis.
<table>
<thead>
<tr>
<th></th>
<th>Alexa Fluor 488</th>
<th>PE</th>
<th>PE/Cy7</th>
<th>APC</th>
<th>Pacific Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 488</td>
<td>N/A</td>
<td>9.180%</td>
<td>2.430%</td>
<td>0.000%</td>
<td>5.880%</td>
</tr>
<tr>
<td>PE</td>
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<td>N/A</td>
<td>1.360%</td>
<td>0.000%</td>
<td>0.021%</td>
</tr>
<tr>
<td>PE/Cy7</td>
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<td>0.463%</td>
<td>N/A</td>
<td>0.000%</td>
<td>0.027%</td>
</tr>
<tr>
<td>APC</td>
<td>0.000%</td>
<td>0.000%</td>
<td>0.000%</td>
<td>N/A</td>
<td>0.000%</td>
</tr>
<tr>
<td>Pacific Blue</td>
<td>5.400%</td>
<td>0.195%</td>
<td>0.047%</td>
<td>0.000%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 5.3 Representative compensation matrix. Data from one sample with compensation analysis completed in FlowJo (Ver 7.6) to assess spill-over.

The use of five fluorochromes allows additional application-specific markers to be introduced as needed. The selection of the antibody/fluorochrome combinations, based on expression levels of the antigen of interest and brightness of fluorophore; with the brighter fluorophores conjugated to lower density antigens, was guided by their commercial availability and possible application using a single-step staining approach. This also ensures that selected antibodies have a level of quality assurance and validation, whilst reducing the number of steps within the protocol.

Following initial isolation of platelet rich plasma (PRP) from whole blood, platelets were treated as described in Chapter 2. Following fixation, a platelet population was first defined on the basis of forward size scatter (FSC) and side scatter (SSC) followed by gating on two platelet surface proteins CD41 and CD61, which allow for platelet subsets to be identified, irrespective of stimulation (Saboor, Moinuddin and Ilyas, 2012).
To determine the activation status of platelets, three platelet activation markers/epitopes were selected; CD62P (Ault et al., 1989), CD42b (glycoprotein Ibα) (Bergmeier et al., 2003, 2004; Gardiner et al., 2007) and an antibody against an epitope expressed on activated but not resting platelets CD41/CD61 (Gardiner et al., 2007).

The panel was designed with particular attention to antibody-fluorophore combinations to ensure that optimal staining was obtained whilst minimising spill-over of background fluorescence. The selection of antibodies was validated using non-permeabilised platelets as benefits of this approach include a simpler and shorter procedure with minimal sample handling and processing. The finalised constructed panel therefore consists of Pacific Blue-CD41, PE/Cy7-CD61, APC-CD41/61, Alexa Fluor 488-CD62P, PE-CD42b.
5.4 Results

5.4.1 Antibody optimisation

In order to optimise antibody concentrations, antibodies were serially titrated on platelets isolated from healthy volunteers ($n=6$) before the scatter profiles were analysed to detect antibody saturation (Figure 5.1). Optimum antibody concentrations were determined at the plateau of signal intensity and are depicted with red boxes.
Figure 5.1 Reagent titrations. Antibodies were serially titrated (1:4) on platelets isolated from healthy volunteers. Individual files were concatenated to allow visualisation of all titrations in a single figure. Unstained controls samples are labelled as zero. Selected antibody titers used are highlighted with red boxes. IgG controls were used at the same concentration as the corresponding antibody. Antibody concentrations are arranged along the X-axis.
5.4.2 Gating strategy

Following antibody optimisation, platelets were treated with ADP (20 μM) before analysis was undertaken with the designed gating strategy and comparative histograms. A representative plot is shown in Figure 5.2. Platelet populations were successfully isolated on CD41, CD61 positivity, with subsequent comparative histograms demonstrating an increased expression of both CD41/61 and CD62P, as well as a decreased expression of CD42b following stimulation with ADP (20 μM).
Figure 5.2 Top Panel: Gating strategy. Following platelet isolation and full blood count to confirm platelet purity and sample treatment, initial platelet population was first identified on basis of forward size scatter (FSC) and side scatter (SSC) (A). Corresponding isotype controls (B) and platelet markers CD41 and CD61 (C) were used to isolate CD41⁺CD61⁺ platelet sub-population (>95% as shown).

Bottom Panel: Comparative histograms. Unstimulated (blue lines) and adenosine diphosphate treated platelets (ADP, 20 μM; stimulated, red lines) illustrate changes in activation markers in relation to levels of CD41⁺/61⁺ (D), CD62P⁺ (E) and CD42b⁺ (F) populations. Sample processing and analysis were completed within four hours of blood collection.
5.5 Adaptation of stimulated population

The optimised multicolour flow cytometry panel was then adapted to allow the simultaneous analysis of bacterial association and stimulation of platelets. Platelets were stimulated with periodontal pathogens (*P. gingivalis* or *T. forsythia*, MOI 1:10) before populations of interest were identified using forward scatter (FSC) and side scatter (SSC) profiles (Figure 5.3a). Platelet purity was selected on CD41 positivity (Figure 5.3b-c).

![Figure 5.3](image.png)

**Figure 5.3** Representative revised gating strategy of platelet isolation. Platelet populations identified through forward-side scatter profiling (A) were selectively gated on CD41 positivity using IgG isotype controls (B) and antibody treated platelets (C).
Association of isolated platelets to bacterial cells was then determined by the relative fluorescent intensity of the bacterial specific antibodies, PGWC1 and TFWC. PGWC1 staining produced a 'smeared' scatter profile, which resulted in a lack of distinct populations (Figure 5.4). To overcome this issue, several methodologies were considered before quadrant gates were allocated on the third contour of both the negative (Figure 5.4a) and positive (Figure 5.4b) controls. This gating strategy allows the separation of bacterial associated platelets (Figure 5.4c, Q2), from platelets that remain unassociated (Figure 5.4c, Q3), with simultaneous analysis of platelet activation markers within both subpopulations (Figure 5.5). No distinct population separation could be achieved with platelets treated with Tannerella forsythia and subsequent staining with TFWC antibody.
Figure 5.4 Isolation of pathogen associated platelets. Example plots of CD41+ platelets (left-hand panel), showing the applied gating strategy (right-hand panel) to untreated PRP (A), PRP treated with 20 μM ADP (B) and P. gingivalis NCTC 11834 treated PRP (C). Due to the 'smeared' profile of bacterial antibody staining (PGWC1), upper gated quadrants were placed above the third contour of untreated PRP and PRP treated with ADP (20 μM). Using these defined gates, platelets treated with P. gingivalis (C) could be separated to define un-associated platelets (C,Q3) and P. gingivalis associated platelets (C,Q2).
Figure 5.5 Comparative histograms of platelet activation following exposure to *P. gingivalis*. Comparative histograms of un-associated platelets (blue lines) and *P. gingivalis* associated platelets (red lines) illustrate platelet activation relative to the expression of CD62P (A), CD41/61 (B) and CD42b (C).
5.6 Discussion

This chapter of work focuses on the development and adaptation of a single-step multicolour flow cytometry panel for the evaluation of platelet activation following exposure to periodontal pathogens. The application of flow cytometry combines the advantages of both microscopy and biochemical analysis techniques allowing for rapid, simultaneous phenotypic analysis of a large volume of cellular events (Muirheadi, Horan and Poste, 1985).

The initial panel development and optimisation demonstrated that platelet sub-population could be identified following PRP isolation and selective gating on both CD41 and CD61 positivity. It was also shown that ADP (20 μM) effectively stimulates platelet activation, which could be quantified through an increased expression of both CD62P and CD41/61 as well as a decreased expression of CD42b. As previously stated, one of the major difficulties surrounding platelet studies is spontaneous activation and a reduction in viability during sample processing (Wallén et al., 1997) and storage (Rinder et al., 1991). Within this study, utilising multiparameter flow cytometric analysis has allowed the detection of surface antigens in a sensitive and specific manner whilst in agreement with previously published guidelines limiting sample processing to a maximum of four hours (Schmitz et al., 1998).

However, adaptation of the newly optimised panel to incorporate the application of bacterial specific antibodies produced a 'smeared' staining scatter profile, resulting in a lack of definition between expected populations. Although spontaneous platelet activation was kept to a minimum, some level of activation was expected in all experimental conditions and appears to result in positive staining throughout all samples incubated with bacterial specific antibodies.
In order to separate bacterial associated platelets treated with these antibodies, several gating methodologies were investigated in detail following previously published guidelines on flow cytometry data presentation (Alvarez et al., 2010).

Initially it was hypothesised that the bacterial associated populations could be separated by comparative histograms but this was challenging due to the heterogeneous populations. Analysis by comparative histograms was also discarded due to reliance on the user to define which two samples were significantly different (Baggerly, 2001) with the resulting subjective selection of gating introducing over manipulation of data and inaccurate analysis (Overton, 1988). Similarly, although dot plots are widely used to view acquired data, this type of analysis also draws considerable limitations. The inherent characteristics of these plots can often mask the density and distribution of the data through overlapping dot placements and a lack of definition between highly populated and sparsely populated regions (Herzenberg et al., 2006).

