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A clinical and microbiological investigation of peri-implant infections

A thesis submitted to the University of Dublin, Trinity College, in fulfilment of the requirements for the degree Doctor of Philosophy by

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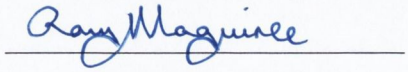

Fergal Rory Maguire

Summary

Osseointegrated oral implants are becoming increasingly more common in restorative dentistry as permanent replacements for lost or missing teeth. Peri-implant infections are a significant area of treatment need but the current evidence base for treatment is low. Peri-implant and periodontal infections share many similarities including clinical and pathological features and both have a microbial aetiology. There is growing evidence that patients with an increased susceptibility to periodontal disease have an increased susceptibility to peri-implantitis, very likely associated with oral bacterial microflora dynamics. The present study was undertaken to further investigate clinical and microbial aspects of peri-implantitis and periodontitis in a cohort of patients attending the Dublin Dental Unversity Hospital.

Aims: The present study was undertaken to further understanding of (i) the risk indicators of oral peri-implant infections including peri-implant mucositis and peri-implantitis, (ii) the bacterial species associated with peri-implant infections and the influence of patient factors on microbiological parameters, (iii) to compare periodontal and peri-implant healing following non-surgical treatment clinically and microbiologically, (iv) to comapre the range and density of bacterial species recovered from subgingival sites using paperpoint and currette sampling, (v) to investigate the relative distribution of *Candida* spp. in periodontal pockets and the oral mucosa and the population biology of the predominant species recovered, *Candida albicans,* from periodontal and oral mucosal sites in patients affected by periodontitis compared to healthy controls.

Methods: Fifty-one patients with dental implant restorations present for five or more years underwent a detailed history, clinical and radiographic examination. The baseline findings were examined in light of the periodontal disease history and smoking status. Patients affected by peri-implant mucositis or peri-implantitis underwent non-surgical treatment, debridement and oral hygiene instruction, and follow up at three and nine months post-treatment. Microbiological sampling was undertaken using paper-points and curettes. Separate paper-point and curette samples from 40 individual sites in 27 patients were tested semi-quantitatively for a panel of 29 bacterial species previously associated with periodontitis and peri-implantitis by checkerboard DNA–DNA hybridisation analysis. For the remaining dental implant patients, paper-point and curette samples were combined prior to checkerboard DNA-DNA hybridisation analysis. For 21 patients without implants, but who were affected with periodontitis, paper-point, curette and oral wash samples were tested for *Candida* spp. by culture on CHROMagar Candida agar. The population structure of selected isolates of *C. albicans*, the predominant species recovered, was investigated using multilocus sequence typing (MLST) using oral carriage *C. albicans* isolates from healthy controls as a comparison group.

Results: No significant association was observed between current smoking status and peri-implantitis or implant loss ($p=0.1$). Severe periodontal disease was found to be significantly associated with peri-implantitis or implant loss ($p=0.03$). For the patients affected by either peri-implant mucositis and/or peri-implantitis and who completed treatment and follow up, a significant decrease in bleeding on probing was noted for both groups ($p<0.05$). Furthermore the percentage of sites with probing pocket depths of ≥6 mm was noted to be significantly decreased for the peri-implantitis group ($p<0.05$) but not for the peri-implant mucositis group ($p>0.05$) nine months following completion of treatment.

On an implant level significant reductions were noted in bleeding on probing and in probing pocket depths, for both peri-implantitis and peri-implant mucositis sites ($p<0.0001$) following treatment. Neither paper-point nor curette sampling yielded markedly greater amounts of bacterial DNA. Paper-point sampling yielded a greater number of species than curette sampling in 55% (22/40) of the sites tested. However in 30% (12/40) of the sites investigated, curette sampling yielded a greater number of bacterial species than paper-point sampling. A combination of both sampling techniques for individual sites produced the best results in terms of the range of bacterial ssepcies detected. *Treponema* spp. were observed to be significantly elevated in peri-implant sites compared to periodontal sites ($p<0.05$). The total of the mean values of the estimated bacterial cell density per individual species detected from all of the samples investigated from

peri-implantitis sites was 33% greater than that of peri-implant mucositis sites. *Tanerella forsythia* and *Porphyromonas gingivalis* levels were significantly elevated ($p<0.01$) and *Neisseria mucosa* percentage levels were elevated to a lesser extent ($p<0.05$) in periodontal and peri-implant sites demonstrating probing depths of >5 mm and bleeding on probing. The levels of *Treponema* spp. were significantly elevated, from periodontal and peri-implant sites in patients with a history of severe periodontal disease, compared to patients with no history of severe periodontal disease ($p<0.05$).

Following non-surgical treatment of patients with peri-implant mucositis or peri-implantitis, significant reductions were observed in the levels of both *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* ($p<0.01$) at peri-implant sites. Almost 50% of peri-implant sites showed an increase of ≥1% in the levels of oral streptococci (28/55), *Capnocytophaga* spp. (28/55) and *Eikenella corrodens* (28/55) following treatment. No significant differences were observed between peri-implant and periodontal sites for levels of *A. actinomycetemcomitans, P. gingivalis, T. forsythia, Treponema* spp. and staphylococcal spp. comparing baseline to nine months following treatment ($p>0.05$).

Candida spp. was recovered from 10/21 (46.7%) periodontitis patients and 16/50 (32%) healthy subjects. *Candida albicans* predominated in both groups and was recovered from all *Candida*-positive subjects. *Candida*-positive periodontitis patients yielded *Candida* from periodontal pockets with average densities from curette and paper-point samples of 3,528 and 3,910 colony forming units (cfu)/sample, respectively, and 1,536 cfu/ml from oral rinses. The majority (18/19) of periodontitis patients' healthy sites sampled were *Candida*-negative. The 16 *Candida*-positive healthy subjects yielded an average of 279 cfu/ml from oral rinses. MLST analysis of 31 *C. albicans* isolates from periodontitis patients yielded 19 sequence types (STs), 13 of which were novel. Eleven STs belonged to MLST clade 1. In contrast, 16 *C. albicans* isolates from separate healthy subjects belonged to 16 STs, with four from clade 1. The distribution of STs between both groups was significantly different ($p=0.04$) and indicated an enrichment of *C. albicans* isolates in periodontal pockets that warrants a larger study.

Conclusions: 1. Patients with a history of severe periodontal disease were significantly more likely to experience peri-implantitis and/or implant loss ($p=0.03$). Current smoking status was not significantly associated with peri-implantitis or implant loss ($p=0.1$). **2.** Non-surgical mechanical treatment resulted in improvements in probing pocket depth and bleeding on probing in patients with peri-implant mucositis and/or peri-implantitis. These findings indicate that non-surgical mechanical treatment may be recommended as a first line treatment for these patient groups. **3.** Markedly differing results were obtained for the detection of microbial species from sub-gingival and sub-mucosal sites using paperpoint and curette sampling and therefore combined samples obtained using both methods should be used to more fully represent the micro-flora of the sub-gingival or sub-mucosal site sampled. **4.** Peri-implant samples yielded significantly higher levels of *Treponema* spp. compared to periodontal sites ($p<0.05$). Furthermore, the levels of *Treponema* spp. were significantly elevated from periodontal and peri-implant sites, in patients with a history of severe periodontal disease ($p<0.05$). These findings indicate that levels of *Treponema* spp. can possibly be used to monitor the health of periodontal and peri-implant sites. **5.** *Candida* spp. were commonly isolated from the sub-gingival sites of periodontitis patients and were significantly associated with active periodontitis. The observed enrichment of clade 1 *C. albicans* isolates from periodontal pockets relative to oral carriage isolates indicates that clade 1 isolates may be predisposed to the environment present in periodontal pockets and may contribute to the periodontal disease process. This research provides dental practice with new and timely evidence-based information on the aetiology, clinical management and outcomes of non-surgical therapy for human peri-implant and periodontal diseases.

Table of Contents

Declaration ..... **II**
Summary ..... **III**
Table of Contents ..... **V**
Acknowledgements ..... **X**
Index of Figures ..... **XI**
Index of Tables ..... **XII**
Abbreviations ..... **XV**
Publications Resulting from Work Described in this Thesis ..... **XVII**

Chapter 1 ..... **1**
General Introduction **1**
1.1 Overview **2**
1.2 Endosseous dental implants: osseointegration **3**
1.3 Problems associated with peri-implant infections **4**
1.4 Peri-implant biofilm formation **7**
1.5 The periodontal tissues in health **9**
1.6 The anatomy of the peri-implant tissues **10**
1.7 Comparison between the peri-implant and periodontal tissues **11**
1.8 Periodontal tissues in disease **14**
1.9 Histopathology of peri-implant infections **15**
1.10 The microbiology of the peri-implant niche **20**
1.11 Risk Indicators **26**
1.11.1 Periodontal disease experience and maintenance care 26
1.11.2 Smoking 36
1.12 Clinical examination of periodontal and peri-implant tissues **40**
1.12.1 Overview 40
1.12.2 Periodontal probing around natural teeth 40
1.12.3 Peri-implant probing 42
1.13 Non-surgical treatment of periodontal and peri-implant infections **45**
1.13.1 Overview of treatment of peri-implant infections 45
1.13.2 The influence of mechanical debridement on sub-gingival biofilms 46
1.13.3 Non-surgical treatment of peri-implant mucositis 50
1.13.4 Non-surgical treatment of peri-implantitis 53
1.14 Investigation **60**
1.15 Research Questions **60**

Chapter 2 ..... **61**
General Materials and Methods **61**
2.1 Patient selection **62**
2.1.1 Ethical approval 62
2.1.2 Inclusion criteria: 62
2.1.3 Exclusion criteria: 62
2.1.4 Dental and medical history and an oral examination 63
2.1.5 Radiographic Examination 63
2.1.6 Patient characteristics in each study 64
2.2 Definitions **66**
2.2.1 Peri-implantitis 66
2.2.2 Peri-implant mucositis 66
2.2.3 Peri-implant health 66
2.2.4 Peri-implant mucositis peri-implantitis patients, patients and peri-implant healthy **patients** **66**
2.2.5 History of severe periodontal disease 66
2.2.6 Patients positive for smoking 67
2.3 Non-surgical treatment of peri-implant infections **67**
2.3.1 Mechanical debridement 67
2.3.2 Oral hygiene instruction 67

2.4 Three, six and nine month examinations **68**
2.5 Microbial sampling **68**
2.5.1 Selection of periodontal and peri-implant microbial sampling sites 68
2.5.2 Paper-point and curette sampling 68
2.5.3 Curette and paper-point sample processing 69
2.6 Microbial analysis **70**
2.6.1 Semi-quantitative determination of microbial species by checkerboard DNA-DNA hybridisation 70
2.6.2 Real-Time PCR analysis 73
2.7 Statistical Methods **74**
2.7.1 Fisher's Exact Test 74
2.7.2 Changes in clinical parameters following non-surgical treatment: descriptive statistics, t-test and regression analysis 74
2.7.3 Percentage relative abundance 75
2.7.4 Comparison of paper-point and curette sampling techniques: Descriptive statistics 76
2.7.5 Observational microbiological study: investigation of peri-implant and periodontal microflora at baseline presesentation 78
2.7.6 Change in the microflora of periodontal and peri-implant sites of patients with peri-implant infections following non-surgical treatment 79

Chapter 3 ..... **81**
The relationship between selected risk indicators and peri-implantitis or implant loss **81**
3.1 Introduction **82**
3.1.1 Peri-implant infections 82
3.1.2 Patients with a history of periodontal disease 82
3.1.3 Smoking status 84
3.2 Aims **87**
3.3 Methods **88**
3.3.1 Patient selection 88
3.3.2 Inclusion criteria: 88
3.3.3 Exclusion Criteria: 88
3.3.4 Ethical approval 88
3.3.5 Clinical history and examination 88
3.3.6 Definitions 89
3.3.7 Statistical Analysis 90
3.4 Results **91**
3.5 Discussion **92**
3.6 Conclusions **94**

Chapter 4 ..... **95**
4.1 Introduction **96**
4.1.1 Peri-implant infections and non-surgical treatment 96
4.2 Aim **97**
4.3 Methods **98**
4.3.1 Patients 98
4.3.2 Treatment 99
4.3.3 Three and nine month examinations 100
4.3.4 Statistical analysis 100
4.4 Results **102**
4.4.1 Bleeding on Probing 102
4.4.2 Percentage of sites with probing pocket depths ≥6 mm 103
4.4.3 Suppuration on probing 103
4.4.4 Unit level analysis 104
4.5 Discussion **105**
4.6 Conclusions **110**

Chapter 5 ..... **111**
A comparison of the bacterial species recovered from sub-gingival sites by paper-point and curette sampling using checkerboard DNA-DNA hybridisation **111**
5.1 Introduction **112**
5.2 Study aim **115**
5.3 Materials and Methods: **116**
5.3.1 Patient selection 116
5.3.2 Microbial Sampling 116
5.3.3 Microbial Analysis 117
5.3.4 Data analysis 117
5.4 Results **120**
5.4.1 Patients 120
5.5 Discussion **123**
5.6 Conclusions **125**

Chapter 6 ..... **126**
A semi-quantitative comparison of the microbiota around natural teeth and oral implants in patients with implants for five or more years determined by checkerboard analysis **126**
6.1 Introduction **127**
6.2 Materials and Methods **130**
6.2.1 Patients 130
6.2.2 Dental and medical history and an oral examination 130
6.2.3 Radiographs 131
6.2.4 Peri-implantitis patients, peri-implant mucositis patients and peri-implant healthy patients 131
6.2.5 Microbial sampling 132
6.2.6 Semi-quantitative determination of microbial species by checkerboard DNA-DNA hybridisation 132
6.2.7 Real-Time PCR analysis 133
6.2.8 Comparison of periodontal and peri-implant microflora 133
6.3 Results **135**
6.3.1 Patients and samples 135
6.3.2 Semi-quantitative detection of microbial species 135
6.4 Discussion **137**
6.4.1 Peri-implant compared to periodontal microflora 137
6.4.2 Peri-implant mucositis compared to peri-implantitis 137
6.4.3 Periodontal health and periodontitis 138
6.4.4 Probing depths of >5 mm and bleeding on probing 138
6.4.5 Smokers compared to non-smokers 139
6.4.6 History of severe periodontal disease 139
6.5 Conclusions **140**

Chapter 7 ..... **141**
Determination of semi-quantitative changes in the microbial flora around natural teeth and implants following non-surgical periodontal therapy **141**
7.1 Introduction **142**
7.1.1 Observed effects of non-surgical mechanical treatment on peri-implant infections 142
7.1.2 Microbial sampling 143
7.2 Aim **143**
7.3 Materials and Methods **144**
7.3.1 Patients 144
7.3.2 Clinical examination and radiographs of peri-implant sites 144
7.3.3 Peri-implantitis, peri-implant mucositis and health implants 145
7.3.4 Treatment 145
7.3.5 Microbial Sampling 146
7.3.6 Semi-quantitative determination of microbial species by checkerboard DNA-DNA hybridisation 146

7.3.7 Quantitative PCR detection of four bacterial species: Real-Time PCR analysis..147
7.3.8 Statistical analysis 147
7.4 Results 149
7.5 Discussion 153
7.6 Conclusions 155

Chapter 8 156
Enrichment of multilocus sequence typing clade 1 with oral *Candida albicans* isolates in patients with untreated periodontitis 156
8.1 Introduction 157
8.2 Materials and Methods 163
8.2.1 Patients included 163
8.2.2 Inclusion and exclusion criteria 163
8.2.3 Sampling of the oral cavity, periodontal pockets and gingival sites for *Candida* spp. 164
8.2.4 Identification of *Candida* isolates 165
8.2.5 Routine *Candida* culture 165
8.2.6 Nucleic acid isolation 165
8.2.7 ABC genotyping of *C. albicans* 166
8.2.8 Genotyping of *C. dubliniensis* 166
8.2.9 Multilocus sequence typing (MLST) 166
8.2.10 Statistical analyses 167
8.3 RESULTS 168
8.3.1 Clinical characteristics of patients with periodontitis 168
8.3.2 *Candida* spp. and cell densities recovered from patients with periodontitis 168
8.3.3 *Candida* spp. and cell densities recovered from healthy volunteers 169
8.3.4 MLST analysis and ABC genotyping of *C. albicans* isolates from periodontitis patients and healthy oral carriers 169
8.3.5 Genetic comparison of oral *C. albicans* isolates recovered from periodontitis patients and from healthy individuals 170
8.3.6 MLST analysis of *C. dubliniensis* isolates recovered from patients with periodontitis **170**
8.4 Discussion 172
8.5 Conclusions 175

Chapter 9 177
9.1 Peri-implant infections 178
9.2 Checkerboard DNA-DNA hybridisation analysis: strengths and weaknesses 178
9.3 Comparison of periodontal and peri-implant microflora 181
9.3.1 Periodontal and peri-implant microflora 181
9.3.2 Periodontal health and periodontitis 183
9.3.3 Peri-implant mucositis and peri-implantitis 183
9.3.4 History of periodontal disease 184
9.3.5 Smoking status 185
9.3.6 Sites with peri-implant probing depths of >5 mm with bleeding on probing 185
9.4 Risk indicators: periodontal disease history 185
9.5 The non-surgical treatment of peri-implant infections: clinical response 187
9.6 Microbial sampling technique 188
9.7 Microbial changes following non-surgical treatment of peri implant infections 189
9.8 Future directions 190
9.9 Concluding remarks 191

References 193
Appendix 1 222
Ethical Approval 222
Patient Information Leaflet 223
Consent Form 224
Ethical Approval 225
Appendix 2 226
Patient Characteristics 226
Table A2.1 Summary of the baseline characteristics of the patients participating in the present investigation. 227
Table A2.2 Summary of patient participation in individual studies in the present investigation 229

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Index of Figures

Page numbers refer to the text page preceding the figure(s)

Figure		Page
1.1a	A photograph of the dental implant restoration affected by peri-implant mucositis.	2
1.1b	A peri-apical radiograph of the dental implant-supported bridge shown in Figure 1.1a.	2
1.2	A peri-apical radiograph of a dental implant-supported bridge affected by peri-implantitis.	2
1.3	Anatomy of the periodontal tissues.	9
1.4	Diagrammatic representiation of periodontal tissues in health and disease adapted from Lindhe *et al.* (2003).	14
1.5	The periodontal tissues in periodontitis: a diagrammatic representation adapted from Lindhe *et al.* (2003).	14
1.6	Repopulation of the sub-gingival habitat post sub-gingival debridement.	48
4.1	Sequence of visits.	98
5.1	Comparison of the frequency individual bacterial species were detected by paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling (n=40) for both periodontal and peri-implant sites.	122
5.2	Comparison of the frequency individual species were detected from each of the peri-implant sites by either paper-point sampling alone, by curette sampling alone or by both of the paper-point and curette sampling techniques.	122
5.3	Comparison of the frequency individual species were detected from each of the periodontal sites by either paper-point sampling alone, by curette sampling alone or by both of the paper-point and curette sampling techniques.	122
7.1	Percentage of peri-implant sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species following treatment.	150
7.2	Percentage of periodontal sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species groupings following treatment.	150
7.3	Percentage of peri-implant mucositis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species groupings following treatment.	150
7.4	Percentage of peri-implantitis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species following treatment.	150
8.1	Neighbour-joining tree based on the polymorphic nucleotides in 19 of the unique STs identified in 31 *C. albicans* isolates recovered from nine different patients with periodontitis, and 16 unique STs identified from oral rinse sampling of 16 periodontally healthy patients.	170

Index of Tables

Page numbers refer to the text page preceding the table(s)

Table	Title	Page
1.1	Similarities and differences between the periodontal and peri-implant tissues.	12
2.1	The 29 bacterial species included in the checkerboard DNA-DNA hybridisation testing panel.	70
2.2	The four reference bacterial species used in real-time PCR.	74
2.3	Oligonucleotides used in real time-PCR experiments to detect *P. gingivalis*, *T. forsythia*, *T. denticola and A. actinomycetemcomitans*.	74
3.1	Number of subjects in each of the studies meeting the inclusion criteria in the systematic review of Renvert & Persson (2009).	84
3.2	Smoking status and peri-implantitis status of the patients.	92
3.3	History of severe periodontitis and peri-implantitis status of the patients.	92
4.1.	Mean Percentage (and standard deviation) of sites demonstrating bleeding on probing for the peri-implant mucositis patients (peri-implant mucositits group) and those affected by peri-implantitis (peri-implantitis group).	102
4.2	Mean percentage of sites demonstrating probing pocket depths of ≥6 mm at baseline (T1), three months post-treatment (T2) and nine months post-treatment (T3) for the peri-implant mucositis and peri-implantitis groups.	104
4.3	Percentage of sites demonstrating suppuration on probing at T1, T2 and T3 for patients with peri-implant mucositis and peri-implantitis.	104
4.4	Multi-level analysis of probing pocket depth (PPD) as a function of unit type (periodontal or peri-implant) and time (T2 and T3 compared to T1).	104
4.5	Multi-level logistic analysis of bleeding on probing (BOP) as a function of tooth type (periodontal compared to peri-implant) and time (T2 and T3 compared to T1).	104
5.1	Comparison of the number of occasions a greater number of bacterial species were detected from individual sites by either paper-point or curette sampling techniques.	120
5.2	Number of bacterial species detected by both paper-point and/or curette sampling from periodontal and peri-implant sites by checkerboard analysis.	120
5.3	Comparison of the means and standard deviations of the estimated bacterial cell density for each of the bacterial species groups investigated identified by paper-point and curette sampling from periodontal and peri-implant sites.	120

5.4	Comparison of the number of times paper-point and/or curette sampling detected each of the 18 bacterial species groupings investigated in either peri-implant or periodontal sites.	122
5.5	Comparison of the frequency individual bacterial species were detected from each of the periodontal sites by either paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling.	122
5.6	Comparison of the frequency individual bacterial species were detected from each of the peri-implant sites by either paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling.	122
6.1	Microbial sampling technique and analysis methods employed for microbiological observational studies, comparing periodontal and peri-implant microflora.	128
6.2	Microbial species identified in microbiological observational studies, comparing periodontal and peri-implant microflora.	128
6.3	The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species/groupings investigated by checkerboard DNA-DNA hybridisation for periodontal and peri-implant sites.	136
6.4	The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for peri-implant mucositis and peri-implantitis sites.	136
6.5	The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings (checkerboard DNA-DNA hybridisation) for sites with probing pocket depths of >5 mm and demonstrating bleeding on probing, and sites not meeting these criteria.	136
6.6	The median (inter-quartile range) value of the percentage relative abundance[1] of selected species investigated by checkerboard DNA-DNA hybridisation for patients with and without a history of severe periodontal disease.	136
6.7	The median (inter-quartile range) value of the percentage relative abundance[1] of each of the 18 species/groupings investigated by checkerboard DNA-DNA hybridisation for periodontal healthy sites and periodontitis sites.	136
6.8	The median (inter-quartile range) value of the percentage relative abundance[1] of each of the 18 species/groupings investigated by checkerboard DNA-DNA hybridisation for patients who are current smokers and non-smokers.	136
7.1	Sampling methods and microbiological analytical techniques employed in studies on the non-surgical treatment of peri-implant infections.	142
7.2	Comparison of change in the microflora of periodontal and peri-implant sites following treatment, estimated bacterial cell density (inter-quartile range) $x10^5$ cfu.	150

7.3	The relative abundance[1] of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Treponema* spp., staphylococcal spp. at peri-implant sites at baseline and nine months following treatment, as determined by checkerboard DNA-DNA hybridisation analysis (n=55).	150
7.4	The relative abundance[1] of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Treponema* spp., staphylococcal spp. at periodontal sites at baseline and nine months following treatment as determined by checkerboard DNA-DNA hybridisation analysis (n=25).	150
7.5	Frequency of peri-implant sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species groupings following treatment (n=55).	150
7.6	Frequency of periodontal sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=25).	150
7.7	Frequency of peri-implant mucositis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=40).	150
7.8	Frequency of peri-implantitis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=15).	150
7.9	Changes in the percentage relative abundance for each of the 18 microbial species investigated[1] between baseline and nine months following treatment for sites demonstrating an increase, no change or decrease in probing depth.	151
7.10	The percentage relative abundance at peri-implant sites at nine months following treatment for each of the 18 microbial species investigated[1] for sites demonstrating an increase, no change or decrease in probing depth.	152
8.1	Clinical characteristics of patients with periodontitis.	164
8.2	*Candida* species and cell density recovered from patients with periodontitis.	168
8.3	MLST STs, allelic profiles and ABC genotypes of oral *C. albicans* isolates recovered from periodontitis patients and healthy carriers.	170

Abbreviations

ATCC	American Type Culture Collection
B	Beta
γ	Gamma
δ	Delta
>	Greater than
<	Less than
=	Equals
≥	Greater than or equal to
≤	Less than or equal to
µl	Microlitre
µM	Micromolar
16S rDNA	Genes encoding 16S ribosomal RNA component of the 30S ribosomal subunit
90°	Ninety degrees (angle)
BOP	Bleeding on probing
cfu	Colony forming unit
cfu/ml	Colony forming units per millilitre
DNA	Deoxyribonucleic acid
90°	Ninety degrees (angle)
e.g.	*Exempli gradita;* for example
et al.	*Et alia*; and others
Etc.	*Et cettera*; and the rest
GCF	Gingival crevicular fluid
h	Hour
i.e.	*Id est*; that is
in vitro	"within the glass"; performing a given procedure in a controlled environment outside of a living organism
in vivo	"Within the living"; performing a given procedure within a living organism
ITI	International Team for Implantology
L	Litre
min	Minute
ml	Millilitre
MLST	Multilocus sequence typing
mm	millimetre
mM	millimolar
OR	Odds ratio
°C	Degrees Celsius

PCR	Polymerase chain reaction
pH	Measure of acidity or alkalinity (hydrogen ion concentration)
PPD	Probing pocket depths
PTFE	Polytetrafluoroethylene
R2	Coefficient of determination
RR	Risk ratio
RAPD	Random amplified polymorphic DNA
RT-PCR	Real time polymerase chain reaction
s	Second
TSA	Trypticase soy broth
UK	United Kingdom
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
v/v	volume/volume
x *g*	x Gravitational force
YEPD	Yeast extract peptone dextrose
e.g.	*Exempli gradita;* for example

Publications Resulting from Work Described in this Thesis

McManus BA, Maguire R, Cashin PJ, Claffey N, Flint S, Abdulrahim MH, Coleman DC. Enrichment of multilocus sequence typing clade 1 with oral *Candida albicans* isolates in patients with untreated periodontitis. *Journal of Clinical Microbiology* 2012; **50**(10):3335-3344.

Chapter 1

General Introduction

1.1 Overview

Peri-implant disease as presented by the 6th European Workshop on Periodontology (Zitzmann & Berglundh, 2008) is defined as "*a collective term for inflammatory reactions in the tissues surrounding an implant*". Peri-implant mucositis is defined as "*the presence of inflammation of the mucosa at an implant with no signs of loss of supporting bone*" (Figure 1.1a and 1.1b), whereas peri-implantitis, in addition to inflammation of the mucosa, is characterised by a loss of implant supporting bone (Figure 1.2).

Approximately 10 million new dental implants are placed annually worldwide (Millenium Research Group: US market for dental implants, 2003), yet our knowledge of peri-implant infections is limited (Furst *et al.*, 2007). In order to inform patients of the possible associated risks of having dental implants, it is crucial that we first understand the aetiology of peri-implant infections and likely contributing factors. This includes determining which microorganisms are associated with these disease processes, what the associated risk factors or risk indicators are and determining the evidence for different treatments of peri-implant infections. The evidence base for the treatment of peri-implant infections is recognised as being low and many treatment approaches are considered empirical (Roos-Jansaker *et al.*, 2006*a*). What is known is that peri-implant infections are going to be a significant area of treatment need for the future (Renvert *et al.*, 2006*a*).