In agreement with Herzenburg and colleagues (2006), it was subsequently concluded that coloured density plots, (zebra plots, quantile contour plots) offer the most accurate representation of the acquired data, with the increased dynamic range and frequency definition allowing for accurate gating placement. Gates placed above the third contour of control samples resulted in P. gingivalis associated populations being successfully isolated and provides a reproducible analysis methodology for the quantitation of platelet activation markers. P. gingivalis association was determined to induce increased CD62P, CD41/61 and CD42b staining when compared to the unassociated populations. The developed methodology however was not effective in separating populations of platelets associated with T. forsythia. It was surmised that
this was due to a lack of association of the bacterium to platelets that resulted in a lack of population definition and was not attributed to limitations within the methodology.

Polychromatic flow cytometry, as with any analytical technique does carry a number of caveats that must be considered. The design and optimisation of multiparameter flow cytometry panels is an extremely labour intensive and costly process. It cannot be overlooked that without carefully constructed panels, extensive antibody titrations, antibody ranking and expression characterisation, as well as dismissal of incorrect antibody combinations and the correct compensation measures, this methodology would lack both accuracy and reproducibility (Mahnke and Roederer, 2007). As well as limitations surrounding instrument and panel set-up, another underlying issue surrounding multicolour flow cytometry is the magnitude of data produced during sample acquisition and the subsequent data analysis that follows.

One of the fundamental advantages to polychromatic flow cytometry is the ability to define and analyse subsets of cells within a population, however by introducing several antibody combinations as well as the possibility of exploring hundreds of different phenotypic characteristic, a pragmatic and sensible approach must be undertaken towards data analysis (Chattopadhyay and Roederer, 2012), which complies with the availability of suitable software (Roederer and Moody, 2008). Within this study, although five phenotypic parameters were applied within the panel, analysis was confined to the comparison of data within two-dimensional plots, meaning that only two phenotypes of interest can be compared at any one time.

Despite these limitations, the polychromatic, multiparameter flow cytometry panel designed within this study provides a robust analysis methodology that provides reliable data acquisition and analysis within this system.
5.7 Summary

This study provides a practical tool to evaluate the status of freshly isolated platelets following treatment with a stimulus in a single-step staining process. The simple and relatively quick protocol described can be used in settings where spontaneous platelet activation needs to be minimised, minimal sample volumes are available and where platelets cannot be stored on-site for an extended period.
Chapter 6: The effect of periodontal pathogens on platelet function and protein expression
6.1 Introduction

It has been postulated that oral pathogens can either penetrate the surrounding tissues and vascular network of the periodontal pocket or release a barrage of biologically active molecules including gingipains and hemagglutinins (Reyes et al., 2013) that can then contribute to or exacerbate several systemic conditions including rheumatoid arthritis (Wegner et al., 2010), diabetes (Borgnakke et al., 2013) and cardiovascular disease (Kerr, 1951, 1962; Kebschull, Demmer and Papapanou, 2010).

Within the vascular system, the role of platelets within homeostasis is well established (Zucker and Nachmias, 1985). Recent studies have highlighted the antimicrobial role of platelets within both the adaptive and innate immunity and how these mechanisms may contribute to atherosclerosis (Ali, Wuescher and Worth, 2015; Hamzeh-Cognasse et al., 2015; Koupenova and Freedman, 2015). Bacterial species are known to interact with platelets, including various streptococcal, staphylococcal and helicobacter strains (Kerrigan and Cox, 2010) with platelets being capable of immobilising and internalising pathogens (Yeaman, 1997, 2010a, 2010b) and exhibiting antimicrobial activity (Zielinski et al., 2001; Lopes-Pires et al., 2012). Several mechanisms have been reported to facilitate bacterial-platelet association including direct interactions via Toll-like receptors (Assinger et al., 2012), glycoprotein (GP) IIbIIIa, GPIb, the fcγRIIa receptor (Cox, Kerrigan and Watson, 2011), or indirect interactions such as lipopolysaccharides (LPS) (Shiraki et al., 2004; Scott and Owens, 2008) or various plasma protein bridges such as fibrinogen or von Willebrand factor (vWF) (Cox, Kerrigan and Watson, 2011).

Periodontal pathogens in the form of biofilms have been reported to induce platelet aggregation (Tu et al., 2016). More specifically, *P. gingivalis* been shown to induce both
platelet aggregation and activation (Curtis et al., 1993b; Afrodite Lourbakos et al., 2001; Li et al., 2008; Assinger et al., 2012; Klarström Engström et al., 2015) which may suggest a possible role of periodontitis in CVD. Although the interaction of P. gingivalis and platelets has previously been reported within the literature the underlying mechanisms as well as the full extent and consequences of these interactions are yet to be elucidated.

6.2 Aims

Previous findings (Chapter 4) demonstrated that the periodontal pathogen P. gingivalis interacts with platelets in a strain-dependant manner and induces platelet aggregation. This chapter focuses on elucidating the mechanisms underlying these platelet/pathogen interactions by addressing the following questions:

- Do periodontal pathogens directly interact with platelets, resulting in the formation of aggregates?
- Does P. gingivalis or T. forsythia interaction result in platelet activation, changes in integrin expression and degranulation of mammalian platelets?
- What are the possible sites of interaction and association between platelets and periodontal pathogens?
6.3 Results

6.3.1 Analysis of platelet-\textit{P. gingivalis} interactions by immunofluorescence microscopy.

Immunofluorescence (IF) microscopy was initially used to determine whether platelets are able to directly interact with the periodontal pathogens, \textit{P. gingivalis} and \textit{T. forsythia}. Platelets were exposed to \textit{P. gingivalis} (MOI 1:10) and stained for F-actin to determine platelet morphology and with the pathogen specific antibody PGWC1 to identify \textit{P. gingivalis} NCTC 11834 (Figure 6.1). The immunostaining demonstrates that \textit{P. gingivalis} associates with, and appears to cause 'clumping' of the platelets when compared to control platelets not exposed to the pathogen. Platelets were also probed with integrin \(\beta1\) (Figure 6.2) and \(\beta3\) (Figure 6.3) specific antibodies, to investigate whether treatment with \textit{P. gingivalis} NCTC 11834 could affect the overall expression of these integrins or whether any co-localisation of bacteria and integrins could be observed. DAPI staining was utilised for identification of \textit{P. gingivalis}. Both integrin \(\beta1\) and \(\beta3\) are expressed across all samples but do not seem to be affected by \textit{P. gingivalis} treatment. Integrin staining was diffused throughout the cytoplasmic space, detailing that with this methodology no specific localisation or changes in expression could be determined.
Figure 6.1 *P. gingivalis* localises and associates with platelets. Isolated platelets were challenged with *P. gingivalis* NCTC 11834 (MOI: 10) for 20 minutes at RT before fixing. Platelets were permeabilised before incubation with pathogen specific antibody, PGWC1 (green) and an F-actin probe (red). Co-localisation and small aggregates of platelets with *P. gingivalis* were observed. Untreated and ADP treated platelets were included as negative and positive control respectively. Data is representative of at least three independent observations.
Figure 6.2 The effect of *P. gingivalis* on platelet integrin β1 expression. Isolated PRP was exposed to *P. gingivalis* NCTC 11834 for 20 minutes at room temperature before fixation. Cells were permeabilised, incubated with an integrin β1 specific antibody before analysis by immunofluorescence microscopy. DAPI staining was also included as a bacterial stain. Uniform diffused staining was observed throughout all treatments, with co-localisation between *P. gingivalis* and platelets (indicated by white arrows). Untreated and ADP treated platelets are included as negative and positive controls respectively. Data is representative of at least three independent observations.
Figure 6.3 The effect of *P. gingivalis* on platelet integrin β3 expression. Isolated PRP was incubated with *P. gingivalis* NCTC 11834 for 20 minutes at RT before fixation. Cells were then permeabilised, incubated with an integrin β3 specific antibody before analysis by immunofluorescence microscopy. DAPI staining was also included as a bacterial stain. Platelets stained positive for integrin β3, with diffused staining throughout all treatments. Co-localisation between *P. gingivalis* and platelets was also observed with platelets treated with NCTC 11834. Untreated and ADP treated platelets are included as negative and positive controls respectively. Data is representative of at least three independent observations.
Platelets were stained with an F-actin probe in combination with an anti-mToR antibody to determine the cytoskeletal rearrangements undertaken during platelet aggregate formation (Figure 6.4). DAPI staining was included to identify bacterial cells. While both the untreated platelet control and the platelet stimulated control (ADP) display a similar phenotype to the other staining figures (Figure 6.1) in relation to F-actin staining, *P. gingivalis* NCTC 11834 treated platelets appear to have formed a large, fibrous matrix that is positively stained for mToR (Figure 6.4). Additionally, distributed throughout this fibrous matrix are platelets that stained positive for F-actin and bacteria suggesting that *P. gingivalis* NCTC 11834 might become trapped within these large fibrous aggregates.
Platelet aggregate phenotyping by immunofluorescence microscopy. PRP was incubated with PG 11834 (MOI: 10) for 20 minutes at RT before being fixed onto glass slides. The effect of *P. gingivalis* was determined by staining two cytoskeletal markers F-actin (red) and mToR (green), with DAPI also being included for bacterial visualisation. Platelets treated with PG 11834 form large fibrous aggregates that are highly positive for mToR staining that show F-actin positivity and bacterial cells distributed throughout. Untreated platelets and platelets treated with ADP (20 μM) were included as negative and positive controls. Data representative of at least three independent observations.
6.3.2 Investigation of platelet aggregates by electron microscopy