The impact of the hygienic and mechanical debridement phase of treatment (initial disease elimination therapy) on the composition of sub-gingival biofilms in patients with peri-implantitis, whilst common to many recommended treatment strategies for peri-implantitis, remains incompletely understood. Peri-implant and periodontal infections share many similarities including clinical, pathological and microbiological features. Both periodontitis and peri-implantitis are chronic inflammatory diseases with a microbial aetiology (Zitzmann & Berglundh, 2008). In both periodontitis and peri-implantitis, the persistent host inflammatory and immune response against the bacterial challenge is responsible for the hard and soft tissue destruction (Zitzmann & Berglundh, 2008). In both periodontitis and peri-implantitis, host susceptibility is considered to be important, however, the evidence base to support this is limited (Renvert & Persson, 2009). There is growing evidence that patients who are at an increased susceptibility to periodontal disease, also have an increased susceptibility to peri-implantitis and subsequent implant loss (Karoussis *et al.*, 2002; Renvert & Persson, 2009). This likely relates both to the microflora present within the patients' mouth (Mombelli *et al.*, 1995)

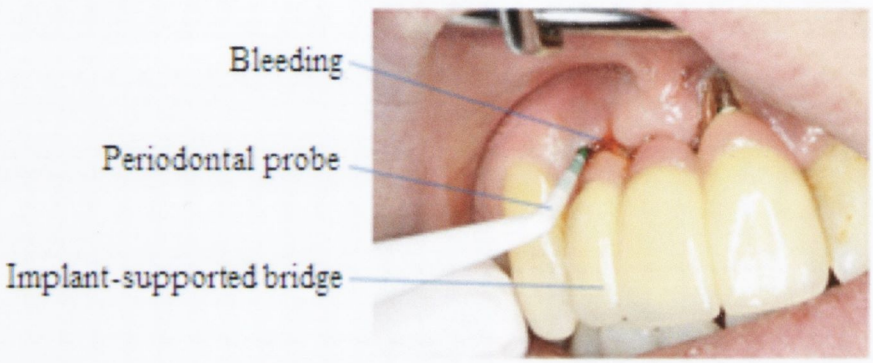

Figure 1.1a A photograph of a dental implant restoration affected by peri-implant mucositis. The periodontal probe (Click Probe, Kerr Hawe, Germany) is in position in the peri-implant crevice of the implant-supported bridge. Note the bleeding on probing present.

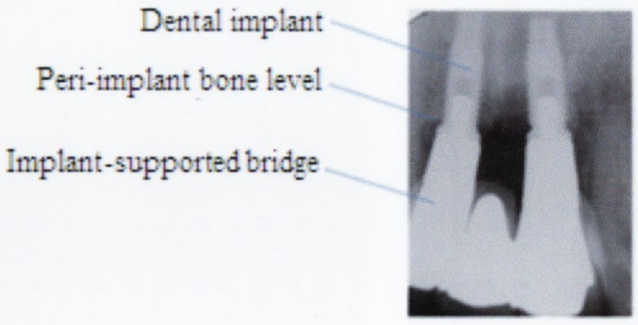

Figure 1.1b A peri-apical radiograph of the dental implant-supported bridge shown in Figure 1.1a. Note the peri-implant bone levels demonstrate little bone loss.

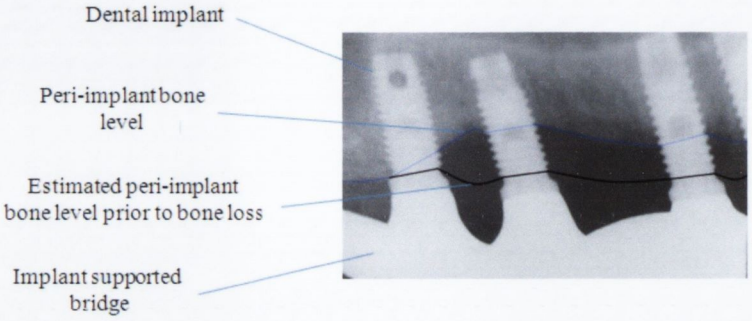

Figure 1.2 A peri-apical radiograph of a dental implant-supported bridge affected by peri-implantitis. Note the peri-implant bone levels demonstrate extensive bone loss.

and genetic factors which determine the manner in which patients respond to peri-implant and/or periodontal infection (Karoussis *et al.*, 2003).

While knowledge of the putative pathogens in periodontitis has been extensively studied, it is of great importance to establish a similar knowledge base for peri-implant diseases. The furthering of knowledge of both the composition of the sub-gingival flora and the changes in the microflora in response to the non-surgical treatment of peri-implantitis is urgently required. The present investigation sets out to increase understanding of the aetiology of peri-implant diseases and provide clinical and microbiological evidence as to the efficacy of non-surgical mechanical treatment in the treatment of peri-implant infections.

1.2 Endosseous dental implants: osseointegration

The osseointegrated dental implant is based on the discovery by Swedish Professor Per-Ingvar Brånemark that titanium can be successfully fused into bone when osteoblasts grow on and into the rough surface of the implanted titanium, forming a direct structural and functional connection between the living bone and the implant (Linder *et al.*, 1983). One group who derived much benefit from the application of osseointegration are fully and partially edentulous patients who regain oral function from new dental implant restorations (Raghoebar *et al.*, 2000).

Endosseous dental implants are titanium screws that are placed within the bone of the jaws and serve as prosthetic anchors to support crowns or bridges or to secure dentures. The bone of the toothbearing region of the jaws forms an intimate interface with the titanium implant surface, firmly holding the implant in place. This form of union between the bone and the implant is termed osseointegration (i.e. the establishment and maintenance of direct bone to implant anchorage). The first report of an osseointegrated dental implant utilised to secure a complete mandibular denture was published in 1977 (Brånemark *et al.*, 1977). Since this time, the practice of dentistry has been transformed and the quality of life of many patients has improved dramatically. Osseointegrated dental implants often enable the optimal restoration of oral function (including eating and speaking), aesthetics and overall well-being of patients in complex cases where previously available treatment approaches were limited or inadequate (Raghoebar *et al.*, 2000). In such instances, dental implants may offer the advantage of being the only predictable way to replace missing teeth with a restoration which is fixed in the mouth, the alternative being a removable denture. The option of a fixed restoration is invaluable,

especially in young patients. Dental implants may also be used to secure a denture which no longer fits in place due to atrophy of the jaw bone following the loss of the patients natural teeth. This is particularly important in the mandible where an elderly patient can frequently no longer wear a complete lower denture during eating or speaking as it moves around the mouth and is uncomfortable and embarrassing. For these patients, two or more implants placed in the previous tooth bearing area of the mandible can secure the denture in place so that the patient may eat, speak and smile without being concerned or uncomfortable (Raghoebar *et al.,* 2000). The patient can remove the prosthesis at night time in the privacy of their own home to facilitate cleaning of the implants and the denture. The stabilisation and retention of a mandibular complete over-denture in the atrophic mandible has been observed to dramatically improve the quality of life of patients who previously experienced great difficulties in coping with a complete lower denture (Feine *et al.,* 1994). Dental implants are also commonly employed to replace missing teeth in simpler cases where they offer advantages over other types of prosthetic restorations such as a fixed (Nevins & Langer *et al.,* 1991) or removable partial denture, which are dependent on the structural integrity and periodontal support of the adjacent natural tooth abutments.

The great contribution of dental implants to dentistry is highlighted by the ever increasing demand for them and over 700,000 implants are placed every year in the USA alone. Just as the supporting structures of the natural teeth can become damaged due to chronic infection and inflammation, so too can the supporting structures of dental implants. Such damage is caused by peri-implant infections, which are sub-divided into peri-implant mucositis and peri-implantitis (Albrektsson & Isidor, 1994). Peri-implant mucositis refers to a reversible inflammatory change of the peri-implant soft-tissues without the loss of the implant supporting bone, whereas peri-implantitis is an inflammatory process affecting the peri-implant tissues which results in loss of the implant supporting bone (Albrektsson & Isidor, 1994).

1.3 Problems associated with peri-implant infections

Peri-implantitis results in the recession and apical shift of the supporting bone and overlying soft-tissues that may result in display of the implant margin leading to aesthetic and psychosocial problems (Yeung *et al.,* 2008). In addition, a decrease in the mechanical stability of the fixture may result in advanced peri-implantitis around short implants (Esposito *et al.,* 2008). Furthermore, peri-implantitis may compromise the long-term

prognosis for the implant restoration. Pjetursson *et al.* (2012) noted in a group of 70 patients with 165 implants placed for a mean period of 7.9 years, that 4.2% of implants experienced a loss of osseointegration due to peri-implantitis. Also, there is no direct evidence of the influence of chronic infection in the peri-implant tissues on patients' systemic health.

However, the systemic impact of periodontopathogens has been elucidated in studies of periodontitis. A statistically significant increase in the risk of atherosclerosis (Desvarieux *et al.,* 2005), myocardial infarction and cardiovascular events (Pussinen *et al.,* 2004) has been reported for patients with periodontitis compared to periodontally healthy controls. Following meta-analyses of the available human data (Meurman *et al.,* 2004), an increased risk of cardiovascular disease and stroke amongst periodontitis patients was noted when compared to periodontally healthy controls. In a study of 628 Pima Indians over an 11-year period, the mortality rate from cardio-renal disease was observed to be 3.5 times higher in diabetic patients with severe periodontitis compared to periodontally healthy diabetic controls (Saremi *et al.,* 2005). Considerable evidence is available linking poor maternal periodontal health and adverse pregnancy outcomes, including preterm birth (Offenbacher *et al.,* 1996). Michalovicz *et al.* (2006) undertook a study of 823 women who received scaling and root planning between 13 and 21 weeks of pregnancy or after delivery. However, the treatment was not observed to significantly influence the pregnancy outcomes of delivery prior to 37 weeks or live preterm birth ($p<0.05$). It is interesting to note that the treatment group had significantly fewer spontaneous abortions than the control group of patients who did not receive treatment ($p=0.08$). However, definitive evidence in terms of improved pregnancy outcomes for patients who receive periodontal treatment are awaited (Michalovicz *et al.,* 2006).

Evidence linking sub-gingival peri-implant biofilms and systemic complications is currently not available. There is no reason to assume that a peri-implant infection is any less detrimental to the host systemic system than periodontitis, in fact quite the contrary. Furst *et al.* (2007) reported an increased prevalence of *Staphylococcus aureus* in periodontal sites following placement of dental implants. *Staphylococcus aureus* can express a diverse array of virulence factors and this organism can cause a wide range of infections and disease syndromes.

The microorganisms associated with endosseous dental implants are often similar to those of the periodontal micro-flora, however, differences have been observed between the composition of the micro-flora of endosseous dental implants and those of

the natural teeth. The extent of this diversity is not yet understood as many microorganisms often found in the oral cavity have not yet been specifically investigated in peri-implant infections.

To the authors' knowledge, the systemic impact of peri-implant infections has not been specifically investigated. From studies of infections of the periodontal tissues, it has been demonstrated that the microorganisms which colonise the pathological periodontal pocket are often found in the blood stream of patients (bacteraemia) and at remote sites from the oral cavity such as within the heart valves or kidneys (Paquette *et al.,* 2007). Also, an upregulation of inflammatory markers throughout the body generally has been observed in patients with active periodontal infection (Paquette *et al.,* 2007). The peri-implant tissues are similar in many ways to the periodontal tissues (Lang & Berglundh, 2011) and it is to be expected that microorganisms from the plaques around endosseous dental implants are also translocated within the blood stream to distant sites where they may mediate adverse affects. It may therefore be hypothesised that the inflammation of the peri-implant tissues may lead to an upregulation of systemic inflammatory markers. The first step in understanding the importance of peri-implant infections on the health of the patient groupings generally is to have evidence as to which microorganisms are present within peri-implant plaques.

By understanding the microorganisms that are present in peri-implant biofilms, one can begin to understand the specific risks to patients' overall medical well-being. Certain microorganisms (e.g. *S. aureus*) that appear to be associated with peri-implant infections (Furst *et al.,* 2007) can express many virulence factors and therefore theoretically pose a systemic risk to the patient. Furthermore, it is of priority to identify which microorganisms are associated with peri-implant infections and which treatment approaches are most effective in the management of peri-implant infections so that one may have the knowledge to make informed treatment decisions and treat peri-implant infections.

As the extent of the problems of peri-implant infections become more evident, more and more emphasis in the dental literature is being placed on the restoration and maintenance of peri-implant health. An Ovid Med-Line search reveals that, up to 1987, there were no articles published in the dental literature using the search term "peri-implantitis or peri-implant mucositis or peri-implant infections". By 1997, there were 58 articles published on peri-implantitis, by 2007, the number of articles published on peri-implant infections had risen to 296 and in September 2012 a total of 662 related articles

were retrieved. This upward trend is expected to continue reflecting the growing interest of the dental community in the topic of peri-implant infections, the difficulties in managing diseases of the peri-implant tissues and the expected increase in treatment requirements for this patient group.

1.4 Peri-implant biofilm formation

Following the introduction of a prosthesis to the oral environment, a biofilm rapidly forms on its surface (Teughels *et al.,* 2011). Biofilms consist predominantly of a highly hydrated exopolysaccharide matrix in which single microbial cells (mostly bacteria) and microcolonies occur heterogeneously interspersed with pores and channels. Biofilms form on all surfaces within an aqueous environment such as the oral cavity (Teughels *et al.,* 2004). Initial bacterial adhesion to prosthetic surfaces is a result of biochemical and physic-chemical forces (Teughels *et al.,* 2011). Initial colonisers provide a pellicle for subsequent colonisers to adhere to (Rosan & Lamont, 2000).

The structure of oral biofilms is such that the outer layers may be influenced by the patient's host cellular and humoral immune defences. Similarly, pharmacological chemotherapeutic agents, disinfectants and antibiotics may only interfere with those microorganisms and their products to which they come into direct contact. Thus, the deeper layers of a biofilm remain relatively undisturbed despite host immune defences, the use of mouth wash disinfectants or administration of systemic or topical antibiotics (Socransky & Haffajee, 2002). The transfer of genes encoding specific virulence factors from one microbial species to another within the biofilm is possible, although such mobile genetic (e.g. bacteriophages, plasmids and tranposons) elements usually only transfer between the same or closely related species (Teughels *et al.,* 2011). Over time, the biofilms mature and may accommodate a more diverse range of microorganisms (Socransky & Haffajee, 2002) To date, much of the literature has focused on bacterial species and little attention has been paid to oral yeasts (Maguire *et al.,* 2008). Traditional techniques employed to investigate the composition of the sub-gingival biofilm in health and disease, include both light microscopy and culture techniques (under aerobic and anaerobic conditions). Thus the microorganisms identified utilising these techniques received greater and greater attention, yet much remains unknown or incompletely understood (Socransky & Haffajee, 2002).

Perhaps there is an inherent bias in continuing to exclusively investigate traditional putative periodontopathogens, in that the microorganisms identifiable by light

microscopy and culture techniques have been limited. It is known that there remains an abundance of microorganisms in the oral environment which have yet to be identified and may be identified using newer techniques (Paster *et al.,* 2006). More than 400 microbial species are reported to exist in subgingival plaque and more than 500 microbial species may be present in the oral environment (Paster *et al.*, 2006). Aas *et al.* (2005) commented that *"the colonisation patterns and bacterial presence around teeth appear to depend on many microorganisms previously not considered in studies".* The microbial flora of peri-implantitis is poorly understood (Furst *et al.,* 2007). Implants demonstrating signs of peri-implantitis demonstrate larger amounts of plaque with increased proportions of Gram-negative anaerobic and facultative anaerobic species compared to that seen in health (Leonhardt *et al.,* 1999). The microflora of peri-implantitis is thought to be similar to that of periodontitis (Lang & Berglundh, 2011).

However, differences may exist between the way microorganisms colonise tooth surfaces and how they colonise titanium implants. When microorganisms previously not considered in oral microbiology studies were investigated in the new peri-implant biofilm by Furst *et al.* (2007), new aspects of the colonisation patterns emerged. *Staphylococcus aureus* is known to have the ability to attach to almost any type of titanium surface (Stenstrom *et al.,* 2009). Up to 15% of implant sites, 12 weeks post insertion of implants, were positive for *S. aureus* (defined by a threshold value of $>1x10^5$ microorganisms) (Furst *et al.,* 2007). Also, it was noted that the peri-implant microflora immediately post-implant insertion is similar to the peri-implant microflora detected 12 weeks post-implant insertion. Furst *et al.* (2007) recommended that the early colonisation pattern of bacteria be investigated in relation to its influence on the future development of peri-implant infections.

There appears to be similarities in the microbiological profiles of periodontitis and peri-implantitis. Differences have been detected between the microbiota of fully and partially edentulous patients. For dentate patients, the periodontopathogens of neighbouring teeth have been observed to colonise implant pockets and three- and six-month data investigating 13 bacterium species appear to be similar (Mombelli *et al.*, 1995).

The risk factors for peri-implantitis and/or implant loss are not yet clear. Whilst patients who have a demonstrated susceptibility to periodontal disease may be treated successfully with osseointegrated dental implants (Nevins & Langer, 1995), controversy remains as to whether this group of patients shows a decreased success rate in the longer

term compared to periodontally healthy patients and whether these patients have an increased requirement for periodontal care. Pathogenic mechanisms proposed include the colonisation of implant surfaces by bacteria from the plaque of neighbouring periodontally compromised teeth which induce peri-implantitis and/or that the host response to the bacterial challenge may mediate the inflammatory destruction of implant supporting tissues (Karoussis *et al.*, 2003).

1.5 The periodontal tissues in health

The periodontal tissues are those that support the tooth and include the gingivae, periodontal ligament, cementum of the tooth's root surface and alveolar bone (Lindhe *et al.*, 2003) (Figure 1.3). The gingival epithelium is divided into the oral epithelium, sulcular epithelium and junctional epithelium. The oral epithelium extends from the gingival margin to the mucogingival junction and is divided into the free gingival margin and the attached gingivae. The attached gingivae describes the oral mucosa between the free gingival margin and the alveolar mucosa. Where the attached gingivae meets the alveolar mucosa is the mucogingival junction, where the pink gingival tissues meet the brighter red alveolar mucosa (Egelberg & Badersten, 2004). The sulcular epithelium is the epithelium which lines the gingival crevice and faces the tooth surface. Below the sulcular epithelium is found the junctional epithelium, which attaches the soft tissues to the tooth root surface (Egelberg & Badersten, 2004).

The oral epithelium is stratified, squamous epithelium and is keratinised. The sulcular epithelium is also stratified squamous epithelium but is non-keratinised and is made up of fewer cell layers than the oral epithelium. The junctional epithelium is non-keratinised and is thinner, being 15-30 cells thick coronally and only a few cell layers thick apically. The junctional epithelium has large intercellular spaces to facilitate the movement of gingival crevicular fluid and immune cells, in particular polymorphonuclear leukocytes. Below the epithelium is found the connective tissues, which consist of: a dense network of collagen fibres; blood vessels; lymphatic vessels; nerves; immune cells and ground substance. The cell of the connective tissues is the fibroblast, which produces both the collagen fibres and the ground substance (Lindhe *et al.*, 2003).

The periodontal ligament ensheaths the entire root surface and is approximately 0.2-0.5 mm in width. The periodontal ligament is made up of bundles of collagen fibres of varying orientation, which extend through the alveolar bone and into the cementum of the root surface. The cementum is deposited directly on the dentine of the root and varies

in thickness between 0.1-1 mm (Lindhe *et al.,* 2003). The cementum is made up of calcium and phosphate crystals formed by the cementoblast cells. Two distinct layers are noted: a) acellular cementum, which is formed during tooth development and b) cellular cementum, where cementoblasts have been engulfed in the cementum they secrete (Lindhe *et al.,* 2003). The cementum acts to tether the periodontal ligament fibres to the root surface. The alveolar bone is made up of thick compact bone, lining the root socket and the outer layer of the buccal/lingual plate, and cancellous bone, between the compact bone plates, being made up of bony trabeculae and bone marrow. The periodontal blood supply consists of large vessels that gradually branch into smaller and smaller vascular networks with interconnections (anastomoses) to each part of the periodontium. The periodontal ligament is nourished by these vessels. Adjacent to the junctional epithelium, there is a network of peripheral blood vessels known as the dento-gingival plexus (Egelberg, 1966).

Clinically, in health, the gingival tissues are pink and may be pigmented. The gingival surface is stippled and the tonus firm. The gingival margin is thin and the depth of the gingival sulcus is shallow, being 3 mm or less. No bleeding is noted on gentle probing, as in health there is little fragility of the vascular tissues (Lindhe *et al.,* 2003).

The periodontal tissues are embryologically derived from the epithelial mesenchymal interactions in the region of the developing tooth root. Epithelial cells of Hertwig's root sheath proliferate into the ectomesenchyme in an apical direction from the dental organ in an outer and an inner layer (Lindhe *et al.,* 2003). The inner layer induces the surrounding ectomesenchyme to form odontoblasts and dentine of the root. When the dentine is formed, the outer layer of the epithelial root sheath becomes fragmented and ectomesenchmal cells of the dental follicle grow through the fenestrations, contact the dentine surface and begin to form cementum (Lindhe *et al.,* 2003). The remaining portions of the periodontium are formed by ectomesenchymal cells from the dental follicle lateral to the cementum, which differentiate into fibroblasts, osteoblasts and cementoblasts, forming periodontal ligament, alveolar bone and cementum respectively (Egelberg & Badersten, 2004).

1.6 The anatomy of the peri-implant tissues

The peri-implant tissues arise as a result of the healing process following implant surgery. The peri-implant soft tissues consist of oral epithelium and connective tissue and alveolar bone. The outer surface of the peri-implant mucosa is made up of stratified, squamous

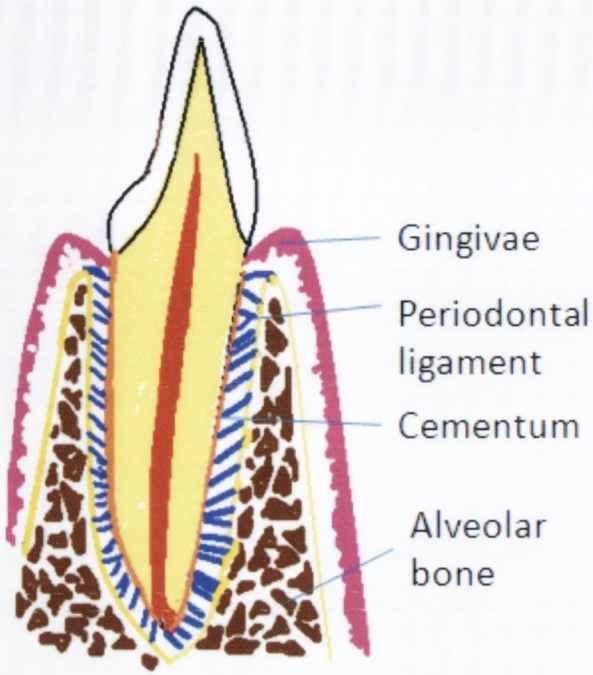

Figure 1.3 Anatomy of the periodontal tissues. The tissues of the periodontium consist of the cementum, the periodontal ligament, the alveolar bone and the gingivae.

epithelium that is keratinised and is continuous with the barrier epithelium facing the implant surface. The oral epithelium facing the implant surface is described as the barrier epithelium and is about 2 mm long and attached at its apical extent to the implant surface by hemi-desmosomes (Gould *et al.* 1984). The barrier epithelium is stratified, squamous epithelium and is not keratinized, being just a few cells thick and stops approximately 1-1.5 mm from the alveolar crest. A connective tissue zone, in direct contact with the titanium dioxide surface of the implant, directly overlies the alveolar bone and is continuous with the barrier epithelium (Liljenberg *et al.*, 1996a). The barrier epithelium and the connective tissue zone is described as the trans-mucosal attachment and consists of approximately 2 mm of barrier epithelium and a zone of connective tissue attachment of approximately 1-1.5 mm in depth (Liljenberg *et al.*, 1996). The peri-implant connective tissues consist of collagen (67%-85%), vascular tissues (0.3%) and fibroblasts (11%-32%) (Gualini & Berglundh, 2003; Berglundh *et al.*, 2004). Collagen fibres, some of which originate from the periosteum of the bone crest, run largely parallel to the implant surface (Buser *et al.*, 1992). The supra-periosteal blood vessels provide the blood supply to the peri-implant soft tissues, contributing to the capillaries of the connective tissues underlying the oral epithelium and the vascular plexus underlying the junctional epithelium. The connective tissue of the trans-mucosal attachment has been observed to contain relatively few vessels, all of which arise from the supra-periosteal blood supply (Abrahamsson *et al.*, 2002).

1.7 Comparison between the peri-implant and periodontal tissues

Anatomical differences have been observed between peri-implant and periodontal tissues (please see Table 1.1). Whilst the periodontal tissues are embryologically derived, the peri-implant mucosa structure and function is as a result of the healing process. Whilst the eruption of a tooth leads to a seal between living tissues, the healing process of the peri-implant tissues result in the attachment of living tissues to a foreign body. The coronal soft tissue seal of the peri-implant tissues is made up both of epithelium and connective tissues. The epithelium from the margins of the incision grows over the post surgical granulation tissues in the regions of a dental implant and upon reaching the surface of the dental implant moves in an apico-coronal direction to seal the implant with a 2 mm long "new junctional epithelium" (Listgarden *et al.*, 1996). The epithelial differentiation is influenced by the signals coming from the underlying connective tissues. Attachment of the epithelial cells to the implant surface results from the basal lamina and the formation

of hemi-desmosomes (Kawahara *et al.*, 1998). The structure and function of the peri-implant's new junctional epithelium has been suggested to be similar to the mechanical, structural and ultra-structural characteristics of the periodontal junctional epithelium (Liljenberg *et al.*, 1996). However, an increase in the collagen to fibroblast ratio and a decrease in the vascularity in peri-implant "interfacial" tissues has been noted in comparison to the periodontal tissues (Lang & Berglundh, 2011). Furthermore, the arrangement of the peri-implant supra-crestal connective tissue fibres is in a predominantly circular orientation parallel and/or vertical to the long axis of the implant abutment (Buser *et al.*, 1992). This differs significantly from that of the periodontal supra-crestal connective tissue fibres. The observed anatomical differences are thought to influence the onset and progression of peri-implant infections. As there is no evidence for structural or functional differences between the periodontal and peri-implant junctional epithelia, it has been proposed that the epithelial sealing of implants may be identical to that of teeth (Lang & Berglundh, 2011). Further evidence is required to contrast the physiological properties of the periodontal and peri-implant junctional epithelia.

The connective tissues between the alveolar bone and the junctional epithelium are in direct contact to the implant surface and are divided into two zones. The inner zone, which is in direct contact with the implant surface, 50-100 μm in width, has been observed to be rich in blood vessels and is thought to appear as scar tissue (Buser *et al.*, 1992). The outer layer is richer in cells and blood vessels (Buser *et al.*, 1992). In an animal-based model, the healthy peri-implant mucosa has been observed to contain more collagen and less immune cells than the gingival connective tissues in health (Berglundh *et al.*, 1991). However, an inflammatory infiltrate has been observed to be present lateral to the sulcular and junctional epithelium of both clinically healthy peri-implant mucosal connective tissue and gingival tissues (Tonetti *et al.*, 2005). Furthermore, there are differences between the microflora and host mediated defence mechanisms of peri-implant tissues and those of periodontitis. Histological examination of the periodontal and peri-implant tissues have revealed important differences, namely:

1. The periodontal ligament space and periodontal fibres are found between the natural tooth root surface and the alveolar bone, whereas a one or two cell layer is interposed between the alveolar bone and the implant surface (Berglundh *et al.*, 1991).
2. The gingival and periodontal connective tissue fibres of the natural tooth are orientated differently to those of the peri-implant tissues (Berglundh *et al.*, 1991).