The underlying characteristics of the platelet aggregates formed following *P. gingivalis* challenge were investigated by electron microscopy using PRP treated with *P. gingivalis* NCTC 11834 or ATCC W50. Untreated and ADP (20 μM) treated PRP were included as negative and positive controls respectively. Scanning electron microscopy (SEM) revealed that untreated platelets remained in an inactivated spherical shape, whereas when stimulated with ADP as positive control, a combination of small platelet aggregates together with individual platelets were observed (Figure 6.5). In contrast, platelets exposed to either wildtype strains of *P. gingivalis* (NCTC 11834 or ATCC W50), showed drastic phenotypic changes in comparison to both the positive and negative controls (Figure 6.5), with the formation of large, fibrous aggregates in which individual platelets can no longer be defined. Bacteria were also identified within the samples treated with *P. gingivalis* ATCC W50 and found embedded within the centre of a platelet aggregate and are isolated to a small area of the overall aggregate. It is suspected that similar findings could be identified within *P. gingivalis* NCTC 11834 treated samples but due to the constraints of SEM and the expanse of the formed aggregates, bacteria could not be identified.
Figure 6.5 Phenotypic analysis of platelet aggregates by scanning electron microscopy (SEM). Platelets were treated with *P. gingivalis* (NCTC 11834 or ATCC W50) for 20 minutes at room temperature before fixation and mounting and analysis by SEM. Platelets treated with *P. gingivalis* form large fibrous aggregates that encase the bacteria. When compared to the negative (untreated PRP) and positive controls (ADP, 20 μM), individual platelets are no longer visible, with the aggregates forming large masses. White arrows indicate small aggregates formed with ADP treatment.
To further probe these findings, bacterially treated platelets were also analysed by transmission electron microscopy (TEM), to identify possible ultrastructural changes and determine whether platelets can internalise *P. gingivalis*. As with SEM, within untreated PRP samples, individual platelets are easily identifiable and are phenotypically comparable to that of a resting platelet state (Figure 6.6). Samples treated with ADP (20 μM) were also easily identifiable and shown to have formed small aggregates with an increase in pseudopodial structures (white arrows; Figure 6.6). Platelets treated with *P. gingivalis* NCTC 11834 and ATCC W50, showed large amounts of cellular debris amongst distinctive bacterial cells (black arrows), with no definitive platelets visible (Figure 6.6). Within the cellular debris are small structures, that could be the remnants of activated platelets but without distinctive phenotype and visible organelles this cannot be confirmed.
Isolated PRP was incubated with *P. gingivalis* (NCTC 11834 or ATCC W50; MOI 1:10) for 20 minutes at room temperature before fixation and processing for imaging by TEM. Within the samples treated with *P. gingivalis*, bacteria surrounded by cellular debris (indicated with black arrows) are clearly visible. Platelets treated with ADP as a positive control show small aggregate formations indicated with white arrows. Untreated PRP was included as a negative control. Each image is shown at two magnifications (left panel 2 µm and right panel 0.5 µm) for increased definition.
6.3.3 Analysis of platelet activation and bacterial association by flow cytometry

Following the visualisation of platelet aggregates, flow cytometry was used to determine whether bacteria associated with platelets and induce platelet activation using the optimised multiparameter, polychromatic flow cytometry panel developed in Chapter 5.

Initial analysis was undertaken to determine wildtype bacterial association to platelets, in relation to bacterial specific antibody staining (PGWC1/TFWC; Figure 6.7). Significant association of both wildtype \textit{P. gingivalis} NCTC 11834 and ATCC W50 was detected within populations of CD41$^+$ platelet ($p<0.001$), whereas no association of \textit{T. forsythia} ATCC 43037 could be determined (Figure 6.7). A slight increase in staining was also observed within samples treated with ADP (20 $\mu$M) but this was not statistically significant.
Figure 6.7 Detection of wild-type periodontal pathogen-platelet association by flow cytometry. Isolated platelets were exposed to periodontal pathogens (MOI 1:10) before fixation, staining and analysis by flow cytometry. Isolated CD41+ platelet populations significantly associated with both *P. gingivalis* NCTC 11834 and ATCC W50. No bacterial association was observed with platelets treated with *T. forsythia* ATCC 43037. Untreated PRP and PRP treated with ADP (20 μM) were included as a negative and positive control respectively. Data is presented as ±SEM, $p<0.001$. n=3.

*P. gingivalis*-associated platelets were further analysed to determine the relative expression of platelet activation markers. Platelets associated with *P. gingivalis* NCTC 11834 showed significantly higher expression levels of CD41/61 ($p<0.05$), CD62P ($p<0.05$) and CD42b ($p<0.01$) when compared with unassociated platelets within the same population (Figure 6.8). Similarly, platelets associated with *P. gingivalis* ATCC W50 were also shown to exhibit significantly higher expression of both CD41/61 ($p<0.05$) and CD42b ($p<0.05$), whilst also inducing an increase in CD62P expression although the latter was not statistically significant (Figure 6.8).
Figure 6.8 Expression of platelet activation markers following association with *P. gingivalis*. Platelet rich plasma was exposed to *P. gingivalis* NCTC 11834 and ATCC W50 (MOI 1:10) before fixation and staining for activation specific antigens. Samples were analysed via flow cytometry. Platelets associated with bacteria showed an increased expression of CD41/61 (A), CD62P (B) and CD42b (C) when compared to associated platelets within the sample population. Data is expressed at ±SEM, n=3. $p = * \leq 0.05$, ** $\leq 0.01$. 
After identifying a possible role for the *P. gingivalis* outer-membrane proteins OMPA1 and OMPA2 within platelet aggregation (Chapter 4), further investigations probed the role of these outer membrane proteins within *P. gingivalis*-platelet association and the induction of platelet activation.

OMPA1 and OMPA2 deficient mutants were shown to significantly associate with platelets (*p*<0.001) with no observable differences between the mutants and the parent strain NCTC 11834 (Figure 6.9). When compared to the unassociated platelet populations, bacterial association was also shown to induce significant increase in expression of CD41/61 (*p*<0.05), CD62P (*p*<0.05) and CD42b (*p*<0.01) within samples treated with the OMP A1 deficient mutant (Figure 6.10). Bacterial association with the OMP A2 deficient mutants also induced an increased expression of CD41/61 as well as significantly increasing the expression of CD62P (*p*<0.05) and CD42b (*p*<0.01) (Figure 6.10).
Figure 6.9 The role of *P. gingivalis* outer membrane protein A (OMPA) in platelet association. Isolated PRP was exposed to *P. gingivalis* (NCTC 11834, NCTC 11834 ΔompA1 or NCTC 11834 ΔompA2; MOI 1:10) and fixed. Bacterial association was determined through staining with a *P. gingivalis* specific antibody (PGWC1) and flow cytometry analysis. Significant levels of bacterial association were observed with wild type *P. gingivalis* and both OMPA subunit deficient mutants, OMPA1 and OMPA2. Untreated PRP and PRP treated with ADP (20 μM) was included as a negative and positive control. Data is expressed as ±SEM, *p* = ***<0.001. n=3
Figure 6.10 *P. gingivalis* outer membrane protein (OMP) deficient mutants induce platelet activation. Isolated PRP was resuspended in modified Tyrode's buffer before being exposed to *P. gingivalis* (NCTC 11834, NCTC 11834 ΔompA1 or NCTC 11834 ΔompA2; MOI 1:10) and fixed. Platelets were stained with antibodies specific for platelet activation associated antigens and analysed on a Gallios flow cytometer. Platelets associated with *P. gingivalis* 11834 and the OMP A1 mutant induced significant expression of CD41/61 (A), CD62P (B) and CD42b (C) when compared with unassociated populations. Similarly, platelet populations associated with the OMP A2 deficient mutant showed an increased expression of CD41/61 (A), as well as significant increases in the expression of both CD62P (B) and CD42b (C). PRP treated with ADP (20 μM) and untreated PRP were included as a positive and negative control respectively. Data is expressed as ±SEM, *p* = *<0.05 **<0.01. n=3.
6.3.4 Induction of calcium mobilisation by periodontal pathogens

Having demonstrated that periodontal pathogens can associate with, and induce platelet activation, the effect on intracellular calcium mobilisation was investigated. Isolated platelets were pre-loaded with the intracellular calcium indicator FURA-2/AM and real-time calcium mobilisation was monitored following exposure to pathogens at an MOI of 1:10 (P. gingivalis NCTC 11834, ATCC W50; T. forsythia ATCC 43037). Untreated PRP and platelets treated with ADP (20 μM) were included as a negative and positive control respectively. A representative plot of intracellular calcium flux is shown in Figure 6.11a. Both P. gingivalis NCTC 11834 and ATCC W50 induced significant platelet calcium mobilisation (p<0.001), whereas no response was recorded with T. forsythia ATCC 43037 (Figure 6.11b). P. gingivalis ATCC W50 induced a significantly higher level of calcium mobilisation when compared to P. gingivalis NCTC 11834 (p<0.001).
Figure 6.11. Platelet calcium mobilisation is induced by wild type periodontal pathogens. Platelets pre-loaded with FURA-2/AM were exposed to either wild type *P. gingivalis* (PG NCTC 11834 or PG ATCC W50) or wild type *T. forsythia* (*T. forsythia* ATCC 43037). Real time intracellular calcium mobilisation was monitored after injection of bacteria as a ratio of fluorescent emission excited at both 340 and 380 nm. (A) Representative calcium mobilisation plot. (B) Both wild type *P. gingivalis* strains significantly induce calcium mobilisation, with no response being recorded with *T. forsythia*. *P. gingivalis* ATCC W50 also induced a significantly higher level of calcium flux when compared to NCTC 11834. Untreated platelets and platelets treated with ADP (20 μM) were included as a negative and positive control. Data is expressed as ±SEM, *p* < 0.001. n=3.
Similarly to wild type *P. gingivalis* strains, the effect of the two outer-membrane proteins OMPA1 and OMPA2 on platelet calcium mobilisation was determined. As with the parental strain *P. gingivalis* NCTC 11834, both OMPA1 and OMPA2 deficient mutants significantly induced platelet intracellular calcium flux (*p*<0.001) (Figure 6.12) with the OMPA2 deficient mutant inducing a significantly higher levels of calcium mobilisation when compared to both the wildtype *P. gingivalis* NCTC 11834 and the OMPA1 mutant (*p*<0.001), whereas no difference was observed between the wildtype and OMPA1.
Figure 6.12 Platelet calcium mobilisation is induced by OMPA deficient *P. gingivalis* mutants. Platelets pre-loaded with FURA-2/AM were exposed to wild type *P. gingivalis* NCTC 11834 or either OMPA deficient mutants (PG NCTC 11834 ΔompA1 or PG NCTC 11834 ΔompA2) and intracellular calcium mobilisation was monitored as a ratio of fluorescent emission excited at both 340 and 380 nm. (A) An example calcium mobilisation plot. (B) Wildtype *P. gingivalis* and both OMPA mutants significantly induce calcium mobilisation. The OMPA2 mutant also induced significantly higher levels of calcium flux when compared to both the wildtype and OMPA1 mutant. Untreated platelets and platelets treated with ADP (20 μM) were included as a negative and positive control. Data is expressed as ±SEM, p<0.001. n=3.
6.3.5 Investigations into platelet degranulation by *P. gingivalis*

Having demonstrated that *P. gingivalis* can trigger intracellular calcium mobilisation, the effects on alpha and dense granule release were investigated.

Isolated PRP was exposed to either *P. gingivalis* NCTC 11834 or ATCC W50 before granular secretion of platelet factor 4 (PF4), serotonin and interleukin 1β (IL-1β) was determined by enzyme-linked immunosorbent assays (ELISA). Untreated and ADP (20 μM) treated PRP were included as a negative and positive control respectively. ELISA assays revealed that both *P. gingivalis* NCTC 11834 and ATCC W50 induced significant secretion of PF4 (*p*<0.001) and serotonin (*p*<0.05), whereas no significant levels of IL-1β were detected (Figure 6.13a-c).
Figure 6.13 Induction of platelet degranulation by wild type *P. gingivalis*. PRP, resuspended in modified Tyrode’s buffer were exposed to *P. gingivalis* NCTC 11834 or *P. gingivalis* ATCC W50 after which the supernatants were removed and analysed by ELISAs specific for platelet factor 4 (PF4), serotonin and interleukin 1β (IL-1β). Both *P. gingivalis* NCTC 11834 and ATCC W50 significantly induced PF4 (A) and serotonin (B) release from platelets whilst no changes in IL-1β release were observed (C). Untreated platelets and platelets treated with ADP (20 μM) were included as a negative and positive control respectively. Data is presented as ±SEM. p= *<0.05, **<0.01, ***<0.001. n=3.
6.3.6 Does the secretome of *P. gingivalis* induce platelet aggregation?

To determine whether proteins secreted by *P. gingivalis* were sufficient to induce platelet aggregation, the bacterial culture supernatants of *P. gingivalis* NCTC 11834 and ATCC W50 was utilised as stimulants in aggregatory assays. An example aggregation plot is shown in Figure 6.14a. Both bacterial supernatants from *P. gingivalis* NCTC 11834 and ATCC W50 failed to induce an aggregatory response (Figure 6.14b). As expected, wildtype whole cell *P. gingivalis* NCTC 11834 is able to stimulate platelet aggregation whereas *P. gingivalis* NCTC 11834 does not. Untreated PRP, PRP treated ADP (20 μM) and PRP treated with bacterial growth media were also included as both positive and negative experimental controls.
Figure 6.14 Platelet aggregatory response following exposure to *P. gingivalis* culture supernatant. Wildtype *P. gingivalis* NCTC 11834 and ATCC W50 were grown as liquid cultures in BHI media until an optical density of 0.8 at 600 nm before centrifugation at 13000 g. The supernatants were removed and used as stimulants in platelet aggregation assays. Sterile BHI media and the relevant WT strains were also included as experimental controls. (A) An example aggregation plot. (B) No aggregatory response was observed with supernatants from either *P. gingivalis* NCTC 11834 or ATCC W50. Untreated PRP and PRP treated with ADP (20 μM) was included as a negative and positive control. Data is expressed as ±SEM, n=3.
It was also hypothesised that lipopolysaccharide (LPS) from *P. gingivalis* could interact with and induce platelet aggregation at higher concentrations. This was determined using commercially available LPS derived from *P. gingivalis* NCTC 11834 at varying concentrations. An example aggregation figure is shown in Figure 6.15a. However as with bacterial supernatants, no aggregatory response was observed with LPS at concentrations of 10, 20 or 40 µg/ml (Figure 6.15b).
Figure 6.15 *P. gingivalis* LPS as a platelet agonist. Isolated PRP was utilised in aggregation experiments where commercial LPS derived from *P. gingivalis* NCTC 11834 was used as a platelet stimulant. **(A)** An example aggregation plot. **(B)** No Platelet aggregation was recorded following stimulation with *P. gingivalis* NCTC 11834 LPS at all tested concentrations (10-40 μg). Untreated PRP and PRP treated with ADP (20 μg) was included a negative and positive control respectively. Data is expressed as ±SEM, n=3.
6.3.7 Investigation of platelet receptor inhibitors on *P. gingivalis* induced platelet aggregation

6.3.7.1 The effect of Toll-like receptor inhibitors on *P. gingivalis* induced platelet aggregation

Having demonstrated that *P. gingivalis* directly associates with platelets, possible mammalian site of interactions were investigated.

The role and contribution of Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4) in platelet aggregation were determined. Isolated platelets were pre-incubated with either a TLR2 or TLR4 inhibitor or a combination of both before being bacterially challenged with *P. gingivalis* NCTC 11834 and aggregation was measured (Figure 6.16a-c). A representative plot including the untreated and ADP treated platelets is shown in Figure 6.16a-b. No significant changes were observed in total percentage aggregation following pre-incubation with either TLR inhibitors or in combination when compared to PRP exposed to wildtype *P. gingivalis* NCTC 11834 (Figure 6.17a). In contrast however, all three of the TLR inhibitory treatments did cause a significantly quicker onset of aggregation (AG0) when compared to the wildtype control (*p*<0.05 or <0.001; Figure 6.17b)
Figure 6.16 The effect of Toll-like receptor inhibitors on *P. gingivalis* induced platelet aggregation. Isolated PRP was pre-incubated with either TLR2, TLR4 or both inhibitors for 15 minutes at room temperature before aggregation assays in the presence or absence of *P. gingivalis* NCTC 11834 were undertaken. (A) An example aggregation plot of the effects of TLR2 and TLR4 inhibitors on untreated PRP aggregation. (B) An example aggregation plot of the effects of TLR2 and TLR4 inhibitors on platelets stimulated with ADP (20 μM). (C) TLR2 and TLR4 inhibitors cause a faster onset of aggregation when compared to untreated PRP stimulated with *P. gingivalis* NCTC 11834. Unstimulated PRP and PRP stimulated with ADP (20 μM) was included as a negative and positive control. n=3.
Figure 6.17 The effects of Toll-like receptor inhibitors on *P. gingivalis*-platelet interactions. Isolated PRP was pre-incubated with TLR2 inhibitor, TLR4 inhibitor or both for 15 minutes at room temperature before aggregation experiments were undertaken. (A) No changes in total percentage aggregation were observed with TLR2, TLR4 or the combination of both TLR2+4 inhibitors. (B) Incubation with TLR2 and 4 inhibitors resulted in a significantly quicker onset of platelet aggregation (AG0) when compared with PRP stimulated with wild type *P. gingivalis* NCTC 11834 only. PRP treated with ADP (20 μM) and untreated PRP were included as a positive and negative control respectively. Data is expressed as ±SEM, *p* = *<*0.05, **<*0.001. n=4.
6.3.7.2 The effects of integrin α₂β₁ and β₃ inhibitors on *P. gingivalis*-induced platelet aggregation