Table 1.1 Similarities and differences between periodontal and peri-implant tissues.

	Periodontal tissues	**Peri-implant tissues**
Origin	Embryologically derived	Healing of oral epithelium following surgery
Collagen to fibroblast ratio	Less in health than peri-implant tissues	Greater in health than periodontal tissues
Vascularity	Greater in health than peri-implant tissues	Less in health than periodontal tissues
Junctional epithelium	Similar	Similar
Supra-crestal connective tissue fibres	Orientation perpendicular to tooth/root surface	Circular orientation parallel to the implant surface
Inflammatory infiltrate	Present in health	Present in health

3. The junctional epithelium of the periodontal tissues is embryologically derived from the reduced enamel epithelium of the tooth germ (Lindhe *et al.,* 2003). The peri-implant epithelium is derived from the oral epithelium (Gould *et al.,* 1984).

As structure and function are intimately related, similarities and differences are therefore likely in the defence mechanisms of the periodontal and peri-implant tissues. As to whether such differences are of clinical importance is not known until further evidence is available comparing physiological and pathological mechanisms of the different tissue types.

The junctional epithelium merits further discussion as it is a unique structure that changes in structure so dramatically in disease and is then described as the periodontal pocket epithelium. Therefore, the differences in the structure of the peri-implant junctional epithelium may by comparison influence health, disease and healing mechanisms of the peri-implant tissues.

The peri-implant soft tissues structure results from the healing processes in the alveolar mucosa following implant placement. A soft tissue seal is established consisting of an epithelial and connective tissue zones and is approximately 3 mm in corono-apical length. However, the new peri-implant epithelium is phenotypically different to that of the alveolar mucosa. It has been hypothesised that as the alveolar mucosa proliferates apically along the implant surfaces, the stimuli coming from the underlying connective tissue affects the phenotypical expression of the oral epithelium (MacKenzie & Hill, 1984). The epithelial cells attach to the implant surface directly via hemi-desmosomes (James & Schultz, 1974). Human histological studies have indicated that the peri-implant epithelium is similar to the gingival epithelium with regard to patterns of differentiation and function. It is recognised that the epithelium lining the peri-implant sulcus share many structural, ultra-structural and functional characteristics with the corresponding gingival tissue (Carmichael *et al.,* 1991; Tonetti *et al.,* 1995; Lilgenberg *et al.,* 1996).

Whilst the junctional epithelium of the periodontal tissues is considered a unique anatomical structure, due to its very high rate of cell turnover and adaptability to protect against microbial stimuli, the gingival connective tissue and the peri-implant crevicular fluid composition and volume were observed to be similar in 31 partially dentate implant patients (Adonogianaki *et al.,* 1995). The epithelial tissues that form the junctional epithelium of the periodontium originate from the remnants of the epithelial root sheath of Hertzwig, whilst the peri-implant junctional epithelium is derived from the oral mucosa. It is hypothesised that the underlying connective tissues may influence the

phenotype of the overlying epithelial cells, resulting in histological changes in the oral mucosa as it encounters the peri-implant connective tissues. The influence of the underlying connective tissues on the epithelium may be elucidated by considering the changes in the gingival epithelium and oral mucosa following the placement of a connective tissue graft in the treatment of a gingival recession lesion. A soft tissue connective tissue graft is harvested from the palate and placed in a prepared recipient bed in the mucogingival region of a mandibular incisor tooth. A successful outcome of the surgery results in the soft tissues in the mandibular incisor region being thicker, a gain in the clinical attachment level. The goal is to help prevent the progression of a recession lesion and the transplanted connective tissues of the palate result in an increase in the durability of the overlying labial gingivae and mucosa in the recipient site (Lang *et al.*, 1990).

1.8 Periodontal tissues in disease

The cause and effect relationship between plaque and gingivitis was established in a classic proof of principle study by Loe *et al.* (1965). Following abstinence of home oral hygiene care, the previously healthy gingival tissues of subjects was observed to become inflamed. Following professional removal of the plaque deposits, a resolution of the inflammation was noted. The process of the development of gingivitis has been observed from histopathological studies (Page & Schroeder, 1976) (Figure 1.4 (a-d), Figure 1.5). Following the accumulation of plaque for several days, inflammation of the gingival tissues is noted and gingivitis diagnosed. Persistence of the inflammation may result in apical migration of the junctional epithelium, an increase in the lymphocyte and plasma cell constituent of the inflammatory infiltrate and eventually periodontal bone loss.

In health, the sulcular and junctional epithelium is thin and demonstrates an even connection with the underlying connective tissues. Immune cells are present but are relatively few in number. Few vascular loops are present in the connective tissues underlying the sulcular and junctional epithelium. The probing depth is shallow and there is an absence of bleeding on probing (Egelberg & Badersten, 2004). Following the accumulation of plaque along the gingival margin and into the sulcus, an increase in the numbers of polymorphonuclear leukocytes in the sulcus and epithelium and connective tissues is noted (Page & Schroeder, 1976). The sulcular and gingival epithelium increase in thickness and downwards projections into the connective tissues (known as rete pegs) form. The ordered blood vessels seen in healthy gingivae are replaced with a vascular bed

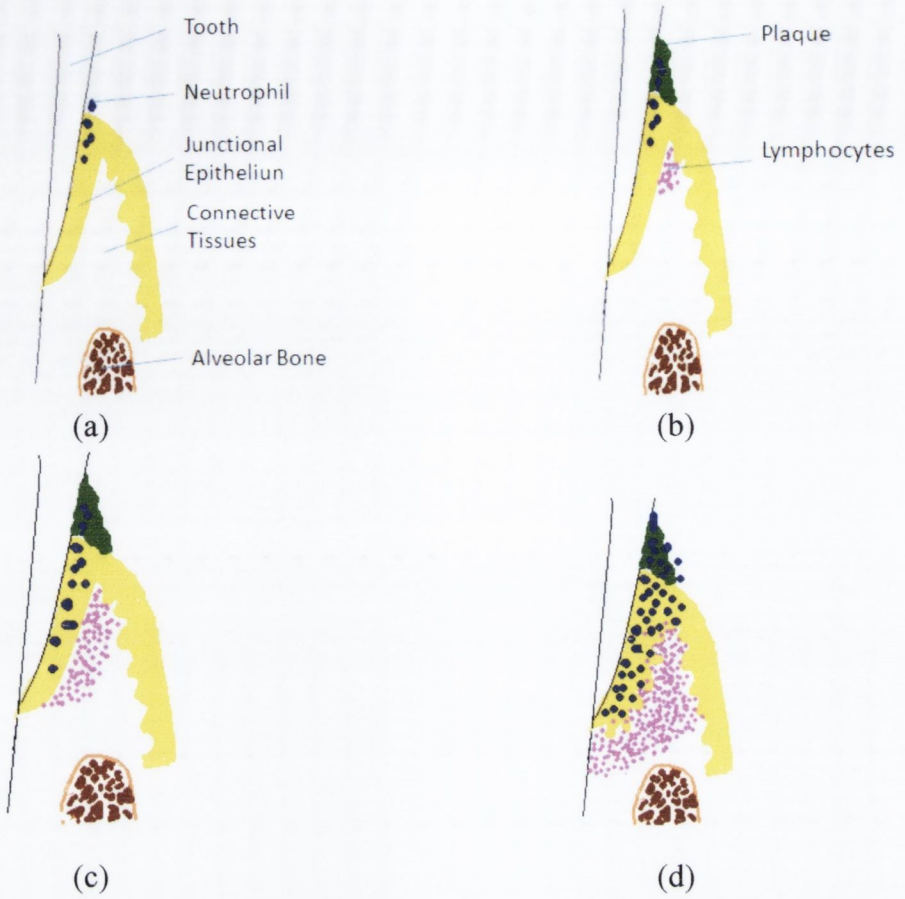

Figure 1.4 Diagrammatic representiation of periodontal tissues in health and disease adapted from Lindhe *et al.* (2003). Panel (a) shows healthy periodontal tissues that are not inflamed and continuous sparse movement of neutrophils takes place from the periodontal connective tissues to the coronal part of the junctional epithelium and the gingival sulcus. Panel (b) shows periodontal tissues in gingivitis (early lesion). In response to microbial colonisation at the gingival sulcus, the periodontal tissues become infiltrated with monocytes/macrophages, lymphoctes and neutrophils. Panel (c) shows the periodontal tissues in gingivitis (established lesion): Increased neutrophil migration and emigration and proliferation of the junctional epithelium. The inflammatory infiltrate increases in size with greater numbers of neutrophils, lymphocytes and monocytes/macrophages. Blood vessels increase in number and collagen is broken down. Few plasma cells are present. Panel (d) shows greatly increased leukocyte infiltrate with plasma cells making up 10-30% of the inflammatory infiltrate. Also, there is a greatly increased neutrophil migration to the gingival sulcus and neutrophil emigration.

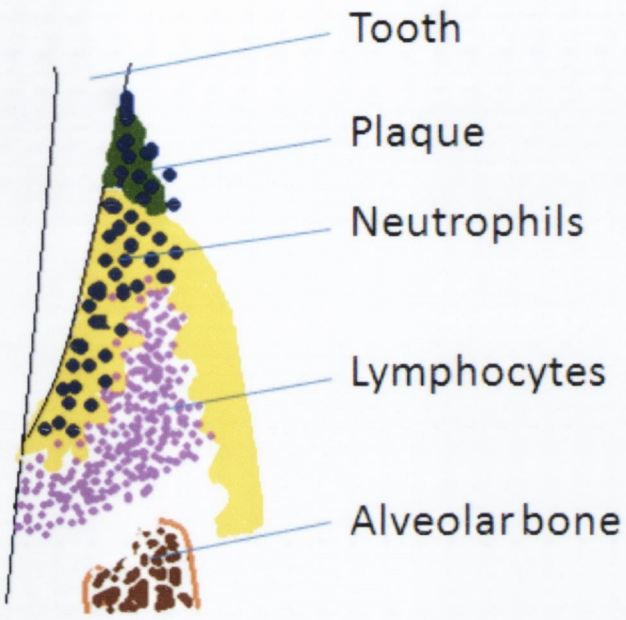

Figure 1.5 The periodontal tissues in periodontitis: a diagrammatic representation adapted from Lindhe *et al.* (2003). Apical migration of the junctional epithelium, increased lymphocyte and plasma cell constituent of the inflammatory infiltrate, periodontal bone loss.

with loop-like formations (Egelberg, 1966). A breakdown of the collagen fibres of the connective tissues is noted and an increase in the vascularity of the connective tissues takes place. The probing depth increases slightly and bleeding on probing is noted (Egelberg & Badersten, 2004). With further accumulation of plaque, a decreased adaptation of the marginal gingivae to the tooth is noted, allowing for an increase in the plaque present below the gingival margin leading to subgingival colonisation. The plaque masses coalesce and are calcified to form subgingival calculus (Lindhe *et al.*, 2003). The sulcular and junctional epithelium increase further in thickness with an increase in intercellular spaces. Epithelial rete-pegs increase in number and penetrate more deeply into the connective tissues. Polymorphonuclear leukocytes and immune cells increase in number (Page & Schroeder, 1976). Further breakdown of the collagen fibres and increase in vascularity of the connective tissues is noted. Deeper probing depths are noted and bleeding on probing is elicited more easily (Egelberg & Badersten, 2004). With continued down-growth of the plaque subgingivally, the epithelial tissues often further distend and the junctional epithelium proliferates along the root surface and becomes ulcerated. The connective tissue degrades further and an increase in vascularity and a greater density of immune cells is observed. A breakdown of marginal alveolar bone is also noted. The probing depth increases and bleeding on probing occurs readily (Egelberg & Badersten, 2004). The periodontal tissues are now referred to as a periodontal pocket (Egelberg & Badersten, 2004). With continued inflammation, initiated and maintained by the persistence of microorganisms within the plaque biofilm, extensive destruction of the periodontal connective tissues and alveolar bone may occur (Page & Schroeder, 1976). The resultant periodontal bone loss may lead to compromised bone support for the tooth and eventual tooth loss (Lindhe *et al.*, 2003).

1.9 Histopathology of peri-implant infections

Histopathological studies have demonstrated that gingivitis and periodontitis lesions share many similarities with peri-implant mucositis and peri-implantitis (Lang & Berglundh, 2011). Important differences have been highlighted between the anatomy of the peri-implant tissues and that of the periodontal tissues and the pathological processes of peri-implant mucositis/ peri-implantitis and gingivitis/periodontitis. However, important similarities have also been noted including:

a) Both peri-implant mucositis/peri-implantitis and gingivitis/periodontitis require plaque as an initiating and stimulating factor for disease progression (Pontoriero *et al.*, 1994; Zitmann *et al.*, 2001).

b) Iatrogenic factors have been shown to influence peri-implant mucositis/ peri-implantitis and gingivitis/periodontitis i.e. access for home care (Serino & Strom 2009; Heitz-Mayfield *et al.*, 2011).

c) Maintenance care has been observed to be important in preventing peri-implant mucositis/ peri-implantitis and gingivitis/periodontitis (Rinke *et al.*, 2010).

d) Both peri-implant mucositis/peri-implantitis and gingivitis/periodontitis share similar constituents of their inflammatory infiltrates - although a differing relative abundance has been recognised (Liljenberg *et al.*, 1996; Gualini & Berglundh, 2003).

e) Both peri-implant mucositis/peri-implantitis and gingivitis/periodontitis have been shown to be influenced by the patient's genetic susceptibility (Laine *et al.*, 2006; Renvert & Persson, 2009).

In a histopathological investigation of 20 partially dentate implant patients comparing the oral alveolar mucosa prior to implant placement and the peri-implant mucosa following implant restoration, Liljenberg *et al.* (1996) noted that prior to implant placement, only scattered inflammatory cells were observed. However, following implant placement and restoration, the healthy peri-implant mucosa demonstrated significantly enhanced numbers of acute inflammatory cells and leukocytes. In a follow up study comparing the histology of periodontal and peri-implant tissues with those of the peri-implant tissues following implant restoration, a greater proportion of B-cells and polymorphonuclear leukocytes were observed in the gingival/periodontal tissues than in the peri-implant mucosa, whilst the proportions of T-cells remained somewhat similar between the two tissue types (Liljenberg *et al.*, 1996). The cause and effect relationship between dental plaque and peri-implant inflammation was demonstrated in a proof of principle study by Pontoriero *et al.* (1994). Following abstinence from oral home care methods (tooth brushing, use of interproximal cleaning aids and mouthwashes) and professional preventative measures (root debridement, scaling and polishing), inflammation was noted in the previously healthy peri-implant tissues and peri-implant mucositis diagnosed. Following the re-institution of professional preventative measures and oral home care measures, the inflammation resolved and peri-implant health was noted. This study

followed the methodology of the "experimental gingivitis" study of Loe *et al.* (1965) and has been termed "*the experimental peri-implant mucositis*" study.

Zitmann *et al.* (2001) investigated the influence of the accumulation of *de novo* plaque formation at teeth and implants in 12 partially dentate patients and noted that the size of the inflammatory cell infiltrate and the proportion of the cells in the infiltrated connective tissue increased at both teeth and implants during the observation period, indicating that the peri-implant tissues do respond to the presence of plaque in a similar manner as the periodontal tissues. Gualini & Berglundh (2003), in a study of 16 human histopathological specimens from 10 patients restored with Brånemark implants for at least five years, noted that peri-implant mucositis sites demonstrated a well defined inflammatory infiltrate in the connective tissues lateral to the barrier epithelium, whilst peri-implantitis sites were observed to have three times greater infiltrated connective tissue with frequent ulcerated pocket epithelium present. T and B cells were not noted to differ markedly in their distribution between peri-implantitis and peri-implant mucositis lesions. However, elastase positive cells and B cells were observed to be significantly elevated in peri-implantitis lesions compared to peri-implant mucositis lesions ($p<0.05$). Also, elastase positive cells were observed to be more diffuse throughout the lesion, especially towards the centre of the infiltrated connective tissue lesion, when compared to the peri-implant mucositis lesion, where these cells were observed to be predominately peripheral, being adjacent to the barrier epithelium. Gualini & Berglundh (2003) hypothesised that the large numbers of B-cells contributed to the aggressiveness and rapid progression of the peri-implant lesions.

However, differences have been noted between periodontal and peri-implant infections. The cellular composition and pattern of cytokine expression has been shown to differ substantially from experimental peri-implant mucositis and experimental gingivitis (Salvi *et al.,* 2012). Matrix metalloproteinase-8 (MMP-8) concentrations were observed to be significantly higher in peri-implant mucositis, than in gingivitis ($p=0.012$). From histopathological observations, peri-implant mucositis and gingivitis early lesions share similarities at three weeks. However, when lesions are present for extended periods, it has been observed that peri-implant lesions are larger, and in direct contact with the underlying alveolar bone and differ in the composition of host defence cells compared to periodontitis lesions (Lang & Berglundh, 2011). The observed differences between peri-implant and periodontal lesions may be understood in light of the differences in the

developmental origin of the cells of the peri-implant and periodontal tissues and the structural differences that exist between them (Sadler, 1985).

It has been observed that peri-implantitis lesions demonstrated a cellular infiltrate more consistent with that of acute inflammation: neutrophils and macrophages have been observed to be present in greater proportions in peri-implantitis lesions than in periodontitis lesions. However, in periodontitis, the cellular infiltrate is predominantly one of chronic inflammation. It is important to note that plasma cells and lymphocytes have been observed to make up the largest constituent of both peri-implantitis and periodontitis cellular infiltrates (Lang & Berglundh, 2011). In a similar manner to gingivitis, peri-implant mucositis is reversible (Lang & Berglundh, 2011) and this has been demonstrated clinically (Pontotiero *et al.,* 1994) and on a molecular level (Salvi *et al.,* 2011) utilising an experimental peri-implant mucositis model. Salvi *et al.* (2011) undertook a clinical study examining 15 patients, employing checkerboard DNA-DNA hybridisation to examine the subgingival microflora for 40 species. That study utilised the mucosal crevicular fluid enzyme linked immunosorbent assay for matrix-metalloproteinase-8 (MMP8) and interleukin-1beta (IL-1beta), prior to and following the abstinence from home or professional oral hygiene for three weeks and subsequently following three weeks of optimal home care. MMP-8 was observed to be significantly elevated in peri-implant sites following three weeks of abstinence from oral hygiene ($p<0.05$), demonstrating the cause and effect relationship between bacterial plaque and peri-implant mucosal inflammation. It is interesting to note that the clinical parameters, as determined by the gingival index, did not recover completely at peri-implant sites compared to periodontal sites following the institution of optimal oral hygiene. The authors suggest that this may indicate that a longer healing period may be necessary for peri-implant tissues compared to the periodontal tissues following treatment.

The major difference between peri-implant mucositis and peri-implantitis is the loss of peri-implant bone beyond that which would be expected as a part of the normal healing of the peri-implant bone, which is referred to as post-surgical bone remodelling. Lang & Berglundh (2011) hypothesised that the progression of peri-implant mucositis to peri-implantitis takes place more rapidly than that of gingivitis to periodontitis, based on the prevalence of peri-implantitis and the prevalence and progression rate of periodontitis from longitudinal studies. The histological findings would also appear to fit this hypothesis. For example, the inflammatory infiltrate of peri-implant lesions has been noted to be larger than the inflammatory lesions of periodontitis (Lindhe *et al.* 1992;

Gualini & Berglundh, 2003; Piattelli *et al.,* 1998; Sanz *et al.,* 1991), occupying up to 65% of the connective tissues (Sanz *et al.,* 1991). Furthermore, the peri-implant inflammatory infiltrate has been noted to be in direct contact with the alveolar bone (Lindhe *et al.,* 1992) in contrast to the periodontitis inflammatory infiltrate where a connective tissue capsule is interposed.

It is also thought that differing anatomical features of peri-implant tissues compared to the periodontal tissues, including the circular arrangement of the supra-crestal connective tissue fibres and the absence of a periodontal ligament, may facilitate the more rapid lateral spread of the peri-implantitis lesion determining the morphology of the peri-implant bone defect (Lang & Berglundh, 2011). Peri-implant bone loss has been observed to usually result in a "four walled circumferential bone defect". Also, it may be hypothesised that presence of microorganisms, such as *S. aureus*, which can express an array of lytic enzymes including hyaluronidase, further contributes to the lateral spread of the peri-implant lesion (Furst *et al.,* 2007; Heitz-Mayfield & Lang, 2010). Hyaluronidase acts to catalyse the hydrolysis of hyaluronan, a constituent of the extracellular matrix, thereby increasing tissue permeability and facilitating spread of the peri-implant infection.

In peri-implantitis, B-cells and elastase positive cells have been observed to occupy a significantly greater proportion of the inflammatory infiltrate, compared to the lesions of peri-implant mucositis. As to whether this difference relates to a different manner of response of the immune system in patients demonstrating bone loss and therefore a susceptibility to peri-implantitis, or a different period of exposure and hence an established lesion with a mature secondary response, remains to be investigated. Inflammatory mediators result in the recruitment and activation of osteoclasts resulting in alveolar bone loss. The specific mechanisms influencing the recruitment and activation of osteoclasts in peri-implantitis and periodontitis have not yet been compared (Lang & Berglundh, 2011).

Ligature induced infections are undertaken in the animal model through placing a ligature around the neck of a tooth or implant in a sub-gingival or sub-mucosal position and allowing time for plaque accumulation. It has been stated that ligature induced peri-implant and periodontal infections share many common features, namely the size of the lesion and the composition of the inflammatory infiltrate, with those of the corresponding clinical infections (Lang *et al.,* 1993, Lang & Berglundh, 2011), making animal studies of

histopathology and treatment approaches relevant to the clinical scenarios observed in peri-implant infections.

Histopathological studies have demonstrated that the peri-implant pocket epithelium is ulcerated and that there is a separation in the adhesion between the connective tissues. Therefore, the translocation of microorganisms from the peri-implant pocket to the tissues and circulation is likely and some authors have suggested that bacterial invasion may occur (Lang & Berglundh, 2011).

1.10 The microbiology of the peri-implant niche

The microflora of peri-implant and periodontal sites share many common species (Pontotiero *et al.*, 1994; Mombelli *et al.*, 1995). However, the newly exposed peri-implant site is a novel niche with perhaps uniquely different ecological characteristics than the periodontal site (Furst *et al.*, 2007). Differences may exist between the colonisation mechanism of microorganisms on tooth surfaces and that around titanium implants. When microorganisms previously not considered in oral microbiology studies were investigated in the new peri-implant biofilm by Furst *et al.* (2007), new aspects of the colonisation patterns emerged. *Staphylococcus aureus* is known to have the ability to attach to almost any type of titanium surface. Up to 39.1% of implant sites, 12 weeks post-insertion, were positive for *S. aureus* (defined by a threshold value defined at $>1x10^5$ microorganisms) (Furst *et al.*, 2007). Accordingly, differences have been observed between the peri-implant and periodontal microflora (Botero *et al.*, 2005; Furst *et al.*, 2007). However, a similar microflora has been isolated from individual patient's periodontal and peri-implant sites, therefore periodontal and peri-implant infections are thought to be intimately related (Quirynen & Listgarten, 1990; Mombelli *et al.*, 1995; Mengel *et al.*, 1996; Leonhardt *et al.*, 2003b; Botero *et al.*, 2005; Furst *et al.*, 2007; Lang & Berglundh, 2011).

Increased proportions of Gram-negative anaerobic and facultative anaerobic species have been isolated from implants demonstrating signs of peri-implantitis, compared to those seen in health (Rams *et al.*, 1983; Sanz *et al.* 1991; Leonhardt *et al.* 1999; Mombelli *et al.* 2002; Botero *et al.*, 2005) including *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia* (Hultin *et al.*, 2002; Luterbacher *et al.*, 2000; Mengel & Flores-de-Jacoby, 2005). An increased microbial load has been observed at peri-implant sites demonstrating peri-implant infections compared to healthy controls (Mombelli & Lang, 1992; Hultin *et al.*, 2002;

Agerbaek *et al.*, 2006). However, in one study, no difference was observed in the microflora of clinically healthy and implants demonstrating signs of peri-implant infection (Renvert *et al.*, 2007).

Edentulous patients have been observed to harbour less *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* than partially dentate patients (George *et al.*, 1994). It has been observed that the removal of all teeth prior to the commencement of implant treatment can result in *A. actinomycetemcomitans* no longer being isolated from the oral flora (Danser *et al.*, 1995). However, in the peri-implant microflora of 37 patients who had had their teeth extracted for periodontal reasons and had implants placed for five or more years, a re-emergence of the traditional periodontopathogens including *P. gingivalis*, *P. intermedia*, *Prevotella nigrescens* and *A. actinomycetemcomitans* was observed (Leonhardt *et al.*, 1999). *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* have been more frequently isolated in patients with a history of chronic periodontitis or aggressive periodontitis than patients with no history of periodontal disease (Mengel & Flores-de-Jacoby, 2005). Staphylococcal spp. and *Candida* spp. have also been isolated from patients displaying signs of peri-implant infections (Leonhardt *et al.*, 1999).

The establishment of the peri-implant biofilm was investigated by Furst *et al.* (2007) in a clinical investigation of the periodontal and peri-implant microflora of 14 partially dentate patients immediately prior to implant placement surgery, immediately post-implant placement, and at 1, 4, 8 and 12 weeks following the implant placement surgery. Significantly higher counts of *S. gordonii*, *S. oralis*, *S. intermedius*, *Lactobacillus acidophilus* and *Actinomyces naeslundii* (type 1 and 2) were isolated from adjacent tooth periodontal sites at four weeks post-implant placement ($p<0.05$) and *Eikenella corrodens* ($p<0.05$), *Fusobacterium nucleatum polymorphum* ($p<0.05$), *Peptostreptococcus micros* ($p<0.05$) and *P. gingivalis* ($p<0.01$) was found. After 12 weeks, 15 of the 40 species examined were observed to have a higher bacterial load at periodontal sites adjacent to the implant compared to pre-operatively. At one-week post-implant placement, only *Veillonella parvula* ($p<0.05$) differed with higher loads at one week. However, at eight weeks post-implant placement, 32/40 species demonstrated higher loads and at 12 weeks, 29/40 microorganisms demonstrated higher loads including *P. gingivalis* ($p<0.05$), *Tanerella forsythia* ($p<0.01$) and *Treponema denticola* ($p<0.001$). Up to 39.1 % of implant sites, 12 weeks post insertion of implants, were positive for *S. aureus* (defined by a threshold value defined at $>1x10^5$ microorganisms) (Furst *et al.*, 2007). Furst *et al.*

(2007) concluded that *"the sub-mucosal dental implant microbiota is already established at the completion of the (implant placement) surgical procedure..."* and that *"the early colonisation pattern may contribute and be explanatory to the development of peri-implant lesions"*. The early colonisers are thought to remain relatively unchanged.

Scanning electron microscopy and culture studies provided the first evidence of the microbiologic aetiology of peri-implant disease. Rams *et al.* (1983; 1984) noted high levels of spirochetes, fusiform bacteria and coccoid rods in deep peri-implant sites demonstrating signs of bone loss, whereas implants with pockets not greater than 5 mm yielded predominantly coccoid microbiota and very few rods. Almost half of the microorganisms cultivable from the deep sites were gram-negative anaerobic rods including *Fusobacterium spp.* and *P. intermedia*, thereby sharing many similarities with periodontal diseases. These findings were confirmed by other investigators using ceramic implants with peri-implant infections where a large selection of Gram-negative anaerobic rods were present in sites demonstrating signs of peri-implant infections whereas low numbers of microorganisms were isolated from healthy peri-implant sites in the same patients and consisted of mainly gram-positive bacteria (Sanz *et al.,* 1990). The microflora associated with per-implantitis has been observed in most cases to be primarily a mixed anaerobic flora containing *Fusobacterium* spp. and *P. intermedia* in high numbers (Mombelli *et al.,* 2002).