The role of the integrins β₃ and α₂β₁ in *P. gingivalis*-induced platelet aggregation was determined using integrin-specific inhibitors. Following pre-incubation of isolated platelets with β₃ and α₂β₁ inhibitors, aggregation assays were conducted. To ascertain whether these integrin inhibitors affected normal platelet function, effect on untreated PRP and PRP treated with ADP (20 μM) were measured. Incubation with either inhibitor did not result in any changes in aggregatory response within unstimulated PRP or PRP stimulated with ADP (20 μM). Representative aggregation plots for both integrin β₃ inhibitor (Figure 6.18) and α₂β₁ (Figure 6.19) are shown below.
Figure 6.18 Representative aggregation plots following incubation with integrin \( \beta_3 \) inhibitor. Isolated PRP was incubated with the \( \beta_3 \) inhibitor for 15 minutes at room temperature before routine aggregation experiments were undertaken. The effects of the inhibitor were determined on untreated PRP as negative control (A) and PRP treated with ADP (20 \( \mu \)M) as a positive control (B). No changes in aggregatory response were recorded in any of the experimental conditions. Representative aggregation plots from three independent observations.
Figure 6.19 Representative aggregation plots following incubation with integrin α2β1 inhibitor. Isolated PRP was incubated with the α2β1 inhibitor for 15 minutes at room temperature before routine aggregation experiments were undertaken. The effects of the inhibitor were determined against untreated PRP as negative control (A) and PRP treated with ADP (20 μM) as a positive control (B). No changes in aggregatory response were recorded in any of the experimental conditions. Representative aggregation plots from three independent observations.
Following incubation with the β₃ integrin inhibitor, no changes were observed in either the total percentage aggregation or the onset of aggregation (AG0) when platelets were exposed to *P. gingivalis* NCTC 11834 at an MOI of 1:10 (Figure 6.20). In contrast, following incubation with the α₂β₁ inhibitor at a concentration of 20 μM resulted in a significant and complete inhibition of platelet aggregation following addition of *P. gingivalis* NCTC 11834 (*p*<0.001; Figure 6.21a-b). At a lower concentration of 10 μM, no significant changes in total percentage aggregation were observed. The α₂β₁ inhibitor did not induce any alterations in the aggregatory response in either the negative (untreated PRP) or the positive (ADP treated) control at both 10 and 20 μM. The α₂β₁ inhibitor also resulted in a significant increase in the onset of aggregation (AG0) at both 10 and 20 μM (*p*<0.001), with a significant increase in lag time also being observed between platelets exposed 20 μM compared with the lower concentration of 10 μM (*p*<0.001; Figure 6.21c)
Figure 6.20 Effects of integrin β₃ inhibitor on *P. gingivalis* NCTC 11834 induced platelet aggregation. Isolated PRP was incubated with the β₃ inhibitor for 15 minutes at room temperature before routine aggregation experiments were undertaken. Representative aggregation plot (A). No significant differences were observed with either total percentage aggregation (B) or the onset time of aggregation (C) following exposure to *P. gingivalis* NCTC 11834. Untreated PRP and PRP treated with ADP (20 μM) were included as a negative and positive control. Data is expressed as ±SEM, n=3.
Figure 6.21 Effects of integrin α2β1 inhibitor on *P. gingivalis* NCTC 11834 induced platelet aggregation.

Isolated PRP was pre-incubated with the α2β1 inhibitor for 15 minutes at room temperature before routine aggregation experiments were undertaken. **(A)** Representative aggregation plot. **(B)** Incubation with the α2β1 inhibitor at 20 μM completely inhibited platelet aggregation following stimulation by *P. gingivalis* NCTC 11834, with no significant differences observed with the lower concentration (10 μM). **(C)** The integrin α2β1 inhibitor significantly increases the lag-time of platelet aggregation following stimulation with *P. gingivalis* NCTC 11834 at both 10 and 20 μM. When incubated with 20 μM of inhibitor *P. gingivalis* NCTC 11834 stimulated aggregation is abolished. Untreated PRP, PRP treated with ADP (20 μM) and PRP treated with *P. gingivalis* NCTC 11834 only were included as comparative experimental controls. Data is expressed as ±SEM, $p=***<0.001$. n=3.
6.3.8 Development of a recombinant protein-based ELISA for the detection of *P. gingivalis*-platelet integrin interactions

Having demonstrated that integrin α2β1 inhibition prevented *P. gingivalis* from stimulating platelet aggregation, a recombinant protein-based ELISA was developed to investigate whether *P. gingivalis* NCTC 11834 or ATCC W50 could directly bind to either integrin α2β1 or αIIbβ3 using immobilised recombinant integrins.

Successful immobilisation of recombinant integrins was confirmed with β1 and β3 specific monoclonal antibodies which showed integrin specific binding (Figure 6.22a-b). *P. gingivalis* NCTC 11834 showed significant levels of binding to the recombinant integrin α2β1 (*p*<0.0001), whereas no significant binding was observed with either *P. gingivalis* NCTC 11834 treated or untreated controls (Figure 6.22c). In contrast no significant binding was observed with integrin αIIbβ3 (Figure 6.22d).
Figure 6.22 Detection of *P. gingivalis*-integrin binding by recombinant protein-based ELISA. 96-well plates were coated with either recombinant αIIbβ3 or α2β1 overnight at 4 °C. Plates were then either incubated with integrin specific antibodies (A-B) to determine successful protein coating or were challenged with *P. gingivalis* (NCTC 11834 or ATCC W50) and then incubated with PG specific antibodies to determine bacterial binding (C-D). Plates were successfully coated with α2β1 (A) and αIIbβ3 (B). *P. gingivalis* NCTC 11834 significantly binds to integrin α2β1, with no significant changes observed with either PG ATCC W50 treatment or untreated controls (C). No binding was observed in any of the experimental treatments with integrin αIIbβ3 (D). Data is presented as ±SEM, *p*** < 0.001. n=3.
6.4 Discussion

Periodontitis and its associated bacterial pathogens have been historically linked to several systemic diseases (Kerr, 1951, 1962). In this study, the interactions of periodontal pathogens and platelets were further characterised. A growing body of research has also documented the importance of platelets within the innate and adaptive immunity in the defence against bacterial infections (Kerrigan, 2015).

*P. gingivalis* induced platelet aggregates were analysed using both SEM and TEM. *P. gingivalis* (NCTC 1834 or ATCC W50) treated platelets resulted in aggregate formation as observed by SEM, whereby bacteria were found encapsulated within the aggregates and the platelets were phenotypically dissimilar to untreated or ADP stimulated controls. TEM analysis also revealed that incubation with *P. gingivalis* resulted in the complete loss of any defined platelet phenotypic structures. These observations are in agreement with those of Li and colleagues (2008) who demonstrated that NCTC 11834 induced the formation of small bacteria-associated aggregates (Li et al., 2008). However as the later study used a lower MOI of .3 and was conducted under stirring conditions, it could explain why smaller aggregates were observed. Further confirmation of the pathogen encapsulated within the aggregates was obtained in TEM imaging. These findings are in agreement with those observed with both *Escherichia coli* and *Staphylococcus aureus* that show the formation of bacterial-platelet clusters (Kraemer et al., 2011; Vieira-de-Abreu et al., 2012).

Studies involving patients suffering from chronic periodontitis have shown the presence of an associated condition of heightened circulatory platelet activation and suggested that this is a direct consequence of the pathogenic bacteria present within the disease (Papapanagiotou et al., 2009). Additionally, an increase in expression level of the platelet activation marker CD62P by *P. gingivalis* has been demonstrated in both
a murine model, (Yu et al., 2011) and in vitro with isolated mammalian platelets exposed to the pathogen (Klarström Engström et al., 2015). In this study, levels of a panel of platelet specific proteins were quantified. Using an optimised in-house flow cytometry, a significant increase in expression of both CD62P ($p<0.001$) and PAC-1 (CD41/61, GPIIbIIIa, αIIbβ3; $p<0.05$) was observed in platelet population which was positive for *P. gingivalis* (NCTC 11834 or W50; $p<0.001$). CD62P is a type1 transmembrane glycoprotein, normally contained within the α-granule but which becomes expressed on activated platelets (Ault et al., 1989). CD41/61, also known as the glycoprotein heterodimer complex GPIIbIIIa and as integrin αIIbβ3 is normally expressed on platelets as a heterodimer in a resting form (Bennett, 2005). However, during platelet activation, CD41/61 undergoes a change in conformation and expresses activation-induced epitopes to which the CD41/61 antibody also known as PCA-1 can bind to (Shattil et al., 1985). The increase in PAC-1 suggest that the integrin CD41/61 has move from its resting state to an activated state (Shattil et al., 1985) and confirms the activation status of platelets.

Surprisingly, an increase in CD42b expression ($p<0.01$) was observed following bacterial exposure. This is in contrast with previous studies which have documented a decrease in CD42b expression following platelet activation due to ectodomain shedding (Tao et al., 2016) but supports the increase in CD42b expression induced on platelets by the Dengue virus (Núñez-Avellaneda et al., 2018). CD42b also known as glycoprotein Ibα (GPIbα) exists within a non-covalent complex of GPIb-IX-V on the platelet surface membrane (Modderman et al., 1992) and plays a role on platelet adhesion to subendothelial matrix, endothelial cells and leukocytes (Berndt et al., 2001). It has also been suggested that in addition to its role in haemostasis, GPIbα
plays a fundamental role within platelet immune response and vascular inflammation (Pitchford, Pan and Welch, 2017) with the common structural motifs of leucine rich repeat regions within each subunit of the GPIb-IX-V complex mirroring those present in all 13 members of the TLR family and suggesting a common ancestry between platelet and leukocytes as well as an involvement with bacterial interaction (Corken et al., 2014). Bacterial species including Streptococcus gordonii (Bensing, López and Sullam, 2004) and Streptococcus sanguis (Plummer et al., 2005) have been shown to exploit the GP1bα ligand in order to bind to platelets and subsequently induce an aggregatory response. The consistent increase in GPIbα expression was observed following P. gingivalis exposure can also be partly attributed to the presentation of the receptor through alpha granule release however this would account for only ~10% of GPIbα expression (Berger, Massé and Cramer, 1996; Maynard et al., 2007).

The increase in expression of both CD62P and CD41/61 and the observed platelet aggregation suggests platelet granule secretion, cytoskeletal rearrangements and further signal transduction (van Velzen et al., 2012). To probe alterations occurring within platelets granular secretion and alteration in calcium mobilisation were investigated. A significant increase in intracellular calcium mobilisation ($p<0.001$), serotonin release ($p<0.05$) as well as PF4 secretion ($p<0.001$) was observed following P. gingivalis stimulation. Intracellular calcium mobilisation is an essential cofactor that drives platelet signalling at a molecular level, resulting in platelet activation, secretion and aggregation (Gerrard, White and Peterson, 1978; Heemskerk et al., 1992, 2001).

Our findings agree with earlier studies which demonstrated that P. gingivalis are able to induce calcium flux in vitro within both epithelial cells and washed platelet preparations (Lourbakos et al., 2001; Klarström Engström et al., 2015). The detection of both serotonin and platelet factor 4 release further confirms that P. gingivalis
stimulates the full signalling cascade of platelet aggregation, with granule release also being directly implicated within the role of platelets within the innate and adaptive immunity (Ali, Wuescher and Worth, 2015). Induction of platelet granular release have reported with several bacterial species with *Streptococcus sanguinis*, *Staphylococcus aureus*, *Streptococcus oralis* and *Streptococcus pneumoniae* stimulating both alpha and dense granule release as an adaptive immune response to bacterial stimulation (Erickson and Herzberg, 1993; Arman et al., 2014). Interestingly, despite previous reports stating that platelets release IL-1β following stimulation with ADP (Sedlmayr et al., 1995; Cha et al., 2000; Brown et al., 2013), both viable *P. gingivalis* and purified LPS failed to show secretion of IL-1β. The release of IL-1β by platelets is currently disputed as other studies (Pillitteri et al., 2007) have suggested that the detectable levels of IL-1β, is a direct consequence of leukocyte contaminants within the platelet isolation process (Pillitteri et al., 2007). Interestingly, *P. gingivalis* has also been shown to degrade several inflammatory chemokines and cytokines *in vitro*, for example; TNF, IL-6, CCL2, CCL5 (RANTES), CXCL1, CXCL8 (IL-8), CXCL10 and IL-2 excreted by fibroblasts (Palm, Khalaf and Bengtsson, 2013), IL-2 produced by jukrat T-cells (Khalaf and Bengtsson, 2012) and isolated mammalian IL-1β, IL-6 and IL-1 receptor antagonist (Fletcher et al., 1998). Thus, further work is required to determine whether IL-1β is being degraded by the well documented protease activity of *P. gingivalis* (Tokuda et al., 1998; Holt, 1999; Lourbakos et al., 2001).

Studies investigating the interactions of *P. gingivalis* with oral epithelial cells identified the subunits of two major outer membrane proteins OMPA1 and OMPA2 of *P. gingivalis* NCTC 11834 as being essential for biofilm formation and a contributory factor to bacterial virulence (Suwannakul et al., 2010; Aruni et al., 2011; Naylor et al., 2016) whilst the sialidase enzyme 0352 as being crucial within *P. gingivalis* virulence
through bacterial adherence and biofilm formation (Li et al., 2017; Yang et al., 2018). The role and importance of these proteins in platelet aggregation was determined and aggregation studies revealed that not only do both OMPA deficient mutants induce a faster aggregatory response but they also induce significantly higher levels of platelet activation and intracellular calcium mobilisation when compared to the parental strain. A similar response was observed with the ΔSia0352 mutants suggesting that disrupting bacterial/cellular interactions, results in a heightened platelet response. As previously discussed (Chapter 4) it is hypothesised that these disruptions within key outer-membrane proteins such as OMPA and the sialidase enzyme possibly reduce the evasive characteristics of P. gingivalis resulting in a faster and more heightened combative platelet activation.

Bacteria are known to bind to oral epithelial cells and platelets via glycoprotein receptors such as integrins. Interactions between bacterial species such as Escherichia coli (Vieira-de-Abreu et al., 2012) and Staphylococcus aureus (Kraemer et al., 2011) with platelets have been previously visualised by both fluorescence and electron microscopy. Using fluorescence microscopy, uniform expression of integrins β1 and β3, as well an F-actin probe was observed in the untreated samples showing a phenotypic discoid platelet shape. Additionally, an increase in clumping of platelets were seen when platelets were treated with P. gingivalis. Disruption in the expression of integrin β1 (Yilmaz, Watanabe and Lamont, 2002; Li et al., 2013), integrin β3 (Li et al., 2013; Boisvert, Lorand and Duncan, 2014) and in actin (Kinane et al., 2012) following P. gingivalis treatment have been documented within epithelial cells. However, reduction in expression of the integrins was not observed with platelets and this could reflect the fact that platelets aggregate within minutes and protein degradation is not possible in
such a small time frame, protein degradation in epithelial cells, usually occurring after
two to four hours following bacterial challenge.

The observed formation of platelet aggregates suggests that platelets must undergo
cytoskeletal re-arrangements (Bearer, Prakash and Li, 2002). This was investigated by
staining for the mammalian target of rapamycin (mTOR), a serine kinase that
integrates with several key process including cell proliferation, protein synthesis and
autophagy (Hay and Sonenberg, 2004) has been identified within previous research by
Stafford and colleagues (2013) as a target for P. gingivalis host cell manipulation.

Within platelets, mTOR has many reported functions including the regulation of mRNA
translation and protein synthesis (Weyrich et al., 1998). In this study, following
exposure to P. gingivalis NCTC 11834, platelets were observed to form large
aggregates consisting of interconnected, fibrous-like structures which stained
positively for mTOR, within which were embedded a combination of pathogen and
discoid platelet structures that are positively stained for F-actin. This is in contrast with
reports of P. gingivalis degrading mTOR following cellular invasion of oral epithelial
cells (Stafford et al., 2013) and is likely to reflect the time taken for P. gingivalis to
invade oral epithelial cells and degrade mTOR via its secreted gingipains (Stafford et
al., 2013) whilst the response of platelets to P. gingivalis happens at much faster rate
within minutes such that protein degradation might not be feasible. The presence of
mTOR within the aggregates might also contribute to aggregate formation as studies
by Aslan and colleagues (2012) have implicated mTOR in lamellipodia formation and
thrombus stability (Aslan and McCarty, 2012). Further to this mToR is also known to be
downstream of integrin/glycoprotein signalling (Watson et al., 2005; Z. Li et al., 2010),
which as previously mentioned is a target for P. gingivalis cellular interactions
Proteases secreted by *P. gingivalis* known as gingipains (Curtis et al., 1993b; A Lourbakos et al., 2001; Naito et al., 2006) and lipopolysaccharide (Zhang et al., 2009) have been shown to induce platelet aggregation within washed platelet preparations. In this study, the effects of both gingipains and LPS on isolated but not washed platelets were investigated and it was observed that neither *P. gingivalis* culture supernatants containing gingipains nor isolated LPS initiated a platelet aggregatory response under the conditions used. This could reflect the different experimental conditions used i.e. isolated platelets contained within plasma as opposed to washed platelets. Despite both PRP and washed platelets being readily utilised within platelet research there are stark differences attached to each platelet preparation. Washed platelets are more resilient against spontaneous activation and allow analysis to be undertaken completely removed from any anticoagulant used within sample acquisition (Cazenave et al., 2004). However one of the major caveats to washed platelet suspensions is the removal of key plasma proteins and the formation of highly selective platelet subpopulations through the centrifugation stages and the use of a physiological buffer (Cazenave et al., 2004). The presence of plasma proteins has also been shown to have a direct impact on platelet function within immunity, with research by Damien and colleagues (2015) demonstrating that soluble CD14 present in plasma facilitates platelet TLR4 stimulation by bacterial LPS.

Previous work has also demonstrated that bacterial species including group B *Streptococcus* (Ma et al., 2009) and *Streptococcus pneumoniae* (Keane et al., 2010) can induce platelet activation through interactions with toll-like receptor 2 (TLR2), whereas bacterial LPS derived from *Escherichia coli* stimulates platelet activation through interaction with toll-like receptor 4 (TLR4) (Andonegui et al., 2005; Ma et al., 2009). *P. gingivalis* has also been shown to activate endothelial and dendritic cells through TLR4,
while epithelial cells were activated through TLR2 (Darveau et al., 2004; Kocgozlu et al., 2009). It was therefore hypothesised as platelets express TLR receptors, inhibiting platelet TLR2 and TLR4 independently as well as in a combined treatment should impede platelet-\textit{P. gingivalis} interaction and consequently reduced or fully inhibit the observed aggregatory responses if \textit{P. gingivalis} interacted with platelets through TLRs. Surprisingly, addition of TLR antagonists resulted in no alteration in the total percentage aggregation induced by the pathogen but caused a significantly quicker onset of aggregation when compared to the untreated \textit{P. gingivalis} control (\(p = \text{NCTC 11834} < 0.05, \text{ATCC W50} < 0.001\)). Although strain variation has previously been reported to impact platelet aggregation lag time (Moriarty et al., 2016), the observed findings cannot be fully explained. One possible explanation could be that antagonising the TLR 2 and 4 receptors results in an upregulation or increased trafficking of further TLR receptors which has been previously reported in dendritic cells following LPS stimulation (Chamorro et al., 2009), however further work would be needed to further explore this premise.