Interestingly, 10 edentulous patients were observed to harbour less *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* than 14 partially dentate patients. Furthermore, implants from which one of the three microorganisms was isolated were at a significantly greater risk for bleeding on probing and demonstrated higher crevicular fluid flow rates, suggestive of inflammation (George *et al.,* 1994). Also, it was observed that in partially dentate patients these three periodontopathogens were often isolated from the peri-implant crevice within 14-28 days following surgical exposure of the dental implant surfaces to the oral environment (Koka *et al.,* 1993). Mombelli *et al.* (1995) observed that the peri-implant microflora of teeth and implants in the same mouth were similar three and six months following surgical exposure of the implant, when 13 microbial species were examined. The treatment concept evolved that it may be better to extract the natural teeth prior to the placement of dental implants in patients with advanced periodontal disease. However, more recently this treatment approach is no longer supported as the evidence from longer observation intervals revealed that the periodontopathogens re-emerge in the microflora of fully edentulous implant patents who have had implants in the

medium term, indicating that it is often not possible to eliminate periodontopathogens completely from the oral flora- even by extracting all of the natural teeth (Lang & Berglundh, 2011). When a suitable ecological niche develops, colonisation and proliferation of pathogenic species may take place in this novel ecological niche (i.e. the peri-implant sulcus). Therefore, the consensus presently is that is not the presence or absence of natural teeth which determines the peri-implant microflora, but the health of the periodontal tissues supporting these natural teeth that is important (Lang & Berglundh 2011). The transmission of microorganisms from the periodontal to the peri-implant niche has been frequently observed and therefore it is important to ensure that the periodontal microflora is predominantly one associated with health.

Quirynen & Listgarten (1990), in a study of 24 partially edentulous implant patients, reported that there were no significant differences in the micro-flora of peri-implant and periodontal sites present within patients, highlighting that the periodontal sites may serve as a reservoir for the bacterial colonisation of titanium implants in the same mouth. Mombelli *et al.* (1995) observed similar distributions of microorganisms when comparing 10 patients with Brånemark implants and 10 with ITI (International Team for Implantology) implants. *Fusobacterium* spp. were isolated from 13 of the 20 implants, whereas *P. gingivalis* was only found in the peri-implant samples of two patients. Interestingly, *A. actinomycetemcomitans* was not isolated from any peri-implant site, and from only one periodontal site, despite all of the patients having had a history of periodontal disease.

Mengel *et al.* (1996) observed in eight periodontitis patients with 36 implants, one year following installation of the implants, no differences between periodontal and peri-implant sites for *A. actinomycetemcomitans*, *P. gingivalis* or *P. intermedia.* Salcetti *et al.* (1997) examined 21 patients with peri-implantitis and eight patients with healthy implants. No significant differences were noted in these patients, between the microbial inflammatory or growth factors mediators when comparing healthy to diseased implant sites. However, greater detection frequencies of *P. nigrescens, P. micros, F. nucleatum ss vincentii,* and *F. nucleatum ss nucleatum*, as well as significant elevations in GCF levels of PGE2, IL-1 beta, and PDGF in mouths with failing implant sites were noted compared to mouths with healthy control implants. The authors concluded that the risk of implant loss appears to be primarily on a patient level.

Leonhardt *et al.* (1999) examined the periodontal and peri-implant microflora of 37 patients who had had their teeth extracted for periodontal reasons and had implants

placed for five or more years. A re-emergence of the traditional periodontopathogens including *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *A.actinomycetemcomitans* was noted. Also, *Staphylococcus epidermidis* and *Candida albicans* were noted in 17% and 10%, respectively, of patients displaying signs of peri-implant infections.

Hultin *et al.* (2000*b*) investigated 15 partially dentate implant patients with implants present for ten or more years. Probing pocket depths were noted to be significantly greater around implants than periodontal sites. No marked differences were noted between periodontal and peri-implant sites. *A. actinomycetemcomitans, P. gingivalis, P. intermedia, B. forsythus,* and *T. denticola*, were frequently detected at implants demonstrating marginal bone loss of ≥2 mm.

Luterbacher *et al.* (2000) observed in 19 patients having a history of moderate to severe periodontal disease that five years following implant placement, the combination of the parameters of bleeding on probing with the detection of either *A. actinomycetemcomitans*, *P. intermedia*, *P. gingivalis* or *T. denticola* resulted in an increased predictability for future peri-implant disease progression as reflected in an increased probing depth or a decrease in the peri-implant bone density. Other investigators have similarly observed that where bleeding on probing was present, pathogenic species were more likely to be isolated (Savitt *et al.,* 1991; Socransky *et al.,* 1991).

Hultin *et al.* (2002) reported on 17 partially dentate implant patients with a total of 98 implants, of which 45 showed marginal bone loss of more than three fixture threads. *Lactoferrin* and *elastase* crevicular fluid concentrations were noted to be higher in peri-implantitis sites than periodontitis sites. Patients with peri-implantitis harboured higher levels of periodontal pathogens including *A. actinomycetemcomitans*, *P. gingivalis, P. intermedia, B. forsythus* and *T. denticola* than controls, suggesting a site-specific bacterial driven inflammation rather than a patient-associated specific host response. In a study of 15 patients with peri-implant lesions, Leonhardt *et al.* (2003b) observed a similar microflora between teeth and implant sites when culture and checkerboard DNA-DNA hybridisation techniques were employed. The authors noted that checkerboard results demonstrated good specificity compared to culture techniques.

Botero *et al.* (2005) conducted a study investigating clinical, radiographic and microbiological parameters in 11 patients with 16 implants demonstrating deep probing pocket depths and in eight patients with 15 stable implants. Samples were cultured for *Enterobacteriaceae* spp. and facultative/anaerobic periodontal pathogens. *P. gingivalis*

was detected in peri-implant sites demonstrating deep probing pocket depths, but not in stable implants. A significant correlation between the sub-gingival microbiota from implants and neighbouring teeth for Gram-negative enteric rods ($p=0.02$) and *P. gingivalis* ($p=0.04$) was noted. This study highlights the mouth as one ecosystem with the microflora of periodontal sites influencing those of peri-implant sites and those of peri-implant sites influencing the composition of the microflora of the periodontal sites.

Mengel & Flores-de-Jacoby (2005) examined 39 partially dentate patients with a total of 150 implants. Fifteen patients had experienced generalised aggressive periodontitis that was well controlled prior to implant placement surgery, 12 patients had been treated for generalised chronic periodontitis prior to implant placement surgery and 12 patients had no experience of periodontal disease. Clinical measures were taken and the sub-gingival microflora composition examined by dark-field microscopy. *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were detected at teeth and implants by DNA analysis. *Aggregatibacter actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were more frequently isolated in patients with a history of chronic periodontitis or aggressive periodontitis than healthy controls. Additionally, a somewhat greater attachment loss was noted for implants and teeth in aggressive periodontitis patients than those affected by chronic periodontitis or with no history of periodontal disease. Microbiologically, generalised aggressive periodontitis subjects have been observed to have fewer cocci and more motile rods and filaments at teeth and implants than periodontally healthy subjects (Mengel *et al.*, 2007).

The question arises as to whether bacterial load is the key determinant of peri-implant disease or health state. This was addressed by Agerbaek *et al.* (2006) in a study of 56 patients (127 implants). A higher bacterial load was observed at implants with probing pocket depths of ≥ 4 mm than those sites with probing pocket depths of ≤ 4 mm. Hultin *et al.* (2002) similarly noted higher bacterial amounts around implants affected by peri-implant bone loss.

Differing findings from individual study groups may in part be explained by differences in the criteria utilised to define health and disease: the patient sample pool; the time since implant placement; sampling techniques employed and the microbial analysis method employed. Paper-point and curette sampling techniques have been noted to differ both qualitatively and quantitatively with regard to species isolated from individual sites utilising both sampling techniques (Keil & Lang 1991; Renvert *et al.*, 1992; Gerber *et al.*, 2005). No study to date has compared the peri-implant and periodontal microflora

utilising both paper-point and curette sampling techniques. The extent of similarities and differences in peri-implant and periodontal microflora in health and disease requires further investigation.

1.11 Risk Indicators

1.11.1 Periodontal disease experience and maintenance care

Are patients with a history of severe periodontal disease at an increased risk of experiencing peri-implant infections? Peri-implant and periodontal infections share many similarities with regard to clinical, pathological and microbiological features. Both periodontitis and peri-implantitis are chronic inflammatory diseases with a microbial aetiology, which is "necessary although not sufficient to initiate the disease" (Sanz, 2011). In both periodontitis and peri-implantitis host susceptibility is considered to be important, however, the evidence base to support this is limited (Schou *et al.*, 2006; Karoussis *et al.*, 2007). Pathogenic mechanisms proposed include: the colonisation of implant surfaces by bacteria from the plaque of neighbouring periodontally compromised teeth which induce peri-implantitis; and/or that the host response to the bacterial challenge may mediate the inflammatory destruction of implant supporting tissues (Karoussis *et al.*, 2003).

It has been demonstrated that the persistence of plaque is the causative stimulus for the peri-implant inflammation (Serino & Strom, 2009). Laine *et al.* (2006) observed a significant increase in the carriage rate for allele 2 in the AL-1RN gene between peri-implantitis patients and controls (56.5% vs. 33.3% respectively) (OR 2.6) and concluded that "*IL-1RN gene polymorphism is associated with peri-implantitis and may represent a risk factor for this disease*". Therefore, it is likely that, similar to periodontitis, patient susceptibility to peri-implant infection may be in part accounted for by genetic factors.

Whilst patients who have a demonstrated susceptibility to periodontal disease may be treated successfully with osseointegrated dental implants (Nevins & Langer, 1995), controversy remains as to whether this group of patients shows a decreased success rate in the longer term compared to periodontally healthy patients and as to whether these patients have an increased requirement for ongoing periodontal care. Wennström *et al.* (2006) noted that 51 patients treated for periodontal disease and restored with dental implants demonstrated little peri-implant bone loss over a five-year observation period. The authors took this to indicate that patients with a history of periodontal disease could be successfully treated with dental implant restorations.

A decreased survival rate of dental implants and an increased prevalence of peri-implantitis has been reported in patients with a history of periodontitis compared to non-periodontitis patients (Hardt *et al.*, 2002; Karoussis *et al.*, 2003; Roos-Jansåker *et al.*, 2006; Mengel *et al.*, 2007; Gatti *et al.*, 2008; Roccuzzo *et al.*, 2010; 2012; Koldsland *et al.*, 2011; Costa *et al.*, 2012; Renvert *et al.*, 2012; Swierkot *et al.*, 2012; Pjetursson *et al.*, 2012; Cho-Yan Lee *et al.*, 2012). However, this is not universally supported as several studies have not observed significantly increased risk of peri-implant infections in patients with a history of periodontal disease (Brocard *et al.*, 2000; Hultin *et al.*, 2000; Rosenberg *et al.*, 2004; Baelum & Ellegaard, 2004; De Boever *et al.*, 2009). Furthermore, peri-implantitis has been observed to affect patients with no demonstrated susceptibility to peri-implant disease (Lang & Berglundh, 2011).

Renvert & Persson (2007) undertook a systematic review of the literature examining whether patients with a demonstrated susceptibility to periodontal disease (i.e. a history of periodontal bone loss) were similarly susceptible to peri-implant bone loss. Unfortunately, due to poor study design and incomplete information being presented, only two studies were considered of sufficient quality for inclusion in the systematic literature review: Hardt *et al.* (2002) and Karoussis *et al.* (2003). The conclusion of this systematic review was that, from the available evidence, there appeared to be an association between a history of periodontal disease and the presence of peri-implantitis, again suggesting a common mechanism of peri-implant bone loss between both disease processes. However, Renvert & Persson (2007) suggested that the quality assessment of both studies indicate that the risk of bias was high and recommend "*further long-term studies involving sufficient number of patients are needed before final conclusions can be drawn about the outcome of implant treatment in patients with a history of periodontitis*".

Hardt *et al.* (2002) described a retrospective study 97 partially edentulous patients with 346 implants, selected from the Gothenburg Brånemark Clinic, placed in the posterior maxilla without bone augmentation. Panoramic radiographs were examined at baseline and at the position of the most coronal portion of the alveolar bone for all teeth, determined as the level where the periodontal ligament space had a normal width. The root length was measured and the distance from the CEJ to the coronal portion of the alveolar bone. Subsequently, the age-related periodontal marginal bone loss score was calculated, employing a formula of Bjorn and co-workers (1969), to describe the severity of the periodontal disease experience. The two tail quartiles of the distribution of the individual age related marginal bone loss scores were chosen to represent the individual

with minimal experience of periodontal breakdown (Non-perio Group) and subjects considered to be susceptible to periodontitis (Perio Group). Twenty-five patients were in each group. The two groups differed with regards to a) mean periodontal bone level (92% compared to 63%, respectively), b) the proportion of teeth with bone level of less than half of the root length (1% compared to 26%, respectively). Patients were reviewed at years one, three and five at the clinic and seen by their general dental practitioners annually during the five year period. In the Non-perio group, 3.3% (3/92) of implants in three of the 25 patients were lost, whereas eight of the 100 implants in seven of the 25 patients of the Perio group patients were lost. In the Non-perio group, 6/25 patients (24%) experienced peri-implant bone loss of ≥2 mm, whereas, 16/25 (64%) of patients in the Perio group demonstrated peri-implant bone loss of ≥2 mm (p=0.029). In total, 44% of implants in the Non-perio group demonstrated peri-implant bone loss of ≥2 mm, whilst 62% of implants in the Perio group demonstrated peri-implant bone loss of ≥2 mm (p=0.055). The authors acknowledged the significant limitations of the study in that the clinical data relating to the periodontal and peri-implant conditions at follow up were not available. Furthermore, the retrospective design provided limited data with regards to smoking status.

Karoussis *et al.* (2003) outlined the findings of a retrospective analysis of 53 partially edentulous patients with 112 hollow screw implants. Eight patients had lost teeth due to chronic periodontitis, while the remaining 45 experienced tooth loss for other reasons. Over a ten-year period patients were maintained in a tailored maintenance program according to the risk of periodontal disease progression. Peri-implant infections were noted and the cumulative interceptive supportive therapy (CIST) protocol employed (Lang *et al.*, 2000). Radiographs were taken following one year and ten years of function. Peri-implantitis was diagnosed when implants met the following criteria: one or more sites with probing pocket depths of 5 mm or more; the presence of bleeding on probing and the presence of radiographic signs of peri-implant bone loss. The authors required that implants met both "success" clinically and radiographically, i.e. an implant was deemed a success only if it met all of the following criteria: no annual bone loss >0.2 mm; no site with a probing pocket depth of greater than 6 mm (PPD >6 mm) and no site with PPD >6 mm and BOP. Peri-implantitis was diagnosed when the following criteria were met: peri-implant probing pocket depth of at least 5 mm (PPD ≥5 mm) with bleeding on probing and radiographic signs of bone loss. The details relating to the classification of periodontitis or non-periodontitis status are limited: The authors stated that if the implants

were to be placed in patients who lost teeth due to periodontitis they were considered as periodontitis susceptible patients, whereas those patients who had lost teeth due to other reasons were considered "without a history of chronic periodontitis". The numbers of patients were eight and 45 respectively, whilst the numbers of implants studied were 91 and 45, respectively. No significant differences in the survival of the implants after five and ten years were noted. However, peri-implantitis was observed to be significantly greater in patients affected by periodontitis in the ten-year observation (Risk Ratio (RR) of 9, 95% CI 3.94-20.57). Also, significantly greater peri-implant marginal bone loss was observed in patients with periodontitis associated tooth loss after five years, with mean differences of 0.5 mm (95% CI 0.06-0.94). Over the 10 year observation period, 71.4% of implants in patients belonging to the periodontitis susceptible group remained free of any biological complication, while 94.2% of the implant in patients belonging to non-periodontitis group did not present any biological complication. A significantly greater incidence of biological complications were observed for implant placed in patients with a history of chronic periodontitis compared to patients without such a history (log-rank test: $p=0.002$: Wilcoxon test: $p=0.0001$). Criteria to define smoking status were not described by Karoussis *et al.* (2003).

During the 10-year follow up period, peri-implantitis was observed in eight of the 21 implants (38%) in the periodontitis group. Peri-implantitis was also observed in five of the 91 implants (4.8%) and in five patients in the non-periodontitis group (Karoussis *et al.*, 2003). Patients in the group with periodontitis associated tooth loss were observed to have a significantly higher prevalence of peri-implantitis compared to the group with non-periodontitis associated tooth loss (RR of 9, CI 3.94-20.57, $p<0.01$). Following 10 years of observation, two implants (9.5%) were lost in two individuals in the periodontitis group and three implants (3.5%) in three individuals in the non-periodontitis group, however, differences were not significant between the two groups (RR of 3.75, 95% CI 0.74-19.02, $p=0.11$).

Rosenberg *et al.* (2004) undertook a retrospective analysis of the records of 334 patients who had 1511 implants placed over a 13-year period. One hundred and fifty-three patients (923 implants) were classified as being periodontally compromised, where as 183 patients (588 implants) were classified as periodontally healthy. Patients who met any of the three criteria outlined below were included in the periodontally compromised group:

b) Tooth loss for periodontal reasons leading to a requirement for implant restoration.

c) Clinical attachment loss (not including buccal or lingual recession). It is worth noting that the clinical threshold employed was not provided.
d) Probing depths of greater than 4 mm at time of implant placement.

Implant failure was classified according to presence of any of the following signs:

a) Clinical mobility.
b) Continuous radiolucency around the implant.
c) Continuous bone loss that necessitated removal or surgical intervention.

The survival of implants placed in periodontally diseased patients was similar to that of patients who were periodontally healthy (90.6% and 93.7% respectively). However, these findings must be viewed in light of the very thorough maintenance undertaken– i.e. all patients were recalled at three-month intervals during the maintenance phase. The authors concluded that "*... with adequate customised recall and maintenance programs following implant placement and loading implants placed in periodontally compromised patients have survival rates similar to those of implants placed in periodontally healthy patients.*"

Costa *et al.* (2012) noted a prevalence of peri-implantitis of 31.2% in 80 partially edentulous patients five years post-implant restoration. Among the 80 patients included, 39 patients had participated in a preventative maintenance program, whereas 41 patients had not. When the prevalence of peri-implantitis was analysed according to whether or not patients had participated in a periodontal maintenance program or not, it was seen that those patients who had participated in the maintenance program had peri-implantitis levels of approximately half that of the no maintenance group (43.9% compared to 18%). The authors observed a significantly elevated risk of peri-implantitis in patients with periodontitis present for the group of patients not attending for maintenance care (odds ratio (OR) of 2.43 CI 1.11-117.33) but not for those who regularly attended for maintenance visits. The lack of preventative, maintenance visits was observed to be significantly associated with peri-implantitis (OR of 5.92, CI 1.09-32.16). Furthermore, if >5% of sites demonstrated probing pocket depths of >4 mm, a significantly elevated risk for peri-implantitis was present for both the no maintenance (OR of 25.49, CI 2.59-251.21) and the regular maintenance groups (OR of 19.65, CI 1.15-334.91).

Renvert *et al.* (2012) undertook an investigation of the prevalence of peri-implant infections in 54 patients seven years post implant restoration, 41 of which who attended subsequently for 13-year examinations. The diagnostic criteria employed were: bone loss ≥1 mm in conjunction with bleeding on probing, with or without suppuration on probing as recommended previously (Sanz & Chapple, 2012). For Nobel Biocare

implants (Nobel Biocare, Gothenburg, Sweden) and Astra implants, the prevalence of peri-implantitis at seven years was 30.4% and 26.6% respectively, and at 13 years, 39.7% and 32.1% respectively. Interestingly, of the implants that experienced peri-implantitis in the first seven years, 19% of these fixtures experienced further bone loss, whereas only 5.9% of implants without peri-implantitis in the first seven years went on to develop peri-implant bone loss. Compliance rates for periodontal maintenance amongst those patients who attended for 13-year evaluation were very high being 94.7% for the Astra implant group and 91.5% for the Noble Biocare implant group. Alarmingly, for this group of patients with high compliance rates for periodontal maintenance over 13 years, approximately 65% of subjects had experienced peri-implantitis. Furthermore, patients with a history of systemic disease demonstrated an elevated risk of developing peri-implantitis (OR of 2.1, 95% CI:1.0-4.4, $p=0.05$). It was also noteworthy that the microbiological profile at seven years failed to be predictive of incidence of peri-implant bone loss at 13 years. The odds ratio of having peri-implantitis for subjects with periodontitis between the year one and year 13 observation time points was a highly predictive 4.1 (95% CI:2.0-8.4, $p<0.001$) for the development of peri-implantitis.

Similarly, Ferreira *et al.* (2006) observed a significantly elevated risk of peri-implantitis in patients with a history of periodontal disease. Ferreira *et al.* (2006) undertook a study in 212 patients who had dental implants for a period of between six months and five years. Periodontitis was diagnosed as the presence of four or more teeth with one or more sites with probing depths of ≥ 4 mm and clinical attachment loss of ≥ 3 mm – these criteria were previously employed by Lopez *et al.* (2002). Of the patients in the study, 14.2 % were diagnosed as having a history of significant periodontal disease, whilst 86.3% of the patients had no such history. Those patients with a history of significant periodontal disease were statistically significantly more likely to demonstrate peri-implantitis when compared to the non-periodontitis patients ($p<0.05$). The respective prevalences were 26.66% and 6.04%. Interestingly, Ferreira *et al.* (2006) noted that the frequency of maintenance visits did not seem to have an influence on peri-implant health status.

Koldsland *et al.* (2011) undertook a follow up study of 109 patients rehabilitated with implant restorations for a mean period of 8.4 years (SD 4.6). The authors subdivided peri-implantitis into overt and detectable. Criteria employed in the diagnosis of overt peri-implantitis included: those implants that demonstrated bleeding on probing; probing pocket depths of ≥ 4 mm and peri-implant bone loss ≥ 2 mm. Detectable peri-implantitis

was diagnosed when peri-implant bone loss of ≥0.4 mm was present in conjunction with clinical signs of inflammation. In total, 20.4% of subjects (11.4% implants) were diagnosed as being affected by overt peri-implantitis. Multi-level statistical analyses revealed that patients a) with a history of periodontal disease or b) were male, were at a significantly greater risk of developing peri-implantitis.

Swierkot *et al.* (2012) undertook a study of 53 patients, 35 of which had a history of periodontal disease and 18 of which had no history of periodontal disease, and followed them for a mean period of 8.25 years. Peri-implant mucositis was noted in 56% of the implants in patients with a history of periodontal disease and 40% of the implants in the patients with no history of periodontal disease, whereas peri-implantitis was noted in 26% of the implants and 10% of the implants for both groups respectively. The authors calculated that patients with a history of generalised aggressive periodontitis were at a five times greater risk of implant failure, a three times greater risk of peri-implant mucositis and a 14 times greater risk of peri-implantitis (p=0.007), than patients with no history of periodontal disease.

Pjetursson *et al.* (2012), in a study of 70 patients (165 implants) who had been treated for periodontal disease and subsequently had dental implant restorations, were followed up for a mean period of 7.9 years. In total, 67.1% (47/70) of patients attended the supportive periodontal therapy program of the University of Berne and 32.9 % (23/70) returned to the referring dentist for supportive care. The authors employed two definitions of peri-implant mucositis or peri-implantitis:

a) Level 1 was defined as sites with probing pocket depths of ≥5 mm and demonstrating bleeding on probing.
b) Level 2 was defined as sites with probing pocket depth of ≥6 mm and demonstrating bleeding on probing.

Implants meeting the criteria of Level 1 or Level 2 presence of peri-implant infection were considered positive for peri-implantitis if they also met the criteria of having bone loss beyond that expected as a part of post-implant insertion remodelling. The implants in the investigation were placed using a transmucosal approach and in order to incorporate a peri-implant bone loss of ≥2 mm into the diagnosis for peri-implantitis, implants with a radiographic marginal bone level of ≥5 mm below the implant shoulder were considered positive for peri-implant bone loss beyond normal remodelling. The prevalence of peri-implantitis (Level 1) was noted to be 38.6% (27/70) on a patient level and 22.2% (37/165) on an implant level. Moreover, when the Level 2 peri-implantitis criteria were applied,

only 17.1% (12/70) of patients and 8.8% (15/165) of implants fulfilled the criteria. Of the patients who were affected by peri-implantitis Level 1, on average 4.1 residual pockets were present for each patient, whereas for the patients who did not meet the peri-implantitis level 1 criterion, the mean number of residual pockets present per patient was 1.9. Differences between the two groups were significant (p=0.022), demonstrating that the presence of residual deep periodontal pockets at the completion of active periodontal treatment, places future implant patients at a significantly greater risk of experiencing peri-implantitis compared to those patients who achieved shallow probing pocket depths throughout their periodontium upon completion of periodontal treatment. This finding is similar to that of Claffey *et al.* (1996), who demonstrated that patients who had residual deep sites at re-evaluation following periodontal treatment, were at an increased risk of periodontal disease progression on a patient level, but not necessarily at the residual deep sites. However, the difference in residual deep probing depths between patients with a positive diagnosis of peri-implantitis (Level 2) and those without were not significantly different (p=0.51).

Of the patients who attended regular maintenance care at the University of Berne, 31.9% (15/47) were affected by peri-implantitis (Level 1), whereas 52.2% (12/23) of patients who underwent maintenance care with their referring dentist were positive for peri-implantitis (Level 1). Similarly, 17.6% (8/47) of patients who attended regular maintenance care at the University of Bern were positive for peri-implantitis (Level 2) and 30.2% (7/23) of patients who returned to their referring dentist were positive for peri-implantitis (Level 2). Therefore, patients who attended a well structured maintenance care program at the specialist centre fared better than those who were returned to their referring dentist for maintenance care, however, the differences observed were not significant (p=0.102). The authors suggested that "*it is reasonable to treat periodontitis to a level of absence of or minimal inflammation as well as to an absence of residual pockets following active therapy to reduce a possible influence of ecological niches on the integrity of implants and their stability.*" The findings of this study are in line with those of Matarasso *et al.* (2010) and Roccuzzo *et al.* (2010), who noted the importance of supportive care in enhancing the long-term outcomes of implant treatment in patients, especially those with a history of periodontal disease. Patients who do not regularly attend for maintenance care are 11 times more likely to experience peri-implantitis than those with good compliance (p=0.001) (Rinke *et al.*, 2010).

Brocard *et al.* (2000) observed similar cumulative survival rates for 1,022 consecutively placed implants for periodontally healthy and periodontally compromised patients over a seven-year period. Hultin *et al.* (2000*a*) observed 143 patients who had implant-supported fixed restorations for five or more years and found no correlation between peri-implant and periodontal bone loss and suggested that *"different interacting mechanisms are involved"*. Following loading, only 2% of the implants were lost. Seven of the nine patients who lost implants were smokers and the majority of the implants lost were in the maxilla.