Having demonstrated that \textit{P. gingivalis} associated with and activated platelets inducing aggregation, which was not abrogated by TLR inhibitors, the interactions of \textit{P. gingivalis} with platelet surface integrins were investigated. Studies using selective platelet inhibitors have led to the identification of potential binding sites for platelet-bacterial interaction: the selective inhibition of GPIIbIIIa (integrin \(\alpha_{IIb}\beta3\)) with Abciximab completely abolished \textit{Streptococcus gordonii} binding to platelets (Petersen et al., 2010) and both \textit{Streptococcus sanguis} (Kerrigan et al., 2002) and \textit{Helicobacter pylori} (Byrne et al., 2003) interact with platelets through GPIIbα. Using aggregation assays, the effect of selected integrin inhibitors on \textit{P. gingivalis} induced aggregation was therefore determined. The addition of the selective \(\beta3\) inhibitor did not affect the
total percentage aggregation or the initiation of aggregatory response, suggesting that
*P. gingivalis* induced aggregation is not dependent on integrin β3 interaction.

In contrast, the selective integrin α2β1 inhibitor, resulted in complete abolishment of
any aggregatory response at 20 μM ($p<0.001$) and a significant increase in lag time
when compared to the untreated *P. gingivalis* control at 10 μM ($p<0.001$). Previous
research has shown that *P. gingivalis* can physically associate with α5β1 integrin
expressed by endothelial cells via major fimbriae (FimA) proteins (Nakagawa et al.,
2002; Yilmaz et al., 2003; Kato et al., 2007; Al-Taweel, Douglas and Whawell, 2016) and
could propose a common pathway by which *P. gingivalis* invades nucleated host cells
(Olsen and Progulske-Fox, 2015) and interacts with platelets inducing platelet
aggregation. This hypothesis is further supported by the ELISA based assays which
revealed that findings that *P. gingivalis* NCTC 11834 but not ATCC W50 bound to
recombinant integrin β1 ($p<0.001$) whilst both *P. gingivalis* strains did not bind to
recombinant integrin β3. Within epithelial cells it is suggested that integrin β1 provides
a functional receptor for fimbriae mediated adherence and invasion for *P. gingivalis*
NCTC 11834 (Amano, 2003).

Interestingly, *P. gingivalis* strains are known to express different types of the major
fimbriae gene *fimA*, with NCTC 11834 expressing type I and ATCC W50 expressing type
IV (Amano et al., 1999). When compared phenotypically, this varying *fimA* expression
results in *P. gingivalis* ATCC W50 displaying much shorter, sparsely populated fimbriae
as opposed to the much longer and widely presented fimbriae of *P. gingivalis* NCTC
11834 (Sojar, Hamada and Genco, 1997). These inter-strain variances could therefore
offer a possible explanation to the differential integrin β1 binding observed and
account for the abrogation of the strain specific aggregation induced by *P. gingivalis*
NCTC 11834 by integrin β1 inhibitors. Taken together, the data suggest that in the case
of by *P. gingivalis* ATCC W50 both platelet activation and calcium mobilisation occurs independently of integrin β1 and additional adhesins or receptors are likely to contribute to *P. gingivalis* ATCC W50-platelet interactions.

Similar studies were conducted with *T. forsythia*, a well-documented periodontal-pathogen shown to be epidemiologically relevant to the progression of periodontitis (Darveau, 2010) and capable of invade epithelial cells (Sabet *et al.*, 2003; Inagaki *et al.*, 2006; Kirschbaum *et al.*, 2010a). Periodontitis patients who are positive for both *P. gingivalis* and *T. forsythia* also have a heightened level of platelet activity (Nicu *et al.*, 2009). However, *T. forsythia* does not associate with isolated platelets or initiate platelet activation via intracellular calcium mobilisation. Collectively, these findings suggest that *T. forsythia* does not interact with isolated mammalian platelets under the given conditions.

Other bacterial species including *Streptococcus sanguinis* and *Staphylococcus aureus* have shown that bacterial interactions with platelets can occur directly as well as indirectly through the dependent synthesis of plasma bridging proteins (Fitzgerald, Foster and Cox, 2006). This could highlight further mechanisms in which *T. forsythia* could interact with platelets but further work would be needed to fully test this hypothesis.
6.5 Summary

To conclude this chapter of work, this data demonstrate that *P. gingivalis* is able to directly associate with mammalian platelets, forming large fibrous aggregates, that stain highly for mToR and encapsulate bacterial cells. The expression of mToR may also be implemented in platelet cytoskeletal rearrangement and thrombus construction following *P. gingivalis* stimulation.

*P. gingivalis* is able to initiate platelet activation though intracellular calcium mobilisation that leads to the release of both alpha and dense granules, independent of bacterial outer membrane protein OMPA. *P. gingivalis* NCTC 11834 but not ATCC W50 actively associates and interacts with integrin α₂β₁ inducing strain dependant platelet aggregation and could suggest a role for bacterial fimbriae within platelet interactions.

Further work is required to determine the extent to which platelet-*P. gingivalis* interactions are dependent on inter-strain variations and could also provide novel pathways to fully characterise how these interactions take place and to identify pathways that could be developed as therapeutic targets.
Chapter 7: General discussion and future directions
7.1 Introduction

The overall aim of this study was to increase current understanding of the mechanism by which periodontal pathogens interact with platelets. The main objectives were to (a) develop an in vitro platelet-like cell model and (b) to optimise and multicolour flow cytometry panel (c) to enable the interactions of two of the red complex oral pathogens P. gingivalis and T. forsythia with platelets to be further characterised. It is anticipated that these findings will contribute to current knowledge of how periopathogens-platelet interactions might contribute to cardiovascular disease, specifically atherosclerotic vascular disease. The findings of this study have been discussed in detail within each relevant chapter. Here a summary of the major findings are presented alongside implications and possible future directions for this work.

7.2 The use of CHRF-288-11 cells as an in vitro platelet model

The CHRF-288-11 cell line originally characterised by Fugman and colleagues (1990) has been proposed as a lucrative model to study megakaryocyte/platelet behaviour (Lev-Lehman et al., 1997). In this study, it was observed that through differentiation, CHRF-288-11 cells adopt a platelet like phenotype that be utilised to study periodontal-platelet pathogen interactions in vitro. PMA (50 ng/ml) over five days resulted in successful CHRF-288-11 differentiation as characterised by the formation of large multi-lobed nuclei and pseudopodia pro-platelet protrusions. Differentiated CHRF-288-11 cells were shown to express the platelet specific glycoprotein CD41, the complex CD41/61, the platelet activation marker CD62P (P-selectin) but not CD42b as quantified by flow cytometry.

CHRF-288-11 cells were shown to associate with and were invaded by P. gingivalis strains NCTC 11834, ATCC W50 and T. forsythia ATCC 43037. Association of P.
*P. gingivalis* resulted in an increase in binding of integrin β1 and β3 specific antibodies which suggest either a change in integrin conformation or potential upregulation of the receptor as well as a reduction in binding of mToR antibodies, whereas association with *T. forsythia* did not.

These characteristics detail the novel application of the CHRF-288-11 cell line as a platelet-like *in vitro* cell model through the expression and utilisation of phenotypic platelet markers.

### 7.3 Development of a multiparameter flow cytometry panel

Following previously published guidelines on flow cytometry panel development (Schmitz *et al.*, 1998), a single step staining and analysis protocol for platelets was designed to simultaneously record platelet-pathogen association and the resulting changes in the expression of platelet activation markers. Using an advanced multiparameter gating strategy, a subset population of platelets associated with *P. gingivalis* were isolated from CD41⁺ platelet populations and the relative expression of CD41/61, CD62P and CD42b were determined. *P. gingivalis* association led to significant increases in CD41/61, CD62P and CD42b when compared to unassociated platelets. This optimised flow cytometry offers a new, practical analysis tool that can be used in settings where spontaneous platelets activation needs to be minimised, minimum sample volumes are available and where platelets cannot be stored on-site for extended period.
7.4 Periodontal pathogens and platelet interactions

Periodontal pathogen-platelet interaction studies revealed that viable *P. gingivalis* directly interacts with mammalian platelets whereas *T. forsythia* does not. Exposure to *P. gingivalis* induced platelet activation via calcium mobilisation and granule release, suggesting an immunological role against *P. gingivalis* infection.

Platelet activation, following platelet association with *P. gingivalis*, resulted in an increased expression of CD41/61, CD62P and CD42b as quantified by flow cytometry as well as the release of serotonin and platelet factor 4, quantified by ELISA. These investigations also revealed that platelet activation induced by *P. gingivalis* occurs independently of interactions with the major bacterial outer membrane subunits OMPA1 and OMPA2.

Surprisingly, although all strains of *P. gingivalis* induced platelet activation, platelet aggregation was only observed in a strain dependant manner by *P. gingivalis* NCTC 11834 and ATCC 381 but not by ATCC W50.