Roos-Jansåker *et al.* (2006) reported a significant association between peri-implantitis and a previous history of periodontitis ($p<0.05$) in 218 patients (1057 implants), who were followed up for 9-14 years after implant placement. De Boever *et al.* (2009) undertook a follow up study of 68 patients with chronic adult periodontitis: 16 patients with generalised aggressive periodontitis and 110 patients not susceptible to periodontitis. A total of 513 implants were observed over a mean period of 48.1 +/-25.9 months. Only patients with fixed partial dentures were included in the study group. Implant loss was recorded at 4.7% for all implants, but at 15.25% in the aggressive periodontitis group. Implants with TPS surfaces had a lower survival rate than implants with SLA surfaces, however, differences were not statistically significant (93% compared to 97%; $p=0.06$). However, for the generalised aggressive periodontitis group, the differences were significant (80% compared to 83%; $p=0.005$). For the aggressive periodontitis group, implants in smokers were observed to have lower survival rates than non-smokers, however, these differences were not statistically significant ($p=0.07$). Periodontal classification ($p=0.012$) and implant surface type ($p=0.027$) were each observed to be statistically significantly related to implant failure. Furthermore, average inter-proximal bone loss was observed to be over twice as much for the aggressive periodontitis group compared to the periodontally non-susceptible patients, being respectively -0.17+/- 0.2 mm mesial and 0.17+/-0.19 mm distal, compared to 0.8 +/- 0.31mm mesial and 0.07 +/-0.3mm distal. Interestingly, for the generalised aggressive periodontitis group, bone loss was observed to be significantly related to the presence of: bleeding on probing; age; inflammation; presence of plaque and probing depth, but this was not the case for other groups.

Gatti *et al.* (2008) observed significant differences in 56 partially dentate implant patients, over a five-year period, with almost twice as much peri-implant bone loss in patients with a history of severe periodontal disease compared to those with no history of

severe periodontal disease. Mengel *et al.* (2007), in a prospective controlled study, noted significantly greater peri-implant bone loss and attachment loss in five patients with a history of aggressive periodontitis compared to five periodontal controls observed over a 10-year period.

Roccuzzo *et al.* (2010 & 2012) observed, in a 10-year prospective study of 112 partially dentate implant patients, somewhat increased, but not significantly different, peri-implant bone loss in patients with history of periodontal disease compared to patients with no history of periodontal disease. However, patients who had implants with at least one peri-implant site with a probing depth of ≥6 mm were observed to be significantly greater in both the patients with a history of severe periodontal disease ($p=0.0001$) and those with a history of moderate periodontal disease ($p=0.005$) compared to patients with no history of periodontal disease. Marrone *et al.* (2013) observed that those patients with active periodontitis were more prone to peri-implantitis (OR of 1.98).

Cho-Yan Lee *et al.* (2012) observed 30 previously treated periodontally compromised patients and 30 periodontally healthy patients who had implant restorations present. Patients who had at least one periodontal site with a probing pocket depth of at least 6 mm at a follow-up examination were allocated to a "residual periodontitis" group, while the remaining patients were assigned to a "no residual periodontitis" group. In comparing the peri-implant probing depths between the periodontally compromised group and periodontally healthy group, no significant differences were observed ($p>0.05$). However, the prevalence of implants with PPD ≥5 mm + BOP was greater in the "residual periodontitis" group, on both an implant level (27% compared to 13%, respectively), and a patient level (37% compared to 17%, respectively). The authors concluded that "*it is the maintenance of periodontal health, rather than a previous history of periodontitis that is the critical determinant of increased risk of peri-implantitis*".

It has been suggested that the incidence of biological complications may depend on the subtype of periodontitis (Mengel *et al.*, 1996; Mengel & Flores-de-Jacoby 2005) and that whilst patients with a history of aggressive periodontitis may be treated successfully with implants in the short term, the prognosis of implants in this patient group in the long term is "open to question" (Karoussis *et al.*, 2007). Serino & Strom (2009) noted in 109 implants in 23 patients that 74% of the implants had "no access/capability" to proper oral hygiene. 48% of the implant patients with peri-implantitis were those with "no access/capability" for proper oral hygiene (65% positive predictive value) whereas only 4% of the implants with access/capability were diagnosed

as being positive for peri-implantitis (82% negative predictive value). Heitz-Mayfield *et al.* (2011) noted less improvement in clinical parameters following non-surgical debridement with or without adjunctive chlorhexidine, for implant restorations affected by peri-implant mucositis with submucosal restorative margins compared to those with supra-mucosal restorative margins. The authors highlighted the importance of giving proper oral hygiene instruction to patients being restored with implant restorations and that the restorations are of proper aesthetic constructions that allow accessibility for oral hygiene.

1.11.2 Smoking

Heitz-Mayfield and Huynh-Ba (2009), from a systematic review of the literature, concluded that smokers were at an increased risk of peri-implantitis compared with non-smokers with reported odds ratios from 3.6 to 4.6, and that the combination of a history of treated periodontitis and smoking resulted in further increases in the risk of implant failure and peri-implant bone loss.

Smoking results in damage to the microcirculation, largely due to vessel wall injury, reduced tissue perfusion and impaired microcirculatory regulation. Smoking specifically compromises endothelial cell function, vasodilation and results in the activation of circulating leukocytes leading to aggregation and adhesion of leukocytes and/or platelets to the microvascular endothelium (Lehr *et al.*, 2000). Furthermore, smoking diminishes the immune response by compromising macrophage and polymorphonuclear leucocyte function through decreasing phagocytosis and delaying margination and diapedesis (Mac Farlane *et al.*, 1992). Therefore, the ability of the immune system to ward off infecting microorganisms is diminished in smokers.

Grunder *et al.* (1999) undertook a study of 74 patients (219 implants) of which 19 of the patients were smokers, with a mean follow up time of 34 months and noted a survival rate of 98.7%. No significant difference in implant survival between smokers and non-smokers was reported, indicating that osseointegration can be preserved in patients who smoke.

However, a "dose-response relationship" appears to be present in relation to smoking and achieving or preserving osseointegration. Bain *et al.* (2003) observed that non-smokers and light smokers demonstrated similar survival rates (95.6% and 95.1% respectively). Whereas a significantly lower survival of osseointegration was noted for both moderate smokers (84.9%) and heavy smokers (87.13%) ($p<0.05$). Koldsland *et al.*

(2011) noted an association between smoking and diagnosis of peri-implantitis status in a group of 104 patients, restored with dental implants for a mean period of 8.4 years.

Levin *et al.* (2008) noted that current smokers demonstrated higher marginal bone loss during all time intervals than former smokers and demonstrated higher marginal bone loss during all time intervals than non-smokers. Levin *et al.* (2008) examined 64 patients for whom 64 implants were placed and bounded mesially and distally by natural teeth and followed up for five or more years. Haas *et al.* (1996) retrospectively examined the clinical and radiographic observations of 366 implants in 107 patients who smoked and these were compared with a group of 1000 implants in 314 non-smoking patients. There was no significant difference in the mean maxillary and mandibular hygienic indices between the group of smokers and that of the non-smokers. Maxillary implant peri-implant mucositis and radiographic discernable bone loss were significantly greater for the smoker group than for the non-smoker group ($p<0.01$). However, for the implants placed in the mandible no significant differences were noted between the groups.

Marrone *et al.* (2013), in a follow up of 103 patients with implant restorations for a mean time of 8.5 years, did not note a significant relationship between smoking and peri-implantitis. Roos-Jansåker *et al.* (2006) reported on 218 patients with 1057 implants followed for 9-14 years on the patient level, and found that smoking was associated with mucositis, bone level and peri-implantitis ($p=0.02$, <0.001 and 0.002, respectively).

Karoussis *et al.* (2003) observed a decreased survival rate and increased complications in periodontally treated patients after 10 years compared with periodontally healthy patients. This was especially heightened in patients with a demonstrated susceptibility to periodontal disease who also smoked. The authors did not outline the criteria describing the level of smoking required to attribute patients to the smoking and non-smoking groups.

Baelum & Ellegard (2004) noted that implants could be maintained for most patients with a history of periodontal disease that were well managed. However, the authors noted an increased risk of failure in smokers compared to non-smokers with an OR of 2.6 (0.9-7.6). The authors did not define peri-implantitis and did not present data relating specifically to this issue. The authors presented data of the peri-implant bone loss at both thresholds of >1.5 mm and >3.5 mm. The peri-implant bone loss of >3.5% is of interest as this criteria is similar to that employed in the present investigation. The one stage system implants with bone loss less than 3.5 mm at five and 10 years were 94.4% (89-97.2) and 86.4% (77-92.2) respectively. Whereas for the two stage implants freedom

from bone loss of greater than 3.5 mm at five and 10 years was noted for 95% (81.5-98.7) and 95% (81.5-98.7) respectively. The one stage implants were hollow screw implants up to 1995, which the authors state were "*virtually impossible to treat once peri-implantitis has developed*". Of the two stage implants, only 94.1% (78.2-98.5) and 75.4% (66.1-82.5) had not experienced peri-implant probing depths of ≥6 mm at five and 10 years respectively. For the one stage implants, 85.4% (78.5-90.2) and 75.4% (66.1-82.5) had not experienced probing depths of ≥6 mm at five and 10 years respectively. The authors highlighted both the need for high quality research into the most efficient way to treat peri-implantitis and the need for clinical tests for the monitoring of peri-implant health to detect peri-implantitis at an early, reversible stage. The authors pointed to the work of Luterbacher *et al.* (2000), which suggested that the combination of bleeding on probing with the detection of either *A. actinomycetemcomitans*, *P. gingivalis, T. denticola* or *P. intermedia* were good diagnostic criteria for peri-implant infection.

Nitzan *et al.* (2005), in a retrospective study, examined the records of 151 patients treated with 646 implants. Patients were divided into three groups: non-smokers, moderate smokers and heavy smokers. Smokers had greater peri-implant bone loss in the mandible compared to non-smokers (0.16 ± 0.17 mm compared to 0.15 ± 0.16 mm) and differences were statistically significant ($p<0.001$). The dose response relationship was evident in the maxillary arch where peri-implant bone loss was seen to be greatest in heavy smokers (0.19 ± 0.18 mm) compared to moderate smokers (0.12 ± 0.16 mm) and non-smokers (0.05 ± 0.07 mm) with differences being statistically significant ($p<0.001$). However, the clinical significance of a 0.07 mm difference in peri-implant bone levels may be questionable. In the mandible, smokers were observed to have more bone loss than non-smokers ($p<0.001$), but no significant differences were present between heavy and moderate smokers ($p>0.05$).

Strietzel *et al.* (2007) undertook a meta-analysis examining survival of dental implants in smokers compared to non-smokers. Twenty nine studies were included in the Meta-analysis and a significantly increased risk of implant failure was observed in the smoking group compared to the non-smoking group (implant related odds ratio OR 2.25; CI 95% 1.96-2.59); patient related OR 2.64; CI 95% 1.7-4.09) compared with non-smokers. Interestingly, the likelihood of failure was enhanced if augmentation procedures were undertaken (OR 3.61; CI 95% 2.26-5.77).

Rinke *et al.* (2010) undertook a cross-sectional retrospective study examining 89 patients with implant restorations for ≥5 years. The authors observed that smokers were at

a significantly elevated risk of peri-implantitis (p=0.023), with 53.3% of smokers being affected by peri-implantitis whilst only 2.8% of non-smokers were positive for peri-implantitis. Compliance was also significantly associated with peri-implantitis (p=0.011). Interestingly, no case of peri-implantitis was diagnosed in the group of non-smokers with regular maintenance care. However, a history of periodontal disease was not significantly associated with diagnosis of peri-implantitis. This study was undertaken in a private practice setting in Germany and demonstrated that with regular maintenance care and absence of smoking, peri-implantitis is unlikely. Therefore, with control of these modifiable risk indicators, the risk of peri-implantitis can be very much decreased. Interestingly, a history of periodontitis was not significantly associated with peri-implantitis diagnosis and this is difficult to interpret. The authors highlighted that, with regular maintenance care, the risk of peri-implantitis was significantly decreased.

Aglietta *et al.* (2010) undertook a retrospective study examining the peri-implant bone levels of periodontally compromised and periodontally healthy smoker patients 10 years following placement of the implant restoration. Ten percent of the dental implants (four of 40) were lost over the 10-year period. Three of the four failures were amongst the periodontally compromised patients and all of the implant failures were after the fourth year of loading, however, differences were not statistically significant. Seventy-five percent of the patients demonstrated bone loss of ≥3 mm after 10 years, highlighting the detrimental effects of smoking on peri-implant bone levels. The authors commented that "*smoking synergistically enhanced the risk of implant failures and marginal bone loss*". These findings were similar to those of Fransson *et al.* (2008), who observed that smokers were at an increased risk of peri-implant bone loss, probing pocket depths of ≥6 mm and suppuration on probing compared to non-smoking patients with comparable oral hygiene levels. Similarly, Koldsland *et al.* (2011) observed that patients with a history of smoking and treated periodontal disease were at an increased risk of peri-implant infections.

Swierkot *et al.* (2012) undertook a study of 53 patients, 35 of which with a history of periodontal disease and 18 of which had no history of periodontal disease. The patients were followed up for a mean period of 8.25 years. Peri-implant mucositis was noted in 56% of the implants in patients with a history of periodontal disease and 40% of the implants in the patients with no history of periodontal disease, whereas peri-implantitis was noted in 26% and 10% of the implants for both groups, respectively. The authors calculated that patients with a history of generalised aggressive periodontitis were at a five times greater risk of implant failure, a three times greater risk of peri-implant

mucositis and a 14 times greater risk of peri-implantitis (p=0.007) than patients with no history of periodontal disease. Smoking was noted to be a significant risk indicator for peri-implantitis, with former and current smokers demonstrating odds ratios of 3.02 and 2.87 with p=0.009 and 0.038, respectively.

Heitz-Mayfield & Huynh-Ba (2009), from a systematic review of the literature, concluded that smokers are at an increased risk of peri-implantitis compared with non-smokers (reported odds ratios from 3.6 to 4.6) and that the combination of a history of treated periodontitis and smoking further increased the risk of implant failure and peri-implant bone loss.

1.12 Clinical examination of periodontal and peri-implant tissues

1.12.1 Overview

The clinical measures considered in assessing peri-implant tissue health include: the presence or absence of plaque on the peri-implant tissues; bleeding on probing of the peri-implant tissues; suppuration on probing of the peri-implant tissues; the depth of the peri-implant pocket; the degree of recession which had taken place and lastly, radiographic assessment for interproximal peri-implant bone loss beyond remodelling. If implants are mobile, the implant is deemed to have failed.

1.12.2 Periodontal probing around natural teeth

Periodontal probing is an essential procedure in the diagnosis of periodontal disease or health and for the evaluation of the response of the periodontal tissues to periodontal treatment (Lang *et al.,* 1996). It is also used to evaluate the prognosis during the maintenance phase of periodontal treatment (Claffey *et al.,* 1995).

For patients entering the maintenance phase of therapy, a new baseline of periodontal parameters should be set (Claffey *et al.,* 1994). Maintenance should be considered a constant re-evaluation, and periodontal parameters evaluated in light of their usefulness in detecting sites, or patients, likely to demonstrate ongoing attachment loss and requiring therapeutic intervention. Risk of periodontal disease progression may be interpreted on a site or patient basis. Individual site data has poor predictive power for ongoing attachment loss in the short term, but improves with extended periods of observation. Patient-based data may be more useful in identifying patients likely to undergo periodontal destruction somewhere in their dentition (Claffey & Egelberg, 1996).

The presence of plaque is a limited predictor of probing attachment loss in periodontal tissues (Vanooteghem *et al.,* 1987; Baderstein *et al.,* 1990; Claffey *et al.,* 1990, Kaldahl *et al.,* 1990). Shallow sites demonstrate the lowest risk of disease progression (Claffey *et al.,* 1990; Baderstein *et al.,* 1990). Sites with residual probing depth >7 mm, show a 25-30% (moderate) predictive value of attachment loss (Claffey *et al.,* 1990; Baderstein *et al.,* 1990). Furcation and buccal/lingual sites with probing pocket depths of >6 mm at 12 months demonstrated a 38% and 36% prediction for further attachment loss at 42 months, respectively. Proximal sites with probing pocket depths in excess of 6 mm at 12 months demonstrated a 15-20 % risk of ongoing attachment loss (Claffey *et al.,* 1990). An improved predictability for ongoing attachment loss was observed for sites with probing depths greater than 6 mm, which also demonstrated bleeding and/or suppuration on probing. Deepening probing depths compared to baseline was strongly associated with periodontal attachment loss (Baderstein *et al.*, 1990).

Surprisingly, there is limited evidence to support the presence of suppuration on probing as a predictor for ongoing attachment loss. This may relate to the limited number of occasions and limited number of sites at which suppuration on probing is observed in the studies examining this relationship. The presence of suppuration on probing at two out of 14 visits may be considered to have the equivalent predictive power of ongoing loss as the presence of bleeding on probing at 11 out of 14 visits (Claffey *et al.* 1990). Kaldahl *et al.* (1990) observed that of the sites at which suppuration on probing was detected on eight to nine occasions, 50% demonstrated ongoing attachment loss.

Bleeding on probing is a moderate predictor for probing attachment loss, with diagnostic values reaching 20 to 40 % (Claffey *et al.,* 1990, Baderstein *et al.,* 1990, Baderstein *et al.,* 1985, Lang *et al.,* 1986, Vanooteghem *et al.,* 1987). The absence of bleeding on probing has been demonstrated to be a good predictor of health on a site basis (Lang *et al.,* 1990). Of sites where bleeding on probing is present at greater than 75% of occasions during maintenance, 40% of these sites show deterioration (Claffey *et al.,* 1990). Baderstein *et al.* (1990) similarly found that of sites demonstrating bleeding on probing at 75% of occasions, 23% of these sites showed further periodontal attachment loss.

Patient level analysis of clinical signs may be more useful than site based analyses at re-evaluation. Patients with a high percentage of residual pocket depth measuring >/=6 mm have a greater risk of developing periodontal attachment loss somewhere in the dentition, than subjects with a low percentage of residual pocket depth

measuring >/=6 mm. However on a site basis a residual pocket depth measuring greater than or equal to 6 mm does not appear to be a risk factor of the same magnitude (Claffey *et al.*, 1996; Rams *et al.*, 1996).

Joss *et al.* (1994) observed a significant positive correlation between the average maintenance bleeding scores and the number of sites at which periodontal attachment loss greater than 2 mm progressed over a period of 53 months. Claffey & Egelberg (1995) observed no significant association between mean bleeding score and periodontal attachment loss over a period of 42 months.

1.12.3 Peri-implant probing

Given the demonstrated value of periodontal probing in the diagnosis, monitoring and evaluating prognosis of the periodontal tissues, it is not surprising that periodontal probing has been employed to evaluate the health of the peri-implant tissues. Peri-implant probing initially was questioned in its value (Mayfield, 2008). Also, clinicians had concerns with regard to the relevance of peri-implant probing as the consistent positioning of the probe within the peri-implant tissues was difficult to achieve in many instances due to the restorative supra-structure present (Lang *et al.*, 1996). Furthermore, clinicians had concerns with regard to the introduction of microorganisms into the peri-implant tissues by the probe tip penetrating the peri-implant tissues (Lang *et al.*, 1996). Additionally, there were concerns with regard to damage of the implant surface with the metal probe tip through corrosion and mechanical scraping of the relatively soft titanium surface (Lang *et al.*, 1996).

However, current opinion holds that peri-implant probing is broadly similar to the probing of periodontal tissues and is just as useful in determining the health or disease status of the tissues (Mayfield, 2008; Lang & Berglundh, 2011). Peri-implant probing is considered to be of value and is recognised as an essential and reliable tool for diagnosing and monitoring for the presence of peri-implant infections (Mayfield, 2008). Valuable information gained includes: the peri-implant probing depth, the level of the mucosal margin relative to the clinical crown margin and especially the presence or absence of bleeding or suppuration on probing (Hammerle & Clauser, 2004). Clinically healthy peri-implant tissues usually do not bleed or demonstrate suppuration on probing and demonstrate stable probing pocket depth measurements relative to a fixed reference point, for example a crown margin (Mayfield, 2008). The presence of bleeding on probing is the key determinant of the presence of a peri-implant infection and associated inflammation (Lang & Berglundh, 2011). Whilst bleeding on peri-implant probing is employed to

disclose peri-inflammation, as inflammation results in the peri-implant vasculature being increasingly fragile, however it should be borne in mind that the peri-implant tissues bleed more readily than the periodontal tissues as a result of a greater blood vessel fragility in health (Bragger *et al.*, 1997). Therefore, the presence of false positive diagnosis for peri-implant inflammation must be considered more likely for peri-implant tissues than for the periodontal tissues.

The differences in the arrangement of the supra-crestal connective tissue fibres results in a different resistance to probing forces between peri-implant and periodontal tissues. Mombelli *et al.* (1997) observed that the probing in the region of dental implants in human subjects is more sensitive to probing force measure than around teeth. The depth of penetration of the probe tip is influenced by the density of the peri-implant tissues, which is influenced by the presence or absence of peri-implant inflammation. In inflamed tissues, the probe tip has been observed to penetrate close to the bone level, whereas if the peri-implant connective tissues were healthy, the probe tip was observed frequently to stop at the histological level of the connective tissue attachment to the implant surface (Lang *et al.*, 1994).

It is recommended that the peri-implant probing force not exceed 0.25 N cm (Mombelli *et al.*, 1992; Lang & Berglundh, 2011). The depth to which the periodontal probe extends into the peri-implant tissues has been observed to relate to the degree of inflammation present in the peri-implant tissues (Lang *et al.*, 1994).

However, it is recognised that the restorative supra-structure often interferes with meaningful peri-implant probing (Hammerle & Clauser, 2004). There are designs of implants and abutments (e.g. platform switching) which make peri-implant probing inaccurate, underestimating the extent of the lesion and therefore the attachment level (Lang *et al.*, 2008; Lang *et al.*, 1996; Heitz-Mayfield *et al.*, 2008). Furthermore, clinical experience demonstrates that probing of peri-implant tissues is often uncomfortable for patients, making accurate probing difficult, especially where there is considerable inflammation present. The degree of discomfort on probing of periodontal tissues has been observed to relate to the degree of inflammation present in the periodontal tissues amongst other factors.

Bleeding on probing (Jepsen *et al.*, 1996) occurs more readily from the peri-implant tissues than from the periodontal tissues (Bragger *et al.*, 1997). Luterbacker *et al.* (2000) reported that sites with bleeding on probing at greater that 50% of recall visits were noted to demonstrate progressive bone loss with 100% predictability. For sites that

demonstrated bleeding on probing on less than 20% of occasions, 50% of these sites remain stable. Interleukin 1β has been observed to increase six fold in early peri-implantitis (Aboyussef *et al.*, 1998). Furthermore, elastase peri-implant crevicular fluid concentrations have been observed to increase in peri-implantitis (Plagnat *et al.*, 2002).

Suppuration is a common finding in peri-implantitis sites (Lang & Berglundh, 2011). Suppuration on probing has been demonstrated to reflect infiltration of the peri-implant soft tissues with large numbers of polymorphonuclear leukocytes in histopathological studies (Buser *et al.*, 1990). Suppuration on probing has been observed to be indicative of peri-implant bone loss of >3 threads in greater than 200 patients followed over a 9-14 year period (Roos-Jansaker *et al.* 2006).

Radiographic examination of implants and periodic monitoring of marginal bone level changes on intra-oral radiographs taken with the paralleling technique has been recommended as inter-proximal bone loss may be well visualized and monitored utilising these techniques (Hollender & Rockler, 1980). Limitations of peri-apical radiographs include the inability to assess peri-implant bone loss on the mid-buccal or mid-lingual region of implant fixtures due to the radiographic supra-imposition of the alveolar bone and the implant fixture and concerns have been raised with regards the sensitivity of conventional radiographic techniques in the detection of early changes in bone density (Lang *et al.*, 1996). Recording baseline data is necessary in order that future comparisons may be made and increasing probing pocket depths/ clinical attachment loss or progressive peri-implant bone loss may be detected. Lang & Berglundh (2011) recommended that where peri-implant probing depths are greater than or equal to 5 mm, radiographic examination is indicated as it is assumed that where crestal bone loss takes place beyond remodelling, that this is mainly due to bacterial infection.

1.13 Non-surgical treatment of periodontal and peri-implant infections

1.13.1 Overview of treatment of peri-implant infections

Peri-implant infections progress rapidly and become increasingly complex to treat as they become more advanced, therefore, it is recommended that peri-implant infections should be treated without delay (Heitz-Mayfield & Lang, 2010). The Cumulative Interceptive Supportive Therapy (CIST) protocol was devised in an effort to guide clinicians empirically in the management of peri-implant infections. The sequence of treatment as outlined by Lang *et al.* (2004) includes, firstly, disease elimination therapy, which comprises of: (a) oral hygiene instruction to prevent re-infection; (b) debridement to decrease the numbers of microorganisms within the plaque biofilm of the sub-gingival peri-implant pocket; (c) decontamination of the implant surface; and (d) a reduction of or elimination of sites that cannot be maintained. Subsequently, if required, the regeneration/ repair of peri-implant supporting tissues can be undertaken.

From a microbiological perspective, the goal of the disease elimination phase of therapy for peri-implant infections is similar to that of periodontal infections: a reduction in the numbers of those species associated with peroidontal/peri-implant disease and "*the establishment of a local environment and micro-flora compatible with health*" (Mombelli *et al.*, 2005). Mechanical disruption of the plaque biofilm may be achieved through supra- and sub-gingival debridement. However, mechanical debridement alone may not be enough to re-establish health (Buchmann *et al.*, 1996). Once inflammation is under control, attempts are made to improve or re-establish osseointegration using regenerative procedures. One key finding from animal studies is that "*Osseointegration can occur to surfaces that were plaque contaminated and cleaned using different methods*" (Kolonidis *et al.*, 2003).

The goal of the treatment of peri-implant infections is to decrease the microbial load below the individual threshold for disease, so that clinical health can be restored (Mombelli *et al.,* 1987; Leonhardt *et al.*, 1999; Shibli *et al.,* 2008;). The treatment of peri-implantitis is largely empirical (Claffey *et al.,* 2008) and at present there is no reliable evidence of the most successful method for the treatment of peri-implantitis (Charralampakis *et al.,* 2011). The recent consensus of the European Workshop on Periodontology highlighted that peri-implant mucositis and early peri-implantitis lesions are often treatable by non-surgical modalities. However, moderate and severe peri-implantitis lesions may not be amenable to treatment by a non-surgical approach (Lang *et*

al., 2011). It is recommended that, even for severe peri-implantitis cases, non-surgical treatment be undertaken initially, including debridement and the establishment of effective oral hygiene measures prior to surgical treatments (Lang *et al.,* 2011; Charralampakis *et al.,* 2011).

The level of evidence to support different protocols for the treatment of peri-implantitis is low. Studies on the treatment of peri-implantitis consist mainly of case reports. Faggion *et al.* (2009) noted that most studies compared several treatment procedures in a small number of patients and reported no power calculation. The authors stated that many serious issues were present in the design, study analysis and reporting of results in investigations of peri-implant treatment outcomes. Faggion *et al.* (2009) highlighted that *"no evidence of difference is however not evidence of no difference"*. Esposito *et al.* (2008), in a Cochrane meta-analysis of human clinical interventions in the treatment of peri-implant infections, observed that "*Sample sizes were too small and follow up too short. This is not to say that currently used interventions are ineffective. Larger well-designed randomised controlled trials are needed.*"

Further understanding of the basic principles of the microbial contribution to the aetiology of peri-implantitis and changes in the micro-flora following mechanical therapy are required. It has been stated that *"Proposed strategies for the treatment of peri-implantitis...must be recognised as empirical"* (Roos-Jansaker *et al.,* 2003). The contribution of mechanical debridement to the re-establishment of peri-implant health has been highlighted as being unknown (Roos-Jansaker *et al.,* 2003). In the absence of randomised controlled trials which examine the efficacy of peri-implantitis treatment modalities, the long term monitoring of consecutive patients utilising individual treatment approaches has been recommended (Roos-Jansaker *et al.,* 2003).

It is recognised that the treatment of peri-implant infections is largely based on the evidence available for the treatment of periodontal infections (Renvert *et al.* 2007). The evidence in relation to the influence of mechanical debridement on peri-implant infections is scant, however, a large body of evidence is available on the influence of mechanical debridement of periodontal sites.

1.13.2 The influence of mechanical debridement on sub-gingival biofilms

The gingival environment consists of multiple, distinct ecological niches. Microorganisms reside in highly ordered ecological systems. The initial colonisers of the exposed cemental surface include mainly streptococci and *Actinomyces* spp. Secondary colonisers include species such as *Prevotella* and *Porphomonas* spp. as these can adhere

to the initial layer through co-aggregation. Within a period of weeks to months, a climax community is established which is highly ordered with different members having different roles and many distinct micro-environments are present.

The sub-gingival plaque has two distinct zones: a zone of Gram-positive cocci and bacilli close to the tooth surface and a zone of Gram-negative organisms next to the gingival crevice (Samaryanake, 2006). Host cellular and humoral immune defences may only influence the most superficial layers of the complex microbial community of the sub-gingival biofilm. Similarly, chemical agents such as disinfectants, biocides and antibiotics, may only interfere with those microorganisms and their products into which they come in contact. Thus the deeper layers of the microbial plaque remain relatively undisturbed despite the host defence, the use of mouth wash disinfectants or administration of systemic or topical antibiotics.

Mechanical disruption of the plaque biofilm may be achieved through supra- and sub-gingival debridement and remains the "gold standard" modality for periodontal treatment. Efficient mechanical debridement results in the removal of the majority of the biofilm complex whilst avoiding deliberate removal of cementum. Complete removal of all deposits is rarely achievable following successful debridement and remnants of the plaque biofilm remain on the root surface and pocket epithelium. The successful outcome for mechanical therapy would be a reduction in the quantity or critical mass of bacterial plaques such that equilibrium between residual microbes and the host defences can be achieved (Cobb, 2002). Removal of sub-gingival plaque and calculus deposits through sub-gingival debridement exposes the root cementum/dentine and pocket epithelium for novel colonisation. Species that may have been successful as climax community members of sub-gingival plaque deposits of established periodontal lesions may find the new habitat less hospitable. The more pathogenic species tend to be climax community members of the biofilm and may be slower growing. This is as a result of being more fastidious and/or requiring ecological factors of the climax community (i.e. anaerobic environment, low pH, nutrients) provided by other neighbouring microbial species. A decrease in pocket depth as a result of a resolution of inflammation, decreased oedema and a re-adaptation of apical connective tissue attachment favours the growth of more aerobic species. Also, a decreased concentration of microbial products, tissue breakdown products and a decrease in the flow of gingival crevicular fluid, along with a more neutral sub-gingival biofilm pH, encourages the growth of less pathogenic species such as

Actinomyces spp. and streptococcal spp. and other Gram-positive aerobic bacterial species which are often early colonisers of tooth and implant surfaces.

Sub-gingival debridement has been observed to result in a decrease in the total number of microorganisms present and a shift in the relative proportion of different microbial species within the sub-gingival plaque biofilm. A decrease in the total bacterial count for sites of 3 mm or greater depth from 91±11 X 10^5 to 23±6 X 10^5 has been observed immediately following sub-gingival debridement (Teles *et al.,* 2006). A decrease in the numbers of *P. gingivalis* (Shiloath & Patters, 1994; Takamatsu *et al.,* 1999; Doungudomdacha *et al.,* 2001; Colombo *et al.,* 2005; Haffajee *et al.*, 1997*a*), *A. actinomycetemcomitans* (Shiloath & Patters, 1994; Doungudomdacha *et al.,* 2001), *P. intermedia* (Shiloath & Patters, 1994; Doungudomdacha *et al.,* 2001; Fujise *et al.,* 2002; Darby *et al.,* 2005), *T. forsythia* (Haffajee *et al.,* 1997*a*; Takamatsu *et al.,* 1999; Colombo *et al.,* 2005; Darby *et al.,* 2005;) and *T. denticola* (Haffajee *et al.,* 1997*a*; Darby *et al.,* 2005) has been reported. A decrease in the numbers of sites colonised by *P. gingivalis* (Shiloath *et al.,* 1994; Haffajee *et al.,* 1997*a*; Takamatsu *et al.,* 1999; Doungudomdacha *et al.,* 2001; Colombo *et al.,* 2005; Cugini *et al.,* 2000), *A. actinomycetemcomitans* (Shiloath & Patters 1994; Doungudomdacha *et al.,* 2001) and *P. intermedia* (Darby *et al.,* 2005; Shiloath & Patters, 1994; Doungudomdacha *et al.,* 2001), *T. forsythia* (Haffajee *et al.,* 1997*a*; Takamatsu *et al.,* 1999; Cugini *et al.,* 2000; Darby *et al.,* 2005; Colombo *et al.,* 2005), *T. denticola* (Ali *et al.,* 1992; Simonson *et al.,* 1992; Shiloath & Patters 1994; Lowenguth *et al.,* 1995; Haffajee *et al.,* 1997; Cugini *et al.,* 2000; Darby *et al.,* 2005) has been observed following sub-gingival debridement. However, the decreased prevalence of these microbial species may not be sustained (Beikler *et al.* 2004).

Repopulation of the new sub-gingival habitat following debridement by the following types of microorganisms may occur: (a) those from residual sub-gingival plaque deposits (Dahlan *et al.,* 2004; Ramberg *et al.,* 1994); (b) those persisting within the radicular dentinal tubules or cementum (Daly *et al.,* 1982; Adriaens *et al.,* 1988; Giuloiana *et al.,* 1997) (c) those from the pocket epithelium and connective tissues (Slots and Rosling, 1983), (d) those from supra-gingival plaque deposits (Magnusson *et al.,* 1984; Ximenez-Fyvie *et al.,* 2000a; Ximenez-Fyvie *et al.* 2000b) and sub-gingival deposits of adjacent teeth and finally (e) from other intra-oral soft tissue sites (von Triol-Linden *et al.,* 1996) (see Figure 1.6). A concomitant increase in the proportions of Gram-positive aerobic cocci and rods is associated with periodontal health (Listgarten *et al.,* 1978; Slots, 1979; Mousques *et al.,* 1980; Magnusson *et al.,* 1984; Henrichs *et al.,* 1985;

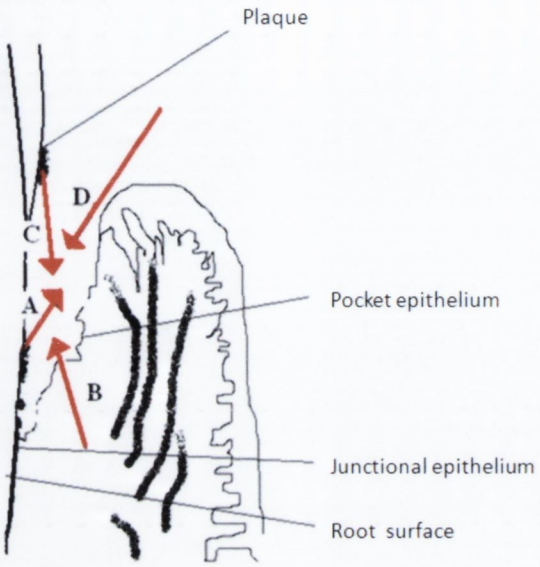

Figure 1.6 Repopulation of the sub-gingival habitat post sub-gingival debridement. Repopulation of the sub-gingival habitat post sub-gingival debridement may be from: (A) residual sub-gingival deposit, (B) soft tissue sites, (C) supra-gingival deposits or (D) deposits present on neighbouring teeth and intra-oral soft tissue sites.

Southard *et al.,* 1989; Sbordone *et al.,* 1990; Haffajee *et al.,* 1997*a*; Stelzel & Florès-de-Jacoby, 2000). Haffajee *et al.* (2006) reported an increase in the proportions of streptococci and *Actinomyces* spp. (including *S. gordonni*, *S. mitis*, *S. oralis* and *S. sanguinis*) as well as *E. corrodens* post sub-gingival debridement. The persistence of *A. actinomycetemcomitans* and *P. gingivalis* following sub-gingival debridement has been attributed to the ability of these microorganisms to invade the pocket epithelium and connective tissues (Slots & Rosling, 1983; Christersson *et al.,* 1985; Renvert *et al.,* 1990; Shiloath & Patters 1994). In the absence of appropriate home care, the re-establishment of pre-treatment microbial plaques, and clinical parameters, will occur in a matter of weeks (Magnusson *et al.,* 1984; Loos *et al.,* 1988; Sbordone *et al.,* 1990). Following debridement and despite appropriate home care, the re-establishment of a pathogenic sub-gingival microbial community and an associated deterioration of clinical parameters in localised sites has been observed from longitudinal investigations examining the impact of this initial phase of periodontal treatment on microbial growth and implant health (Becker *et al.,* 1984; Kocher *et al.,* 2000; Konig *et al.,* 2001; Checchi *et al.,* 2002). Haffajee *et al.* (2006) observed at three months post-debridement similar microbial profiles for those sites that went on to display decreased, stable or increased pocket depth measurements at one year following debridement. However, distinct differences were observed during the study for those sites that showed decreased, stable or increased pocket depth measurements at one year following debridement. Sites that remained stable or which had an increased probing attachment level at 12 months, maintained microbial profiles over the 12-month period. However, for those sites which demonstrated decreasing probing attachment levels in this time, a re-emergence of species of the red and orange complexes was detected in the three to 12 month period. The red complex includes *P. gingivalis, T. forsythia, T. denticola* and the orange complex includes *F. nucleatum, P. intermedia, P. nigrescens, Peptostreptococcus micros, Streptococcus constellatus, Eubacterium nodatum, Campylobacter showae, C. gracilis, C. rectus* as described by Socransky *et al.* (1998) who observed species which were commonly detected together by undertaking microbial population analysis and community ordination analysis. This confirmed the importance of the maintenance phase of periodontal therapy. In the absence of professional maintenance, an increase in the prevalence and counts of periodonto-pathogens is to be expected (Westfelt *et al.,* 1983; Renvert *et al.,* 1990; Shiloath & Patters, 1996).

1.13.3 Non-surgical treatment of peri-implant mucositis

The goal of treatment of peri-implant infections is the control of infection and prevention of disease progression (Mayfield, 2008). It may be suggested that compared to the volume and quality of the research to date on the response of periodontitis to non-surgical treatment, there is a paucity of data investigating the efficacy of non-surgical treatment of peri-implant infections (Renvert *et al.* 2006 & 2008). The treatment of peri-implant mucositis requires that infection and inflammation are addressed through removal of the causative stimulus, namely the plaque biofilm, and the subsequent resolution of the peri-implant inflammation or peri-implant mucositis. Effective removal of the plaque biofilm is achieved through mechanical debridement of the available implant surfaces and indeed any teeth present. Targeted oral hygiene instruction can then be recommended and the recall interval adjusted as appropriate to minimise the likelihood of recurrence of the peri-implant mucositis. Animal and clinical investigations have been undertaken and demonstrate the efficacy of this treatment approach, especially if combined with the use of topical anti-microbial agents, in achieving peri-implant health. The available evidence is outlined below.

It has been demonstrated in both animal and clinical investigations that non-surgical root debridement and oral hygiene instruction are effective in the treatment of peri-implant mucositis (Renvert *et al.,* 2008; Lang & Berglundh, 2011). In a study investigating the response of experimentally induced peri-implant mucositis lesions in nine cynomolgus monkeys to (a) mechanical debridement alone, (b) mechanical debridement and adjunctive use of 0.2% chlorhexidine gel and irrigation with 0.12% chlorhexidine and (c) no treatment (control group). Results demonstrated an improvement in both probing depth and clinical attachment levels (Trejo *et al.*, 2006). Histopathological examination revealed significantly smaller peri-implant inflammatory lesions in treatment groups compared to the control group ($p<0.01$). No significant difference was observed between the peri-implant inflammatory lesions of the two treatment groups. Therefore, it has been demonstrated that non-surgical debridement is effective in the treatment of peri-implant inflammatory lesions of peri-implant mucositis, resulting in improvements in clinical attachment level, probing depth and the resolution of inflammation.

Clinical studies also confirmed the efficacy of non-surgical root debridement of peri-implant mucositis lesions with or without adjunctive antimicrobial agents. Both essential oil-containing mouthwashes (Ciancio *et al.,* 1995) and self-administered

chlorhexidine irrigation (Felo *et al.,* 1997) have been shown to result in clinical improvements in probing pocket depths and reduced bleeding on probing. Felo *et al.* (1997) undertook a clinical investigation of the adjunctive administration of 0.06% chlorhexidine (Periogard, Colgate Palmolive, Surrey, UK) subgingivally by a powered oral irrigator (WaterPik, Water Pik, Inc., Colorado, USA) compared to rinsing with a 0.12% solution of chlorhexidine gluconate once daily. The authors noted that those patients who utilised the subgingival administration of the chlorhexidine 0.06% showed statistically significant improvements in their modified gingival index and bleeding index, whilst patients who rinsed with chlorhexidine 0.12% did not. De Araujo Nobre *et al.* (2006) noted reductions of probing pocket depths and bleeding scores for seven of nine patients who underwent mechanical debridement, oral hygiene instruction and subgingival irrigation with chlorhexidine gel. Porras *et al.* (2002) noted improvements for 16 patients treated with debridement and oral hygiene instruction with or without the adjunctive administration of chlorhexidine irrigation or topical gel administration. Strooker *et al.* (1998) undertook a clinical trial in the treatment of peri-implant mucositis, which compared the efficacy of non-surgical mechanical debridement utilising carbon fibre curettes (and polishing the implant and bar surfaces using a rubber cup and prophylactic paste) to the application of 35% phosphoric acid (pH 1) to the peri-implant sulcus in 16 patients, employing a split mouth design. Microbial paper-point samples were taken from the peri-implant sites prior to and following treatment on each of three occasions: baseline, one month post-treatment and five months post-treatment. Both treatment modalities were observed to result in a significant reduction of the number of colony forming units (cfu) on each of the three occasions. However, the effects of the change in the peri-implant microflora was short lived, as at each timepoint (baseline, one month and five month visits), no differences were observed in the number of cfu prior to treatment on each of the three occasions. The samples were examined for the frequency of detection of selected microorganisms prior to and following treatment. The frequency of detection of *F. nucleatum* and *P. micros* was observed to change little before and after treatment on each of the three occasions. However, the frequency of detection of *B. forsythus, C. rectus, P. gingivalis* and *P. intermedia* were very low prior to each of the treatments. *Aggregatibacter actinomycetemcomitans* was not recovered from any sample. Interestingly, the authors observed a reduction in mean probing depths for both treatment modalities. A probing force of 0.65 N was employed, utilising a force controlled probe (Brodontic Dentsply Ash, Surrey, UK). The initial probing depth was observed to be

shallow (2.97 mm and 2.83 mm for the test and control sides, respectively). At the five-month visit, the mean probing depths were decreased, with an average of 0.63 mm on the test side and 0.35 mm on the control side. No significant differences were observed between the test and control side for probing pocket depth (PPD) or percentage sites bleeding on probing (% sites BOP). Strooker *et al.* (1998) recognised that the treatments were undertaken in patients with "*relatively shallow pocket depths*". This has implications for the interpretation of the data with regards the changes in the peri-implant microflora and the efficacy of the intervention. Patients had peri-implant probing depths at baseline of 2.97 (±0.68) mm and 2.83 (±0.57) mm for the test and control side, respectively, which were seen to improve following treatment by approximately 0.5 mm to 2.34 (±0.54) mm and 2.48 (±0.49) mm, respectively. Similarly, the percentage of peri-implant sites bleeding on probing at baseline was quite low at approximately 30% (30.5% and 29.2% for tests and controls, respectively). This was observed to decrease to 22.7% (± 23.31) and 19% (± 17.27), respectively, at one month and 9.7% (± 10.97) and 14.3% (± 22.47) at five months. Thus, the tissues were relatively healthy at baseline and the margin for improvement was small. It is not surprising that the number of colony forming units did not demonstrate a sustained change between visits, as it is likely that the number of colony forming units associated with relative health were restored in the intervening time interval. This study demonstrated that it is possible, in cases of peri-implant mucositis, through mechanical debridement employing Teflon curettes or through the application of 30% phosphoric acid, to disrupt the peri-implant biofilm sufficiently, to cause a decrease in the number of colony forming units. However, the investigators observed that the changes observed post operatively were of short duration, as at one month later, and four months following the second round of treatment, no changes in the peri-implant microflora were observed to be sustained. Clinically, the authors observed that there was a trend towards a gradual small reduction in both probing depth and bleeding on probing over time.

A randomised controlled trial involving 29 patients affected by peri-implant mucositis, showed that at one and three month intervals post-mechanical debridement and oral hygiene instruction, no significant beneficial effect was noted by patients using chlorhexidine gel (0.5%) twice daily to brush their teeth compared to those using a placebo (Heitz-Mayfield *et al.* (2011). Heitz-Mayfield *et al.* (2011) undertook a double blind randomised controlled trial examining the influence of debridement, with the adjunctive use of chlorhexidine gel (0.5%), compared to placebo, in the treatment of peri-

implant mucositis in 29 patients. Probing was undertaken using a graduated periodontal probe with a light probing force (approximately 0.2-0.3 N) (Gerber *et al.*, 2009). The deepest peri-implant probing depth sites were sampled at baseline and one month post-treatment. Thirty-eight percent of patients had complete resolution of peri-implant inflammation following the treatment. A significant reduction in bleeding on probing was observed for both groups from baseline to one-month post-treatment ($p<0.05$), with no significant difference between groups ($p>0.1$). No significant changes in peri-implant probing depths were observed between baseline and one-month post-treatment or between baseline and three-months post-treatment. Significant reductions in total units of DNA isolated were observed between baseline and one-month post-treatment ($P<0.1$). A reduction in peri-implant inflammation was observed following both interventions, however, there was no adjunctive benefit to the administration of chlorhexidine gel (0.5%). The authors concluded that mechanical debridement and oral hygiene instruction was effective in reducing peri-implant mucositis. The authors noted that peri-implant sites where crown margins were placed sub-gingivally, were more difficult to maintain plaque free and more likely to demonstrate signs of peri-implant inflammation.

1.13.4 Non-surgical treatment of peri-implantitis

Macro- and microscopic features of dental implants (screw shaped designs and their roughened surfaces), engineered to improve osseointegration, when exposed to the oral environment through bone loss, facilitate plaque accumulation (Renvert *et al.*, 2008) and therefore contribute to progressive peri-implant bone loss. It has been suggested that "*Treatment models such as scaling and root planing, effectively used to treat root surfaces of teeth with periodontal disease, are not suitable for peri-implantitis treatment.... infection control by elimination or significant reduction in bacterial load to a level allowing healing is therefore difficult to accomplish with mechanical means alone*" (Renvert *et al.*, 2008). Few studies have been undertaken to examine the response of the peri-implantitis lesions to non-surgical debridement. This is surprising as this is the most basic of treatments and, even if not found to be efficacious, would present a baseline of comparison for other modalities of treatment. One of the challenges that must be overcome if mechanical debridement is to be successful in achieving a reduction in microorganisms with virulence factors in the submucosal peri-implant niche, is the effective debridement of titanium surfaces. *Staphylococcus aureus* has been shown *in vitro* to be very difficult to remove from titanium surfaces by mechanically scraping alone (Bjerkan *et al.*, 2009). Systemic antimicrobials, combined with local adjunctive measures,

have been observed to result in an improvement in probing pocket depths, gingival indices and microbial parameters (Mombelli & Lang, 1992; Khoury & Buchmann, 2001). Mechanical debridement in combination with the adjunctive use of topical or systemic anti-microbials has been observed to be effective in the resolution of peri-implant infections for the majority of patients (Heitz-Mayfield & Lang, 2004).

Mombelli & Lang (1992) undertook an investigation of nine patients with titanium hollow implants demonstrating bone loss and probing depths of at least 5 mm, six months following implant placement. The authors highlighted that the treatment was directed towards decreasing the "*sub-gingival bacterial mass and suppressing the anaerobic segment*". Implants underwent mechanical debridement and sub-mucosal irrigation with 0.5% chlorhexidine gel and patients were prescribed systemic ornidazole 1000 mg orally for ten days. A significant improvement in bleeding score was observed immediately following the treatment, which was maintained over one year. Significant improvements in peri-implant probing pocket depths were observed gradually over the one year observational interval - from 5.89 to 3.43 mm ($p<0.001$) and a significant reduction in peri-implant bleeding score from 89 to 43% in the one year interval compared to baseline values ($p<0.01$). Furthermore, one year following the treatment, significantly improved microbial profiles were observed (where microbial samples underwent culture, utilising continuous anaerobic culture techniques). Significant reductions in the frequencies of isolation of *P. intermedia, Fusobacterium* spp., *Actinomyces odontolyticus* and *A. Wolinella* were observed, comparing baseline microbial profile data to those gained at one year ($p< 0.01$). The authors highlighted that *"In some cases, this approach may lead to sufficient improvement of clinical conditions, in others it may serve as a basis for further therapy, such as guided tissue regeneration procedures*".

De Araujo Nobre *et al.*(2006) undertook a study of 20 patients with moderate to advanced peri-implantitis who underwent treatment with either an Er:YAG laser or mechanical debridement and subgingival irrigation with chlorhexidine gel (0.2%). Reductions in clinical attachment levels were noted for both the laser and chlorhexidine groups, the mean change in the CAL for the chlorhexidine sites was from 6.2 ±1.5 mm at baseline to 5.6 ±1.6 mm after six months ($p<0.001$), whilst the Er:YAG laser group demonstrated a mean change in CAL from 5.8 ±1 mm to 5.1± 1.1 mm.

Leonhardt *et al.* (2003) undertook a case series examining the outcome of administration of systemic antibiotics (according to susceptibility testing), following open surgical debridement and application of hydrogen peroxide to peri-implantitis sites in

nine patients (26 implants) and followed up for six months, one year and five years post-treatment. In total, 7/26 implants were lost and 5/26 implants demonstrated progression of peri-implant bone loss in the five-year follow up. Mombelli *et al.* (2005) investigated 25 patients with peri-implantitis (30 implants). Implants underwent debridement as "soft and hard deposits were removed from all accessible implant surfaces" with a plastic scaler and fibres containing tetracycline HCl were packed into the sulcus and glued in place with a cyanoacrylate adhesive. Following 10 days, the fibres were removed. Patients were placed on chlorhexidine 0.2% twice daily for two weeks. Patients were re-examined at one, three, six and 12 months post-treatment. Two patients with persisting peri-implantitis and suppuration were dismissed from the study. One further patient demonstrated deepening probing depths, whilst six patients demonstrated no change in probing depth. All other patients demonstrated reductions in probing depths of their deepest site that was "*quite remarkable in some cases*". The mean bleeding index was observed to decrease significantly over the 12-month observation interval ($p<0.0001$), whilst the probing pocket depths also decreased significantly ($p<0.001$). Treatment resulted in a reduction of bacterial load by a factor of 6.3. Furthermore, treatment resulted in significant reductions of *P. intermedia/nigrescens*, *Fusobacterium* spp., *B. forsythus* and *Campylobacter rectus* ($p<0.01$). *Fusobacterium* counts were observed to be elevated in the two cases of progressive peri-implantitis with suppuration.

Persson *et al.* (2006) examined the changes in the microflora in a prospective study of 25 patients (31 implants) affected by peri implantitis. Implants demonstrating signs of peri-implant bone loss and probing depths of ≥5 mm underwent debridement with adjunctive administration of 0.2% chlorhexidine and minocycline microspheres (Arestin OraPharma, Inc, Warminster, Pennsylvania, USA). Patients were observed for one year and demonstrated significant reductions of probing pocket depths ($p<0.001$) and bleeding on probing ($p<0.001$). DNA-DNA checkerboard hybridisation techniques were employed to examine the changes in the peri-implant microflora. At 180 days post-debridement, a statistically significant decrease in the total bacterial load was observed. Furthermore, for 27/40 microorganisms investigated, a significant decrease in their numbers was noted, these included *A. actinomycetemcomitans* and *P. gingivalis* ($p<0.01$). However, at 270 days post-debridement the reduction in bacterial load was no longer significant ($p<0.12$), but significantly lower levels of 12/40 microorganisms were present, including *A. actinomycetemcomitans* ($p<0.05$). At 12 months post-debridement, the bacterial load was not significantly different from baseline, nor was there a significant

reduction for any complex or bacteria other than *A. actinomycetemcomitans* ($p<0.05$). Sampling was undertaken utilising sterile curettes. The authors examined the baseline data for each of the implant sites that failed to respond to treatment, either by losing integration or demonstrating a large increase in microbial load compared to baseline. None of the investigated species were observed to be associated with implant loss ($p>0.01$). A dichotomous rating was employed to further elucidate the effects of the treatment on the peri-implant microflora. At baseline, four patients had no microbial species greater than an arbitrary cut-off point of 10^5 bacterial cells Ten days after the administration of the local antibiotics, 13/25 patients had no detectable bacteria based on the 10^5 detection level. Three subjects who had baseline positive findings of 15 or more pathogens at the 10^5 level, had <4 pathogens detectable at the 10^5 level at day 360.

Maximo *et al.* (2009) investigated the treatment of peri-implant infections by mechanical debridement of implants. Open-flap debridement of implants affected by peri-implantitis in 13 patients was carried out, employing teflon curettes and abrasive sodium carbonate air powder. Ten patients with healthy implants served as a control group. For 12 patients with implants affected by peri-implant mucositis, non-surgical debridement was undertaken with teflon curettes and abrasive sodium carbonate air powder. A mean reduction in probing depth of 1.3 ± 1.2 mm was observed for peri-implant mucositis implants and a mean reduction in probing depth of 3.1 ± 1.7 mm was observed for peri-implantitis implants following the treatments. For both peri-implantitis and peri-implant mucositis groups, significant improvements were observed in each of the following parameters: the number of implants demonstrating one or more sites with probing pocket depths of ≥5 mm; those demonstrating bleeding on probing and those demonstrating suppuration on probing. Curettes were employed to take submucosal microbial samples and samples were analysed employing checkerboard DNA-DNA hybridisation techniques. For the peri-implantitis group, following treatment, significant differences were observed in the mean bacterial counts of: *Parvimonas micra* (orange complex); *Tanerella forsythia*; *Porphyromonas gingivalis*; *Treponema denticola* (red complex) and *Treponema socranskii* (red complex) ($p<0.05$). An increase in counts of *Neisseria mucosa* was observed following treatment ($p<0.05$). Furthermore, the total bacterial counts were observed to be significantly decreased in the peri-implantitis group following treatment ($p<0.05$). For the peri-implant mucositis group, significant reductions were observed between the mean bacterial counts of *Fusobacterium nucleatum ss. nucleatum*, *P. gingivalis* and *T. socranskii* prior to and following treatment ($p<0.05$), whilst an increase

was observed in the mean bacterial counts of *Gemella morbillorum* following treatment ($p<0.05$). *Tanerella forsythia* and *Actinomyces gerencseriae* numbers were observed to be higher in the peri-implantitis group compared to the peri-implant mucositis and the healthy implants ($p<0.05$). *Capnocytophaga ochracea* was observed to be significantly increased in the peri-implant mucositis group when compared to the other groups. The healthy implants demonstrated very low levels of the three red complex pathogens.

In a study of 32 peri-implantitis patients, a reduction of peri-implant probing depths was observed following mechanical debridement and application of minocycline microspheres or 0.1% chlorhexidine gel (Renvert *et al.*, 2008). A diverse selection of microbial species were investigated utilising both anaerobic culture and DNA-DNA hybridisation techniques. Both treatments resulted in a reduction in numbers of indicator bacteria, however, no differences were noted in the mean total viable counts. Persson *et al.* (2010) observed no difference in the peri-implant microflora for implants demonstrating signs of peri-implantitis, comparing baseline and one year post-treatment. Renvert *et al.* (2009) undertook a study of 31 patients who completed non-surgical, periodontal treatment either with titanium hand instruments or ultrasonic device (Durr Vector system, Durr Dental AG, Beitigheim-Bissingen, Germany), adapted with a tip designed for debridement of dental implants (LM Instruments Oy, Parainen, Finland). Subsequently, all implants were polished and patients received oral hygiene instructions individually at each time point. No differences were observed between the two groups and the data was merged. Probing depths demonstrated no differences between baseline and six months ($p=0.3$). However, significant differences were noted at six months for bleeding on probing at the worst peri-implant sites between time points ($p<0.01$). Renvert *et al.* (2009) noted that only 15% of sites that underwent mechanical debridement by hand instruments or ultrasonic devices, had ceased bleeding on probing six months following treatment.