Investigations into the underlying mechanisms of *P. gingivalis* induced platelet aggregation demonstrated that within the applied methodology, both *P. gingivalis* secreted proteome and isolated *P. gingivalis* lipopolysaccharide (LPS) were unable to stimulate platelet aggregation, under the experimental conditions used in this study, possibly further emphasising a dependency on direct platelet to pathogen interactions.

Screening of *P. gingivalis* mutants deficient in either the major outer membrane protein subunits OMPA1/OMPA2 or the virulence associated sialidase 0352 resulted in no impact on the total percentage aggregation induced by *P. gingivalis* but did result in a faster onset of the aggregatory response. Similarly, screening of specific platelet
receptor inhibitors found that platelet aggregation occurred independently of interactions with toll-like receptor 2 and 4 as well as integrin β3 but all inhibitory experiments resulted in a faster onset of the aggregatory response.

Interestingly, platelet aggregation induced by \textit{P. gingivalis} NCTC 11834 was completely abolished following incubation with a specific integrin α2β1 inhibitor, with \textit{P. gingivalis} also being shown to directly associate with recombinant integrin β1 within an ELISA. Collectively, these findings suggest that platelet aggregation induced by \textit{P. gingivalis} NCTC 11834 is dependent on interactions with integrin α2β1, specifically the β1 subunit and could reveal independent virulence mechanisms such as fimbriae exhibited by \textit{P. gingivalis}.

These findings provide novel data which demonstrate that \textit{P. gingivalis} induces platelet aggregation and activation via granule release within platelet rich plasma, with the observed aggregatory response occurring in a strain dependant manner.

### 7.5 Future Directions

Within this body of work, it was postulated that through the associated localised bleeding and increased bacterial load found within chronic periodontitis, pathogenic bacterial species, specifically \textit{Porphyromonas gingivalis} and \textit{Tannerella forsythia}, opportunistically penetrate the vascular system. During this invasive process, these pathogenic bacteria would interact with platelets, giving rise to an altered platelet state, the formation of bacterial-platelet aggregates and ultimately contribute to the progression of atherosclerotic vascular disease.

As detailed above, this study does not only demonstrate that periodontal pathogenic bacteria are able to interact with platelets and megakaryocytic-like cells but may also
provide novel pathways in which periodontitis is able to contribute to the progression of CVD's.

The CHRF-288-11 cell line provides a unique niche in which to study bacterial-platelet interactions and with further expansion could reduce the need for regular blood acquisition for platelet studies. Although within this study, CHRF-288-11 cells have been shown to express a number of platelet markers, further analysis of protein expression could reveal a plethora of platelet markers expressed by the CHRF cells adding further benefit to the reported applications of these megakaryocytic like cells. It is also possible that through varied differentiation protocols such as those detailed by Lepage and colleagues (2000) that utilise cytokines as opposed to PMA could reveal varying phenotypes within the cell line further adding to its applications within a platelet-like cell model. Novel developments within the field of in vitro megakaryocyte culture and in vitro platelet production could abrogate the need for blood donations entirely (Moreau et al., 2016; Strassel, Gachet and Lanza, 2018), possibly replacing the CHRF-288-11 cells and allowing for platelet methodologies to become routine and high throughput.

Interestingly, it was found that although T. forsythia is able to interact with and invade the megakaryocytic cell line, it was not observed to interact with platelets within any investigations. However from the investigations undertaken within this body of work it cannot be concluded that T. forsythia is unable to interact with platelets and could suggest an alternative pathway in which periodontal-pathogens and platelets interact.
Further work could be used to highlight whether *T. forsythia* is able to interact with mammalian platelets, whether it actively evades platelet response or whether *T. forsythia*-platelet interactions are reliant on the synthesis of cofactors such as those observed with *Staphylococcus aureus* aggregation (Yeaman, 2010b).

Similarly, it is well established that pathogenic bacterial species within the oral biofilm are able to coaggregate (Kolenbrander, Andersen, Blehert, *et al.*, 2002), with suggestions that this may facilitate synergistic behaviour between the pathogens, increasing virulence activity (Kolenbrander, 2000; Kesavalu *et al.*, 2007; Kirschbaum *et al.*, 2010b). Exposing platelets to co-cultures of *P. gingivalis* and *T. forsythia* could expose mechanisms by which *T. forsythia* is able to interact with platelets and reveal further aspects of *P. gingivalis*-platelet interactions thereby providing a closer representation of the biofilm present with the periodontal pocket.

In addition, the inter-strain variances of *P. gingivalis* induced platelet aggregation and activation suggest that bacterial genetics as well as their associated virulence factors such as fimbriae contribute to the combative platelet response.

Interestingly, phylogenetic comparisons of the *P. gingivalis* strains utilised within this study reveal that NCTC 11834 and ATCC 381 are highly similar, whereas ATCC W50 is comparatively highly divergent (Griffen *et al.*, 1999). This is further emphasised through full genome comparisons as illustrated by a circular genome representation (Figure 7.1). In order to expand on the initial findings within this thesis, full screening of the ATCC 381 strain as well as the introduction of further *P. gingivalis* strains such as ATCC W83 which is closely related to ATCC W50 would further explore the strain specific nature of platelet aggregation in response to *P. gingivalis*. This study also found strain specific interactions with integrin α2β1, with the full extent of *P.*
*P. gingivalis*-platelet interactions as well as the underlying mechanisms remaining inconclusive. It is hypothesised that through the application of pull-down assays, the specific mammalian protein targets for *P. gingivalis* could be identified and allow the proposal of a comprehensive mechanistic pathway.

These pull-down assays could then be coupled with an extensive proteomic study of both the mammalian platelets and the bacterial pathogens. With regards to platelets, it could be used to identify specific pathways in which platelets respond to varying pathogenic bacterial species and could implicate further roles for platelets within both the innate and adaptive immune system. Further work could also highlight the exact proteinous nature of the fibrin-like structures which form following platelet exposure to *P. gingivalis*. These investigations could utilise fluorescently labelled fibrinogen to determine whether the formed matrix is similar to that of a haemostatic fibrin mesh (Holinstat, 2017) as well as being coupled with further immunofluorescent microscopy and western blotting to confirm the presence of further proteins such as mToR and actin which are known to stabilise thrombus formation (Aslan and McCarty, 2012).

As a separate approach, analysing the bacterial strains could also reveal specific bacterial genetic features and proteomic differences, which not only contribute to the observed variances in platelet activation and aggregation but could also highlight highly virulent pathogenic strains in relation specifically to CVD.
Figure 7.1 Circular representation of the \textit{P. gingivalis} NCTC 11834, ATCC 381 and ATCC W50 genomes. \textit{P. gingivalis} NCTC 11834 (431947.7) and ATCC 381 (1403335.5) are genetically highly similar whereas comparatively ATCC W50 (1125722.3) is highly divergent. This representation was generated using the PATRIC online genome comparison tool.
In order to expand on the findings surrounding platelet-pathogen interactions, there are many potential investigative avenues that could be explored. Initially it would very interesting to investigate as to whether periodonto-pathogens influence platelet adhesion, which is a known contributor to atherosclerosis and atherothrombosis (Finn et al., 2010; Badimon, Padró and Vilahur, 2012; Anlamlert, Lenbury and Bell, 2015). At the principle level, this could be undertaken using platelet adhesion assays which quantitate platelet adhesion to either glass coverslips or a collagen coated surface. This methodology could then be applied to a microfluidics system, which would investigate whether platelets can interact with periodonto-pathogens under shear flow and whether these interactions interfere with platelet adhesion properties under physiological conditions.

Further to this, platelet interactions with the vessel wall, specifically dysfunctional or activated endothelium are thought to be crucial in the role of platelets within the development of the atherosclerotic legion as well as the terminal thrombotic events (Sachais, 2001). Recent developments within microfluidic chambers allow the study of the platelet endothelium interface and could be utilised to investigate whether periodontal pathogens promote the adherence to the endothelium, resulting in atherosclerotic disease-like phenotypes (Zilberman-Rudenko et al., 2017).

These future studies could be coupled with patient blood samples and clinical isolates of periodonto-pathogens to ascertain whether the presence of specific strains of *P. gingivalis* is directly associated with the progression of CVD or whether patients with periodontitis behave differently to the healthy blood samples applied within this study, specifically addressing whether patients with an increasing severity of periodontitis
also present with an increase in platelet activation and aggregation, as well as an increased risk of CVD.

Used in combination, the findings within this thesis as well as further work could identify novel contributory factors to the development of CVD, aid in the identification of risk factors associated with periodontitis and help develop a personalised medical approach to the treatment of patients through the use of platelets as complex biomarkers. Here it is postulated that through the identification of specific 'at risk' patients as well as the application of preventative periodontal monitoring and treatments could hold substantial prognostic promise for the future treatment of CVD.
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Ethical approval

21st May 2015

Dr P Stafford
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Sheffield Hallam University
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Sheffield S1 1WB

Dear Dr Stafford

Ethics Application No. HWB-BIO-03

Thank you second version of your project entitled: Oral pathogen and cardiovascular disease: can oral pathogens contribute to cardiovascular disease by altering platelet behaviour and function. Following re-review we are delighted to give you ethics permission to undertake the project.

Yours sincerely

KM

Dr N Jordan-Mahy
Chair of the Bioscience and Chemistry Ethics Committee
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