Persson *et al.* (2010) examined the peri-implant microflora of 31 patients before and six months following non-surgical mechanical treatment. Non-surgical mechanical treatment was undertaken on 17 patients employing titanium curettes and on 14 patients usingthe Durr Vector system (Durr Dental AG, Germany), which were adapted with a tip designed for debridement of dental implants, as in the previous study. Semi-quantitative analysis was undertaken for 79 bacterial species utilising checkerboard DNA-DNA hybridisation techniques. Interesting differences were observed between the peri-implant microflora detected in those patients for whom debridement was undertaken with curettes

and those for whom ultrasonic devices were employed. Thirty minutes post-debridement, significant decreases in the counts of bacterial species were observed for *A. actinomycetemcomitans* (serotype a), *L. acidophilus*, *Streptococcus anginosus* and *Veillonella parvula* ($p<0.01$) for the curette group. For those implants that underwent debridement using the ultrasonic device, no significant differences or trends towards a decrease in particular microbial species were observed 30 minutes following debridement. The authors noted that at six months post-debridement, bacterial reductions had been lost and the peri-implant microflora was similar to the baseline microflora. Persson *et al.* (2010) hypothesised that the lack of improvement in microbial profiles following mechanical debridement and oral hygiene instruction may have resulted from:

a) The difficulties in achieving adequate debridement of implant exposed surfaces non-surgically.
b) The efficacy of patients' oral hygiene were not as effective as hoped for.
c) The biofilm of peri-implant infections may include pathogens that were resistant to treatment.

Schwartz *et al.* (2011) carried out a study of 32 patients affected by advanced peri-implantitis, who were treated with flap surgery implantoplasty (changing of the shape of the dental implant fixture surface to decrease plaque retention) and debridement with either plastic curettes, cotton pellets and sterile saline or a erbium-doped yttrium aluminium garnet laser (Er:YAG laser). The intra-bony component was augmented with natural bone mineral and covered with a collagen membrane. No significant differences were noted between groups ($p>0.05$). The authors noted no significant impact of method of surface debridement on the clinical outcome following combined surgical treatment of advanced peri-implantitis lesions ($p>0.05$). Sahm *et al.* (2011) undertook a randomised controlled trial of 30 patients, comparing mechanical debridement outcomes for either air abrasive or carbon curettes, with subsequent chlorhexidine digluconate application. At six months, both groups demonstrated similar improvements in probing pocket depths, 0.6 +/- 0.6 mm and 0.5 +/- 0.6 mm, respectively, and these differences were not significant ($p>0.05$). Bleeding scores were observed to decrease by 43.5 +/- 27.7% and 11 +/- 15.7% for the air abrasive and carbon curette groups, respectively, with a significantly greater reduction in bleeding scores observed for the air abrasive group.

Thone-Muhling *et al.* (2010) undertook a randomised controlled trial comparing the efficacy of full mouth debridement of 13 patients with 36 3i™ *Osseotite* BIOMET (3i UK Ltd., Berkshire, UK) implants affected by peri-implant mucositis, with and without

the adjunctive administration of chlorhexidine 1% gel subgingivally. Patients were recalled on four occasions during the observation period and followed up clinically and microbiologically for eight months. A standardised plastic periodontal probe (Plast-o-Probe, Dentsply Maillefer, Chemin du Verger 3, 1338 Ballaigues, Switzerland) was employed in examination of the peri-implant tissues. Size 30 paper-points were taken from each implant and the samples pooled and analysed employing PCR to examine the 16SrRNA. Significant reductions were noted with regards to probing pocket depths for both groups (test group: -0.65 mm p=0.033; control group -0.58 mm, p=0.004), with no difference between groups. Reductions were observed in bleeding on probing for both groups, however, differences were not statistically significant at eight months (p>0.05). *Porphymonaas gingivalis, P. intermedia* and *C. rectus* levels remained below the threshold level (limit 10^2 cells) throughout the study. The authors commented that the additional value of chlorhexidine was not clear.

Machtei *et al.* (2012) undertook a randomised controlled clinical trial including 60 peri-implantitis patients and examined the adjunctive administration of chlorhexidine gluconate 2.5 mg topical antimicrobial agents, as prepared in the Perio Chip (Dexcel Pharma GmbH, Or-Akiva, Israel) and control topical preparations which did not contain the chlorhexidine gluconate 2.5 mg active agent, which were prepared for the investigation by Dexcel Pharma GmbH and referred to as Matrix C. Patients were either administered Perio Chip (Dexcel Pharma GmbH, Israel) or Matrix C (Dexcel Pharma GmbH, Israel), on seven occasions within an 18 week period. Patients were examined at six months post-treatment and compared. Seventy percent of implants in the Perio Chip group and 54% of implants in the Matrix C group had reductions of ≥2 mm in probing depth (mean reductions of 2.19 ±0.24 mm and 1.59 ± 0.23 mm respectively), with differences between the two groups not being significant (p=0.08). Bleeding on probing was reduced by half in both groups. Gradual reductions in both probing pocket depth and bleeding on probing were noted for both groups (from 100% to 43.3% and 59% for the test and control respectively). It is interesting to note that the Matrix C group, in whom the matrix of the Perio Chip was administered without the active ingredient, also demonstrated significant probing depth reduction and reductions of bleeding on probing. The authors suggested that the mechanical debridement and the degradation of the chip that released glutaraldehyde probably resulted in some improvement. The authors also highlighted that the repeated application protocol may be acting to disrupt the biofilm, thus exerting a mechanical effect that was beneficial. The authors similarly observed that

the clinical response to the a similar protocol of placing chlorhexidine chips around natural teeth, resulted in "incredibly similar" reductions in probing pocket depths – 2.09 mm compared to the 2.13 mm reduction noted around dental implants.

1.14 Investigation

The present investigation sets out to further understanding of the aetiology of peri-implant diseases and provide clinical and microbiological evidence as to the efficacy of non-surgical mechanical treatment in the treatment of peri-implant infections.

1.15 Research Questions

1. Are patients who smoke or have a history of severe periodontal disease at a greater risk of developing peri-implantitis than patients who do not smoke or demonstrate severe periodontal attachment loss?

2. Does non-surgical mechanical treatment (i.e. oral hygiene instruction and mechanical debridement) of peri-implant infections result in changes in the peri-implant clinical parameters of probing pocket depth, bleeding on probing and suppuration probing?

3. Does the method of collection of the sub-mucosal microbial sample (i.e. using paper-point or curette) influence the composition of microbial communities isolated from periodontal and peri-implant sites?

4. Are there differences in the microflora around natural teeth and implants; around implants affected by mucositis and those affected by peri-implantitis; between patients who are smokers and those who are non-smokers; and patients with a demonstrated susceptibility to periodontal disease and those with no demonstrated susceptibility to periodontal disease?

5. Is it possible to influence the composition of the peri-implant microflora in cases of peri-implant mucositis and peri-implantitis by non-surgical mechanical treatment alone (i.e. without adjunctive antimicrobial agents or surgical interventions)?

Chapter 2

General Materials and Methods

2.1 Patient selection

2.1.1 Ethical approval

Approval for the present study was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Dublin, Trinity College Dublin, Ireland. At the initial screening and baseline examination visit, all patients were provided with the Patient Information Sheet and the Patient Consent Form (see Appendix 1). At their second visit, at least one week following the initial consultation, patients signed the Patient Informed Consent Form prior to being included in the present investigation.

Patients were recruited from the Division of Restorative Dentistry and Periodontology of the Dublin Dental University Hospital list of patients who had one or more dental implants placed five years previously. The following inclusion and exclusion criteria were applied:

2.1.2 Inclusion criteria:

- Patients over the age of 18 years.
- Patients with dental implants that have been in place for five or more years.
- Patients with one or more implant restorations, with a diagnosis of peri-implantitis or implant loss. This was a requirement for both of the interventional studies, which examined the response of peri-implant infections to non-surgical treatment (Chapters 5 and 7), but not for the two observational studies, which examined a) the risk indicators for peri-implantitis and implant loss (Chapter 4) and b) the periodontal and peri-implant microflora in health and disease (Chapter 6).
- Provision of a signed Patient Informed Consent Form.

2.1.3 Exclusion criteria:

- Requirement for prophylactic medication.
- Pregnant or lactating women.
- Patients having taken antibiotics, antifungals or steroid medication (which may affect the microbial flora), within two months of enrolment.
- Individuals under 18 years of age.
- Adults with learning disabilities and unable to provide informed consent.
- Patients with a terminal illness.

2.1.4 Dental and medical history and an oral examination

At the baseline consultation, a detailed dental and medical history and an oral examination were undertaken. This included a detailed periodontal examination, involving full mouth probing pocket depths measurement and measurement of recession and bleeding on probing. The periodontal probe was inserted into the periodontal or peri-implant sulcus at six points for each unit, values were recorded for the deepest probing depth at each of the following sites: the distobuccal, midbuccal, mesiobuccal, mesiolingal, midlingual and distolingual. All sites demonstrating bleeding or suppuration on probing were recorded. A plastic periodontal probe with a pressure sensitive indicator corresponding to 0.2 – 0.25 N force was used for all periodontal and peri-implant probing (Click Probe, Kerr Hawe, Bioggio, Switzerland). A peri-implant probing force of between 0.2–0.3 N has been recommended as the most reliable in disclosing the connective tissue adhesion level (Schou *et al.,* 2002). Recession values were recorded by measuring the distance from the most coronal aspect of the gingival margin to the cemento-enamel junction for teeth and from the mucosal margin to the portion of the ceramic deemed to represent the commencement of the clinical crown.

Periodontal examination was undertaken by one of two experienced examiners (Ms. Karin Nylund, an experienced dental hygienist and the author. The examiner who undertook the detailed periodontal examination for an individual patient was always the same. Additionally, both examiners undertook repeated examination of five separate patients. Differences in measures recorded did not vary by greater than 1 mm for over 90% of the sites for either operator, indicating good intra-examiner reproducibility (Schwartz *et al.*, 2005).

2.1.5 Radiographic Examination

Peri-apical radiographs were undertaken utilising a Rinn holder (Dentsply Rinn, Dentsply UK, Surrey, UK) and employing the long cone paralleling technique, i.e. the peri-apical radiograph film, the implant and the collimator face were aligned as parallel as possible and the radiograph exposed for the recommended time interval dependant on the implant's anatomical location (Kelly *et al.,* 1975). Radiographs included both conventional and digital radiographs. Conventional radiographs were viewed on a light box in a darkened room and a photograph was taken at a shutter speed of 0.32 s on a Cannon 30 D camera with a Macro 250 mm lens (Canon UK Ltd., Surrey, UK). Similarly, where phosphor plates (Sidexis Dental Systems, Charlotte, North Carolina,

USA) were employed to capture a digital image of the radiograph, images were viewed in a darkened room and the settings adjusted to optimally view the peri-implant bone levels. Digital images were imported to Windows XP Paint Shop (Microsoft Corporation, Washington, USA), cropped and pasted to Microsoft Office PowerPoint 2007 (Microsoft Corporation, USA) and coded according to the patient's investigation number and the implant's anatomical location. The diagnosis of peri-implantitits required that >2.5 mm of peri-implant interproximal bone loss was observed (see Section 2.2). Where radiographs that had been taken one year following delivery of the implant restoration were available, these were compared to the radiograph taken at the baseline examination as part of this investigation. However, in many instances, such one-year post implant placement radiographs were not available and in these cases the extent of the bone loss from the first implant thread was measured. Measurements were made by counting the number of implant threads between the peri-implant bone level and the reference point (either the first thread or the bone level on the one year post operative radiograph, where available). As the inter-thread distance was known, bone loss could then be calculated. All radiographs were examined by two experienced periodontist examiners, with regard to diagnosis of peri-implant bone loss of ≥2.5 mm and Kappa analysis (Viera *et al.*, 2005) revealed good agreement between examiners ($p<0.001$). Where discrepancies were present between examiners, a third experienced periodontist examiner was employed and the majority vote determined the diagnosis reached. As all radiographs were coded according to the patient number and the tooth number, examiners were blinded to the identity of radiographs and the clinical parameters.

2.1.6 Patient characteristics in each study

Patient demographics are described in Appendix A2

2.1.6.1 Observational clinical (risk indicator) study

In the observational clinical (risk indicator) study (Chapter 3), 51 patients were included in total. Of these, 13 (25.5%) were male and 38 (74.5%) were female. Forty-five per cent (23/51) were non-smokers, 23.5% (12/51) were smokers and 31.4% (16/51) were ex-smokers. Causes of tooth loss were as follows: 9.8% (5/51) were due to failed bridgework; 31.4% (16/51) due to periodontitis; 21.6% (11/51) due to hypodontia; 19.6% (10/51) due to caries; 13.7% (7/51) due to trauma; 3.9% (2/51) due to unerupted teeth.

2.1.6.2 Interventional clinical study

In the interventional clinical study (Chapter 4), there were 47 patients in total. Of these, 12 (25.5%) were male and 35 (74.5%) were female. In total 46.8% (22/47) were non-smokers, 36.2% (17/47) were smokers and 12.8% (6/47) were ex-smokers. Causes of tooth loss were as follows: 12.8% (6/47) were due to failed bridgework; 25.5% (12/47) due to periodontitis; 27.7% (13/47) due to hypodontia, 12.8% (6/47) due to caries; 17% (8/47) due to trauma; 4.3% (2/47) due to unerupted teeth.

2.1.6.3 Sampling technique comparison study

In sampling technique comparison study (Chapter 5), there were 28 patients in total. Of these, seven (25%) were male and 21 (75%) and were female. In total 7.1% (2/28) were non smokers, 53.6% (15/28) smokers, 39.3% (11/28) were ex-smokers. Causes of tooth loss were as follows: 32.1% (10/28) were due to periodontitis, 17.9% (4/28) were due to hypodontia; 14.3% (4/28) were due to caries; 14.3% (4/28) were due to trauma; 14.3% (4/28) were due to failed bridgework and 7.1% (2/28) were due to an unerupted tooth.

2.1.6.4 Observational baseline microbiological comparison study

In the observational baseline microbiological comparison study (Chapter 6) there were 41 patients in total. Of these, 8 (19.5%) were male and 33 (80.5%) were female. 53.7% (22/41) were non-smokers, 34.1% (14/41) were smokers and 9.8% (4/41) were ex-smokers. Causes of tooth loss were as follows: 14.6% (6/41) were due to failed bridgework; 29.3% (12/41) due to periodontitis; 24.4% (10/41) due to hypodontia, 12.2% (5/41) due to caries; 14.6% (6/41) due to trauma; 4.8% (2/41) due to unerupted teeth.

2.1.6.5 Interventional microbiological changes study

In the interventional microbiological changes study (Chapter 7), there were 28 patients in total. Of these, five (17.9%) were male and 23 (82%) were female. 64.3% (18/28) were non-smokers, 21.4% (6/28) were smokers and 14.3% (4/28) were ex-smokers. Causes of tooth loss were as follows: 14.3% (4/28) were due to failed bridgework; 28.6% (8/28) due to periodontitis; 18% (5/28) due to hypodontia, 14.3% (4/28) due to caries; 21.4% (5/28) due to trauma; 3.6% (1/28) due to an unerupted tooth.

2.2 Definitions

2.2.1 Peri-implantitis

Implants were classified as being affected by peri-implantitis using the recommendations of the Peri-implantitis Working Group of the European Workshop on Periodontology (2002) if they had: bleeding or suppuration on probing; probing pocket depth of ≥6 mm and radiographic bone loss ≥2.5 mm (Berglundh *et al.*, 2002).

2.2.2 Peri-implant mucositis

Implants were classified as being affected by peri-implant mucositis, using the recommendations of the Peri-implantitis Working Group of the European Workshop on Periodontology (2002) if they had peri-implant bone loss <2.5 mm and demonstrating at least one site with probing pocket depths of ≥6 mm, but demonstrating bleeding or suppuration on probing.

2.2.3 Peri-implant health

Patients with implants not demonstrating bleeding or suppuration on probing were diagnosed as being healthy implants.

2.2.4 Peri-implant mucositis peri-implantitis patients, patients and peri-implant healthy patients

For the purposes of this investigation, patients with implants positive for peri-implant mucositis were classified as "peri-implant mucositis patients", whereas patients with implants positive for peri-implantitis were classified as "peri-implantitis patients". It is important to note that, where patients had implants affected by peri-implantitis they were classified as peri-implantitis patients, even if they also had healthy implants or implants affected by peri-implant mucositis. Similarly, patients who were positive for peri-implant mucositis, who also had healthy implants, were classified as peri-implant mucositis patients. Finally, patients who only had healthy implants were classified as "peri-implant healthy patients".

2.2.5 History of severe periodontal disease

The lack of a clear definition of periodontitis consistently employed across studies, limits the interpretation of available evidence for the relationship between periodontitis and

peri-implantitis (Renvert & Persson, 2009). Following the recommendations of the European Workshop on Periodontology (2005), patients were classified as having experienced severe periodontal disease if ≥30% of sites demonstrated inter-proximal clinical attachment loss of ≥5 mm (Tonetti & Claffey, 2005).

2.2.6 Patients positive for smoking

Patients who were active smokers, or had ceased smoking within the 12 months prior to baseline examination, were classified as smokers. Patients who reported not smoking in the 12 months prior to examination were classified as non-smokers.

2.3 Non-surgical treatment of peri-implant infections

2.3.1 Mechanical debridement

Full mouth debridement was undertaken for each patient at separate visits to the baseline examination. Debridement was undertaken over one to four visits, according to the patient's ability to tolerate treatment and the difficulties of the instrumentation required. Local anaesthesia was employed when necessary. The goal was to achieve the end point of deposit free implant and tooth/ root surfaces. Debridement of implant fixture surfaces was undertaken using titanium tipped curettes (Salvin Dental Specialties, Charlotte, North Carolina, USA) as follows: gross supra-mucosal plaque and calculus deposits were removed firstly, if present. The curette tip was then placed in contact with the implant surface in the region of the most coronal threads. The curette tip was utilised to debride the implant surface in the region of the threads until it was deposit free, subsequently deeper threads were instrumented until the base of the pocket was reached. Following debridement, the implant surface was carefully checked utilising plastic periodontal probe (Click Probe, Kerr Hawe, Switzerland) to locate any residual deposits. All residual deposits were removed and the implant surface repeatedly checked until a deposit free surface had been achieved. The natural teeth were debrided in a similar manner employing only Gracey Curettes (Hu-Friedy, Chicago, Illinois, USA).

2.3.2 Oral hygiene instruction

Oral hygiene instruction was tailored to individual patient requirements. Patients were instructed to use either manual or battery powered toothbrushes according to their manual dexterity and preference. Where patients had manual dexterity difficulties or if they stated a preference for an electric toothbrush, an electric toothbrush was recommended and

patients were instructed in its safe and efficient use. Where inter-proximal spaces were large enough to accommodate inter-proximal brushes (Te Pe, Malmö, Sweden), appropriate sizes were selected and the patients were instructed in the use of these. Where inter-proximal spaces were too small to accommodate inter-proximal brushes, patients were instructed in the use of super-floss (Oral B, Procter & Gamble, Surrey, UK).

2.4 Three, six and nine month examinations

Detailed periodontal examinations were undertaken three and nine months following completion of active treatment. Patients were also seen for an interim maintenance visit, six months following completion of active treatment. At each of these visits, selective debridement was carried out at those sites that demonstrated bleeding on probing. Oral hygiene re-instruction was also provided.

2.5 Microbial sampling

2.5.1 Selection of periodontal and peri-implant microbial sampling sites

Sampling sites included the deepest site of each implant present. In addition, samples were taken from the deepest site of one periodontally healthy tooth (the contra-lateral unit to the implant restoration where present, or the nearest unit to this) and one periodontally diseased tooth (the tooth with the deepest periodontal probing pocket depth).

The samples selected for analysis were a subset of the larger collection of microbial samples taken from each patient participating in the investigation and were selected by a third party not involved in the investigation, in a manner similar to throwing a dart at a list of numbers.

2.5.2 Paper-point and curette sampling

At least one week following the initial baseline examination, microbial sampling was undertaken. Both curette and paper-point sampling techniques were employed following careful isolation of teeth and implants. Prior to sampling, saliva was removed using low volume suction. Sites were isolated with cotton wool rolls and the supragingival plaque removed with cotton wool pellets. Paper-point sampling was undertaken first and curette sampling subsequently, as follows: paper-point samples were obtained using three no. 35 sterile paper-points (Coltene Roeko, Altstätten, Switzerland) per site. Each of the three paper-points were individually inserted into the periodontal or peri-implant pocket until all three were present in the pocket together for 30 s, they were then removed and placed

in a sterile 1.5 ml plastic tube (Sarstedt Ltd., Wexford, Republic of Ireland) containing 1 ml of yeast extract peptone dextrose YEPD (Yeast Extract Peptone Dextrose) broth (per litre: 10 g yeast extract [Sigma-Aldrich Ltd., Dublin, Republic of Ireland] 20 g bactopeptone [Difco, Michigan, USA] and 20 g glucose (pH 5.5) (Fisher Scientific, Massachusetts, USA), and sealed. A curette sample was also taken of the subgingival plaque deposits from each site. Curette samples from peri-implant sites were obtained using titanium tipped curettes (Salvin Dental Specialties, USA). The curette tip was utilised to gain deposits from the implant fixture surface, initially from the coronal threads and subsequently from the deeper threads. Following sampling, the tip of the curette was removed from the peri-implant pocket and rotated vigorously in a 1 ml sterile YEPD broth in a 1.5 ml tube, in order to disperse the microorganisms. Curette samples were gained from the periodontal sites utilising the same technique, except employing Gracey Curettes (Hu-Friedy, Illinois, USA).

2.5.3 Curette and paper-point sample processing

Curette and paper-point samples were transferred to the microbiology laboratory as soon as possible after collection (within 1-3 h of sampling) and were stored at 4°C until processing. Each sample was assigned a unique sequential number as it arrived in the laboratory for processing, allowing anonymous processing. Each sample's details (patient number, date of sampling, sampling method, periodontal or peri-implant site and state of health) were recorded with the sample number in a database (Microsoft Office Access 2003, Microsoft Corporation, Washington, USA), for future reference. Samples were stored at 4°C for 1-24 h until processing. All samples were processed in a Class II biological safety cabinet (Astec Microflow, Hampshire, UK). Each paper-point and curette sample was vortexed for 40 s to 1 min using a Heidolph Reax Vortex (Heidolph Instruments GmbH & Co., Schwanbach, Germany) at a maximum speed, to disperse the microorganisms present in the paper-point and curette samples. Samples were then stored at -80°C until required for analysis.

2.6 Microbial analysis

2.6.1 Semi-quantitative determination of microbial species by checkerboard DNA-DNA hybridisation

2.6.1.1 Preparation of clinical samples for checkerboard analysis

Samples were removed from storage at -80°C and allowed to thaw at ambient temperature. Each sample was then vortexed thoroughly as described above to resuspend the sample material. Each sample underwent centrifugation at 16,100 x g for 10 min using an Eppendorf 5415R bench top centrifuge (Eppendorf, Hamburg, Germany) and vortexed for 40 s to 1 min using a Heidolph Reax Vortex (Heidolph Instruments GmbH & Co., CITY, Germany) at a maximum speed, to disperse the microorganisms present in the paper-point and curette samples. For each site investigated, a 100 µl aliquot was taken from both the curette sample and the paper-point sample using a 50-250 µl Gilson Pipetman pipette (Gilson Inc., Wisconsin, USA) fitted with a sterile 1–200 µl StarLab TipOne graduated filter tip (StarLab Ltd, Milton Keynes, UK) and combined in a single 1.5 ml Eppendorf Safe-Lock microfuge tubes (Eppendorf, Germany). The exceptions were sites where samples which were to be analysed separately in order to compare the efficacy of paper-point and curette sampling techniques (as described in Chapter 5, Section 5.3.2), in which case 200 µl of each paper-point and curette samples from individual sites were pipetted into two separate sterile 1.5 ml microfuge tubes.

The microfuge tubes containing the aliquots of each sample selected for checkerboard DNA-DNA hybridisation analysis were then transported on dry ice to the Oral Microbiology Laboratory, University of Bern, Bern, Switzerland.

2.6.1.2 Preparation of bacterial whole genomic DNA probes

All samples were analysed by the checkerboard DNA-DNA hybridisation technique. Whole genomic DNA probes were prepared for a total of 29 bacterial species (Table 2.1) that were included in the checkerboard panel and sample DNA preparation was undertaken. The majority of bacterial strains used in checkerboard analysis were purchased as lyophilised stocks from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) (exceptions are noted in Table 2.1). Bacterial stocks were rehydrated in Mycoplasma broth (Difco Laboratories, State, City USA) and grown on Trypticase Soy Agar (TSA) with 5% defibrinated sheep blood (BBL, Baltimore Biological Laboratories, Cockeysville, Maryland, USA). Apart from the staphylococcal spp. and the streptococcal spp., the bacteria were grown at 35°C under anaerobic

Table 2.1 The 29 bacterial species included in the checkerboard DNA-DNA hybridisation testing panel.

Bacterial reference strains[1,2]	[3]Species groupings
Staphylococcus aureus ATCC 25923	Staphylococcal spp.
Staphylococcus epidermidis GUH 130381	
Actinomyces naeslundii ATCC 12104	*Actinomyces* spp.
Actinomyces viscosus ATCC 43146	
Actinomyces odontolyticus ATCC 17929	
Aggregatibacter actinomycetemcomitans (Y4) ATCC 43718	*A. actinomycetemcomitans*
Aggregatibacter actinomycetemcomitans ATCC 29523	
Campylobacter gracilis ATCC 33236	*Campylobacter* spp.
Campylobacter rectus ATCC 33238	
Capnocytophaga gingivalis ATCC 33612	*Capnocytophaga* spp.
Capnocytophaga ochracea ATCC 33596	
Eikenella corrodens ATCC 23834	*E. corrodens*
Fusobacterium nucleatum spp. *nucleatum* ATCC 25586	*Fusobacterium* spp.
Fusobacterium periodonticum ATCC 33693	
Lactobaccilus acidophilus ATCC 11975	*L. acidophilus*
Leptotrichia buccalis ATCC 14201	*Leptotrichia* spp.
Neisseria mucosa ATCC 19696	*Neisseria* spp.
Parvimoas micra ATCC 33270	*P. micra*
Porphymonas gingivalis ATCC 33277	*P. gingivalis*
Prevotella intermedia ATCC 25611	*Prevotella* spp.
Prevotella melaninogenica ATCC 25845	
Selenomonas noxia ATCC 43541	*Selenomonas* spp.
Streptococcus gordonii ATCC 10558	Oral streptococci
Streptococcus oralis ATCC 35037	
Streptococcus mitis ATCC 49456	
Tanerella forsythia ATCC 3037 (338)	*T. forsythia*
Treponema denticola ATCC 35405	*Treponema* spp.
Treponema socransky D40DR2	
Veillonella parvula ATCC 10790	*Veillonella* spp.

[1]ATCC, American Type Culture Collection (http://www.lgcstandards-atcc.org).[2] *S. epidermidis* GUH 130381 GUH: Ghent University Hospital Collection, Ghent, Belgium (www.lmg.ugent.be) and *T. socransky* D40DR2 D: sample from Forsyth Institute Massachusetts, USA (www.forsyth.org). [3]Bacterial species belonging to specific species groupings were grouped together for data analysis.

conditions (85% (v/v) N_2, 5% (v/v) CO_2, 10% (v/v) H_2) for three days on TSA supplemented with 3% (v/v) defibrinated sheep blood. Several bacterial strains were grown on supplemented or enriched media. *Bacteroides forsythus* was grown on TSA supplemented with 5% defibrinated sheep blood and 10 µg/ml N-acetyl muramic acid (NAM) (Sigma-Alrdich Chemical Co., Missouri, USA). *Porphymonas gingivalis* was grown on TSA supplemented with 5% defibrinated sheep blood, 0.3 µg/ml menadione (Sigma) and 5 µg/ml haemin (Sigma). *Eubacterium* spp. and *Neisseria* spp. were grown on Fastidious Anaerobic Agar (BBL, USA) with 5% (v/v) defibrinated sheep blood. *Treponema denticola* and *T. socranskii* were grown in Mycoplasma broth supplemented with 1 mg/ml glucose, 400 µg/ml nicatinamide, 150 µg/ml spermine tetrahydrochloride, 20 µg/ml sodium isobutyrate, 1 mg/ml L-casteine, 5 µg/ml thiamine pyrophosphate and 0.5% (v/v) defibrinated sheep blood. Staphylococcal spp. were grown on TSA at 37°C under aerobic conditions.

The bacteria were then placed in 1000 µl TE buffer (10 mM Tris-HCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6) and washed twice by centrifugation in TE buffer at 1027 x *g* for 10 min. The pelleted cells were resuspended by 15 s of sonication and incubated at 37°C for 1 h. The cells were resuspended and lysed with either 10% (w/v) sodium dodecyl sulphate (SDS) and proteinase K (20 mg/ml) for Gram-negative strains or in 150 µl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma) and 5 mg/ml achromopeptidase (Sigma) in TE buffer for Gram-positive strains. DNA was isolated and purified using the method of Smith *et al.*, 1989. The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of the absorbances at 260 and 280 nm. DNA precipitation was performed as described by Socransky *et al.* (1994). Whole genomic DNA probes were prepared from each of the 29 bacterial strains by labelling 1 µg DNA with digoxgenin antibody conjugated with alkaline phosphatase (Amersham Life Science, Illinois, USA) using a random primer technique as outlined in Section 2.6.1.4.

2.6.1.3 DNA extraction from clinical samples

A 150 µl volume of TE buffer (10 mM Tris-HCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6) was added to each 200 µl sample-containing Eppendorf tube (Eppendorf) followed by 150 µl of 0.5 M NaOH and the samples boiled in a water bath for 5 min (Haffajee *et al.*, 1997*a*). The samples were neutralised by adding 0.8 ml of 5 M ammonium acetate. Then, 100 µl aliquots of the suspension were placed in lanes on a

nylon membrane (Boehringer Mannheim, Indianapolis, USA) using a Minislot device (Immunetics, Massachussettes, USA). The DNA was fixed to the membrane using a vacuum and cross-linking achieved by exposure to ultraviolet light (Stratalinker 1800, Stratagene, California, USA) followed by baking at 120°C for 20 min. The Minislot device permitted the deposition of extracted DNA from 28 different microbial clinical samples in individual lanes on a single 15 x 15 cm nylon membrane as well as two control lanes containing 10^5 or 10^6 cells of each test species. The membrane with fixed DNA was placed in a Miniblotter 45 (Immunetics, USA), with the lanes of DNA at 90° to the channels of the device. A 30 x 45 checkerboard pattern was produced. Each channel was used as a hybridisation chamber for separate DNA probes. Signals were detected by chemiluminescence using a Storm Fluorimager (Molecular Dynamics, California, USA). The 29 probes employed are listed in Table 2.1.

2.6.1.4 Pre-hybridisation and hybridisation

The membranes were prehybridised at 42°C for 1 h in 50% (v/v) formamide, 5 X SSC (1 X SSC = 150 mM NaCl, 15 mM Na 2-hydroxypropane-1,2,3-tricarboxylic acid, pH 7.0), 1% casein (Sigma), 5 X Deinhardt's reagent, 25 mM sodium phosphate (pH 6.5) and 0.5 mg/ml yeast RNA (Boehringer). Digoxigenin-labeled whole genomic DNA probes were prepared for 28 bacterial species using a random primer technique (Feinberg & Vogelstein, 1983).

In the present study, the probes and hybridisation buffer were placed in individual lanes of the Miniblotter and the whole apparatus placed in a sealed plastic bag. Membranes were hybridised overnight at 42°C in a hybridising solution containing 45% formamide, 5 X SCC, 1 x Denhardt's reagent, 20 mM Na phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 20 ng/ml of labeled probe, 10% dexatran sulphate and 1% casein (Sigma). The probes were denatured by heating each probe-containing hybridisation buffer at 100°C for 5 min prior to cooling on ice. Membranes were washed at low stringency to remove loosely bound probe and then at high stringency (68°C, 0.1 SSC, 0.1% SDS, 20 min, twice) in a Disk Wisk apparatus (Schleicher and Schuell, Keene, New Hampshire, USA).

To detect hybrids, membranes were then blocked and incubated with a 1:2,000 dilution of anti-digoxgenin antibody conjugated with alkaline phosphatase (Amersham Life Science) using the modification described by Engler-Blume *et al.* (1993). The membranes were washed at high stringency and were incubated in AttoPhos (Amersham Life Science) overnight at room temperature and the DNA probes were detected using

chemifluorescence. Using a Storm Fluorimager (Molecular Dynamics), the chemifluorescence detected the antibody to digoxigenin conjugated with alkaline phosphatase AttoPhos substrate (Amersham Life Science). The probes and their source strains were compared (Ximenez-Fyvie *et al.*, 2000). Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit detection of 7.5×10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were converted to semi-quantitative estimates by comparison with the standards on the same membrane.

As the checkerboard DNA-DNA hybrisdisation method employs whole genomic probes, cross-reactivity from closely related species was possible, leading to concerns with this analysis approach. Therefore, real time PCR was undertaken for selected species. The selected species were *A. actinomycetemcomitans, T. forsythia*, *P. gingivalis,* and *T. denticola* as these were four of the species most commonly associated with periodontal and peri-implant infections. In the analysis of the checkerboard data, only where the real-time PCR had confirmed the presence of each of these species, was the result of the checker- board DNA-DNA hybridisation considered valid and included in the analysis. Furthermore, in order to mitigate any possible decreased specificity of the checkerboard DNA-DNA hybridisation technique, compared to culture or other molecular techniques, closely related species taxa were combined and used in data analysis to give a result for an individual species or a group of species, as outlined under the species groupings heading in Table 2.1.

2.6.2 Real-Time PCR analysis

Additional to the checkerboard technique, real-time PCRs were undertaken for four bacterial species including *A. actinomycetemcomitans, T. forsythia*, *P. gingivalis,* and *T. denticola* (Tables 2.2 and 2.3) as described by Eick *et al.,* (2011) and modified by using GoTaq qPCR Master mix (Promega Corporation, Wisconsin, USA). In the final analysis, only results confirmed by real-time PCR for these species were considered valid and included in the analysis.

DNA was extracted from 100 µl of each sample using the Chelex method (Yang *et al.*, 2008). Real-time PCR was carried out using a real-time analyser (7500 Real Time PCR System, Life Technologies, California, USA). Oligonucleotides were synthesised by Microsynth (Balgach, Switzerland) (Table 2.3). The primers targeting 16S rDNA regions of *P. gingivalis*, *T. forsythia* and *T. denticola* were designed as described by Ashimoto *et*

al., (1996) and those for *A. actinomycetemcomitans* were designed as described by Tran & Rudney (1999) (See Table 2.3).

PCR amplifications were carried out in Real-Time PCR analyser (7500 Real-Time PCR System) in a reaction volume of 25 µl, consisting of: 2.5 µl template DNA and 22.5 µl of reaction mixture (containing 12.5 µl GoTaq qPCR Master Mix (Promega), 0.5 µmol/L (1.25 µl) of each primer and 7.5 µl dH_2O). Negative and positive controls PCRs were included in each experiment. The positive controls consisted of 2.5 µl genomic DNA in concentrations equivalent to 10^2 to 10^7 cfu of the reference strains: *P. gingivalis* ATCC 33277; *T. forsythia* ATCC 43037; *A. actinomycetemcomitans* ATCC 33384; *T. denticola* ATCC 35405). The negative control was 2.5 µl of sterile water, added to 22.5 µl reaction mixture. The cycling conditions comprised an initial denaturation step at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min according to the GoTaq qPCR Master Mix Protocol (Promega).

2.7 Statistical Methods

2.7.1 Fisher's Exact Test

In Chapter 3, the Fisher's Exact Test, utilising the approximation of Katz *et al.* (1978), was employed to investigate the relationship of:

a. Smoking to diagnosis of peri-implantitis
b. Experience of severe periodontal disease to diagnosis of peri-implantitis.

2.7.2 Changes in clinical parameters following non-surgical treatment: descriptive statistics, t-test and regression analysis

In Chapter 4, the data was analysed on both a patient level and an implant level. On a patient level, patients were divided into those affected by peri-implant mucositis (Peri-implant mucositis Group) and those affected by peri-implantitis (Peri-implantitis Group). For each patient, periodontal and peri-implant clinical data were collated both together and separately. Comparisons were made both of the individual peri-implant and periodontal data and the combined peri-implant and periodontal data for each of the following clinical parameters: percentage of sites bleeding on probing (% sites BOP); percentage of sites demonstrating probing pocket depths of ≥6 mm (% sites ≥6 mm PPD); percentage of sites demonstrating suppuration on probing (% SOP). A formal model was employed comparing the clinical data within groups, and between groups, over the three time-points: baseline (T1), the three month examination (T2) and the nine month

Table 2.2 The four reference bacterial species used in real-time PCR.

Bacterial reference strains[1]
A. actinomycetemcomitans ATCC 29523
T. forsythia ATCC 43037
P. gingivalis ATCC 33277
T. denticola ATCC 35405

[1]ATCC, American Type Culture Collection (http://www.lgcstandards-atcc.org)

Table 2.3 Oligonucleotides used in real time-PCR experiments to detect *P. gingivalis*, *T. forsythia*, *T. denticola and A. actinomycetemcomitans*.

Species	Primer sequences 5'-3'	Expected amplimer size (base pairs)
A. actinomyctemcomitans	F-ATTGGGGTTTAGCCCTGGTG R-ATGTCATCCCCACCTTCCTC	557
T. forsythia	F-GAGTATGTAACCTGCCCGCA R-TGCTTCAGTGTCAGTTATACCT	641
P. gingivalis	F-AGGCAGCTTGCCATACTGCG R-ACTGTTAGCAACTACCGATGT	404
T. denticola	F-TAATACCGAATGTGCTCATTTACAT R-TCAAAGAAGCATTCCCTCTTCTTCTTA	316

examination (T3). The within-patient clustering was controlled for by a patient-specific model using random effects. A logistic regression model that allowed for patients nesting within time was utilised (Agresti, 2013).

Analysis was also undertaken on a unit level for the tooth with the deepest probing pocket depthat baseline and each of the implants present. The following measures were analysed: probing pocket depth, bleeding on probing and suppuration on probing. Investigation for statistically significant changes was undertaken employing the unpaired t-test, as the data was normally distributed and comparable at two time points.

The change in the deepest probing pocket depth between baseline and the nine months examination was compared for individual teeth and implants. Similarly, the presence or absence of bleeding on probing at a unit level was undertaken comparing the baseline (T1) and nine (T3) month data. Coding of the unit level bleeding on probing data was undertaken as follows: if any of the six points around the implant or tooth examined demonstrated bleeding on probing, the unit was recorded as positive for bleeding on probing. If no site bled on probing, the unit (either tooth or implant) was recorded as negative for bleeding on probing. Similarly, for suppuration on probing, if suppuration on probing was detected at one or more sites, the unit was recorded as positive for suppuration on probing. If no suppuration on probing was detected, the unit was recorded as negative for suppuration on probing.

2.7.3 Percentage relative abundance

The percentage relative abundance measure (a measure of the proportion of each bacterial species grouping within the overall sum of bacterial species identified in a particular sample or group of samples) was employed as it provides an insight into the composition of the periodontal or peri-implant plaque biofilm. Furthermore, as the volume of plaque (and peri-implant or periodontal crevicular fluid) may vary on differing sampling occasions, the same estimated bacterial cell density of a particular species grouping would represent a different proportion of the overall microbial species detected, if different volumes of plaque (and peri-implant or periodontal crevicular fluid) were recovered on different sampling occasions. By undertaking comparisons of the relative proportions of each bacterial species groupings (percentage relative abundance) within individual samples, or groups of samples, the variance of the plaque (and peri-implant or periodontal crevicular fluid) volume recovered was taken into account. Other investigators appear to have employed a similar measure (Jervoe Storm *et al.*, 2007) in

which they examined the "total target pathogen proportion", although they did not provide details on how this proportion was calculated (See Chapter 5, Section 5.1).

In the present investigation, the relative proportions of DNA of individual species that were identified from individual sites utilising paper-point or curette sampling techniques were referred to as the percentage relative abundance (% relative abundance). The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species groupings as a proportion of all of the species detected at an individual site.

Only 15.19% of all the relative abundance figures calculated in the present investigation were >10%, therefore a 1% change in relative abundance, represented a greater than 10% change in the proportion of any individual species grouping within the plaque/ crevicular fluid biofilm, on approximately 85% of occasions. Furthermore, Socransky *et al.* (2004) previously demonstrated that whole genomic probes employed in checkerboard DNA-DNA hybridisation analysis showed essentially no cross reactions to other species at a level of >10% (See Chapter 9, Section 9.2) - based on this a safe margin of error for the detection of microbial species by checkerboard DNA-DNA hybridisation may be taken to be 10%. As stated, 85.16% of all relative abundance measures calculate in the present investigation were <10%. As a 10% margin of error on 10% is 1%, a 1% threshold of change was selected for the analysis. This is a novel approach to the analysis of changes in relative abundance and a >1% also provided a simple starting point for employing such a threshold in an investigation which examined the shifts in the proportions of different bacterial species groupings within periodontal/peri-implant plaque biofilms.

2.7.4 Comparison of paper-point and curette sampling techniques: Descriptive statistics

In Chapter 5, data from checkerboard DNA-DNA hybridisation analysis of individual paper-point and curette samples were examined to investigate qualitative and quantitative differences between the microbial species detected using both sampling techniques. Descriptive statistics were employed to compare the estimated bacterial cell density per species grouping (based on DNA-DNA hybridisation), detected from all of the samples gained using both paper-point and curette sampling techniques, under the following headings:

1. Comparison of the number of occasions a greater number of bacterial species were detected from individual sites by either paper-point or curette sampling techniques.

2. Comparison of the estimated number of bacteria cell densities of each of the examined species groupings detected by both paper-point and curette sampling, by paper-point sampling alone or by curette sampling alone, from periodontal and peri-implant sites.

3. Comparison of the estimated number of bacteria cell densities of each of the examined species groupings detected under the following headings:

   A. Periodontal sites:

      a) paper-point samples

      b) curette samples

   B. Peri-implant sites:

      a) paper-point samples

      b) curette samples

4. Comparison of the number of times paper-point and/or curette sampling from peri-implant and periodontal sites yielded each of the 18 bacterial species groupings investigated.

5. Comparison of the frequency individual species were detected by paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling (n=40) for:

      c) The periodontal and peri-implant sites

      d) The periodontal sites

      e) The peri-implant sites

The relative amounts of DNA of individual species that were identified from individual sites utilising paper-point or curette sampling techniques were referred to as the percentage relative abundance (% relative abundance). The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species

groupings as a proportion of all of the species detected at an individual site. Comparisons were made of the percentage relative abundance of DNA of individual species that were identified from individual sites utilising paper-point or curette sampling techniques using descriptive statistics. The estimated bacterial cell densities of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Treponema* spp. and staphylococcal spp. were compared between paper-point and curette samples utilising the Wilcoxon Rank Sum test.

2.7.5 Observational microbiological study: investigation of peri-implant and periodontal microflora at baseline presesentation

The relative amounts of DNA of individual species groupings that were identified from individual sites utilising paper-point or curette sampling techniques were referred to as the percentage relative abundance (% relative abundance). The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species groupings, as a proportion of all of the species detected at an individual site. Comparisons were made of:

a) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for periodontal and peri-implant sites. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

b) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for peri-implant mucositis and peri-implantitis sites. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia* and *Treponema* spp.

c) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for periodontal healthy sites and periodontitis sites. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

d) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA

hybridisation technique for patients who have a history of severe periodontal disease and those without a history of severe periodontal disease. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

e) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for patients who are current smokers and non-smokers. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

Descriptive statistics were employed to compare the total estimated bacterial cell density per individual species $x10^5$ (based on DNA-DNA hybridisation) from peri-implantitis sites compared to the peri-implant mucositis sites.

2.7.6 Change in the microflora of periodontal and peri-implant sites of patients with peri-implant infections following non-surgical treatment

The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species groupings, as a proportion of all of the species detected at an individual site.

The median bacterial cell density from the checkerboard DNA-DNA hybridisation analysis, as compared between baseline and nine months post-treatment for the 18 microbial species groupings, was investigated. Owing to the problems associated with multiple hypothesis testing (namely, decreasing degrees of freedom and decreasing p-values accordingly), only five of the 18 species groupings investigated were analysed using the Wilcoxon Rank Sum Test. These included: *A. actinomycetemcomitans P. gingivalis, T. forsythia*, *Treponema* spp. and staphylococcal spp.

The changes in the relative abundance of *A. Actinomycetemcomitans, P. gingivalis, T. forsythia*, *Treponema* spp. and staphylococcal spp. between baseline and nine months following treatment were compared for the periodontal and peri-implant sites. The Wilcoxon rank sum test with continuity correction was applied

Descriptive statistics were employed to examine:

a) The frequency of peri-implant sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 species groupings following treatment.

b) The frequency of periodontal sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 species following treatment.

c) The frequency of peri-implant mucositis demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 species following treatment.

d) The frequency of peri-implantitis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 bacterial species groupings following treatment.

Chapter 3

The relationship between selected risk indicators and peri-implantitis or implant loss

3.1 Introduction

3.1.1 Peri-implant infections

Dental implants enable restoration of missing teeth, where previously available treatment approaches were limited or inadequate (Stegenga *et al.*, 2000). However, peri-implant tissues may become infected (Berglundh *et al.*, 2002), and there is growing concern with respect to the success of implant restorations in patients with a demonstrated susceptibility to periodontal disease (Renvert & Persson, 2009). This concern has important implications for restorative treatment planning and scheduling of maintenance care. Peri-implant disease is a collective term for inflammatory processes in the tissues surrounding an osseointegrated dental implant (Albrektsson & Issidor, 1994). Peri-implant mucositis refers to a reversible inflammatory change of the peri-implant soft-tissues without the loss of the implant supporting bone, whereas peri-implantitis refers to an inflammatory process affecting the peri-implant tissues which results in loss of the implant supporting bone (Albrektsson & Issidor, 1994). The Peri-implantitis Working Group of the European Workshop on Periodontology (2002) recommended the following criteria for the diagnosis of peri-implantitis: bleeding/ suppuration on probing; probing pocket depth of ≥6 mm and radiographic bone loss >2.5 mm (Berglundh *et al.*, 2002). Peri-implant mucositis has been reported to affect over 90% of dental implant patients (Fransson *et al.*, 2005), while peri-implantitis affects between 1.8% and 28% of dental implant patients (Buser *et al.*, 2012; Fransson *et al.*, 2005). Proposed risk indicators for peri-implant infections include smoking and a history of periodontal disease (Heitz-Mayfield, 2008). However, the evidence to support these associations is limited.

3.1.2 Patients with a history of periodontal disease

Patients demonstrating advanced periodontal bone loss are considered susceptible to periodontal disease. The presence of deep periodontal pockets at periodontal re-evaluation has been associated with periodontal attachment loss (Claffey *et al.*, 1996). As to whether such an increased susceptibility to periodontal bone loss results in peri-implant bone loss, remains to be established (Renvert & Persson, 2009). A decreased survival of dental implants and an increased prevalence of peri-implantitis has been reported in patients with a history of periodontitis compared to patients without a history of periodontal disease (Hardt *et al.*, 2002; Karoussis *et al.*, 2003; Ferreira *et al.*, 2006; Roos-Jansåker *et al.*, 2006; Mengel *et al.*, 2007; Gatti *et al.*, 2008; Roccuzzo *et al.*, 2010, 2012;

Costa *et al.*, 2012; Renvert *et al.*, 2012; Swierkot *et al.*, 2012; Pjetursson *et al.*, 2012; Cho-Yan Lee *et al.*, 2012). However, this is not universally supported, as several studies have not observed a significantly increased risk of peri-implantitis in patients with a history of periodontal disease (Broccard *et al.*, 2000; Hultin *et al.*, 2000; Rosenberg *et al.*, 2004; Baelum & Ellegaard, 2004; De Boever *et al.*, 2009). Furthermore, peri-implantitis has been observed in some patients with little or no periodontal bone loss (Lang & Berglundh, 2011).

Renvert & Persson (2009) undertook a systematic review of the available dental literature examining whether patients with a demonstrated susceptibility to periodontal disease (i.e. a history of periodontal bone loss) were similarly susceptible to peri-implant bone loss. Unfortunately, due to poor study design and incomplete information being presented, only three studies met the inclusion criteria (Table 3.1) (Hardt *et al.*, 2002; Karoussis *et al.*, 2003; Mengel *et al.*, 2007).

Hardt *et al.* (2002) described a retrospective study of 97 partially edentulous patients with 346 implants present for ≥5 years. Panoramic radiographs were examined and the age-related periodontal marginal bone loss scores calculated according to the formula of Bjorn and co-workers (1969) to describe the severity of the periodontal disease experience. The authors selected the two tail quartiles of the distribution of the individual age related marginal bone loss scores to represent a) the individuals with minimal experience of periodontal breakdown and b) those subjects considered to be susceptible to periodontitis. Twenty-five patients were in each group. The two groups differed with regards to the mean periodontal bone levels remaining, being 92% and 63%, respectively. In the group of patients with minimal experience of periodontal breakdown, three out of 92 implants were lost in three of the 25 patients. Eight out of 100 implants were lost in seven of the 25 patients with a demonstrated susceptibility to periodontitis. In the group of patients with minimal experience of periodontal breakdown, six of the 25 patients (24%) experienced peri-implant bone loss of ≥2 mm, whereas 16 of the 25 (64%) of patients considered to be susceptible to periodontitis demonstrated peri-implant bone loss of ≥2 mm ($p=0.029$).

Karoussis *et al.* (2003) outlined the findings of a retrospective analysis of 53 partially edentulous patients with 112 hollow screw implants. The authors employed the following criteria: if the implants were placed in patients whose tooth or teeth were lost due to periodontitis they were considered a "periodontitis susceptible patient", whereas those patients who had lost teeth due to other reasons were classified as "without a history

of chronic periodontitis"- the numbers of patients were eight and 45 respectively, whilst the numbers of implants were 21 and 91 respectively. Over the 10 year observation period, only 71.4% of the periodontitis susceptible patient group did not experience peri-implantitis or implant loss, whilst 94.2% of patients without a history of chronic periodontitis did not experience peri-implantitis or implant loss. The criteria to define smoking status were not described, however. After 10 years, Karoussis *et al.* (2003) noted a decreased survival rate and an increased complication rate in periodontally treated patients compared to the periodontally healthy patients and observed that this was especially heightened in those patients with a demonstrated susceptibility to periodontal disease who also smoked.

Mengel *et al.* (2007) undertook a prospective controlled study of five patients with a history of generalised aggressive periodontitis and five patients who were periodontally healthy, over an observation period of 10 years. The dental implant survival rates were 100% in the periodontally healthy group and 83.33% in the generalised aggressive periodontitis group. The authors observed no difference in the plaque index between the two groups, but the peri-implant gingival index of the generalised aggressive periodontitis group was significantly higher than that of the periodontally healthy group, throughout the study period. Differences in the periodontal probing depths were also noted between the two groups, with deeper values being recorded for the generalised aggressive periodontitis group compared to the periodontally healthy group. Surprisingly, the peri-implant probing depths between the two groups were comparable throughout the observation period, remaining ≥4mm throughout. Mengel *et al.* (2007) noted significantly greater peri-implant bone loss and attachment loss in the generalised aggressive periodontitis group compared to the periodontally healthy group.

Renvert & Persson (2009) concluded that patients with a history of periodontitis may be at an increased risk of peri-implantitis.However, they suggested that the quality assessment of studies indicated that the risk of bias was high and recommended "*further long-term studies involving sufficient number of patients are needed before final conclusions can be drawn about the outcome of implant treatment in patients with a history of periodontitis*".

3.1.3 Smoking status

The relationship between smoking history and peri-implant bone loss is not clear. Smoking damages the microcirculation, leading to vessel wall injury, reduced tissue perfusion and impaired microcirculatory regulation (Lehr *et al.*, 2000). Specifically,

Table 3.1 Number of subjects in each of the studies meeting the inclusion criteria in the systematic review of Renvert & Persson (2009).

Study	Periodontitis (n)	Non-periodontitis (n)
Hardt *et al.*, 2002	25	25
Karoussis *et al.*, 2002	8	45
Mengel *et al.*, 2007	5	5

smoking compromises endothelial-dependent vaso-relaxation, endothelial cell function and contributes to artherosclerosis (Lehr *et al.,* 2000). Furthermore, smoking compromises polymorphonuclear leukocytes and macrophage function (Mac Farlane *et al.,* 1992), thereby limiting the immune system's ability to efficiently ward off infecting microorganisms.

Significantly decreased dental implants survival rates have been noted for patients who smoke compared to those who do not (Wilson & Nunn, 1999; Hultin *et al.*, 2000*a*; Kourtis *et al.,* 2004). Wilson & Nunn (1999) undertook a retrospective investigation of 65 patients, 27 of whom had experienced implant failure. A significantly higher dental implant failure rate was observed in the patients who smoked compared to those who did not ($p=0.24$). In 143 patients who had implant-supported fixed restorations for five or more years, nine patients experienced dental implant loss (Hultin *et al.*, 2000*a*). Importantly, seven of the nine patients who lost dental implants were smokers and the majority of the dental implants lost were in the maxilla. In a study of 405 patients, Kourtis *et al.* (2004) also noted a significantly higher dental implant failure rate in patients who smoked compared to those who did not smoke ($p<0.001$). Ortorp & Jemt (2004) undertook a randomised controlled trial observing the dental implant failure rate for smokers compared to non-smokers over a five year period. A significantly higher dental implant failure rate was observed in smokers compared to non-smokers ($p<0.01$).

However, some studies did not note an increased risk of dental implant failure in smoking patients compared to non-smoking patients (Grunder *et al.*, 1999; Bain *et al.,* 2002; Lemmerman & Lemmerman, 2007). Grunder *et al.* (1999) undertook a study of 74 patients, (219 implants) of which 19 of the patients were smokers, followed up for a mean time of 34 months and noted a dental implant survival rate of 98.7%. No significant difference in the dental implant survival rate between smokers and non-smokers was reported, indicating that osseointegration can be preserved in patients who smoke. Bain *et al.* (2002) undertook a Meta-analysis of nine prospective investigations examining the three year cumulative survival rate of dental implant restorations in smokers compared to non-smokers. No significant difference was observed in the dental implant survival rate for patients who smoked compared to those who did not. Lemmerman & Lemmerman (2007) undertook a retrospective study examining 1003 dental implants in 376 patients over a mean period of function of 5.3 years. No correlation was noted between implant loss and smoking status ($p=0.95$)

With regards to peri-implantitis, many studies have observed a significant relationship between smoking status and the prevalence of peri-implantitis (Hass *et al.*, 1996; Karoussis *et al.*, 2003; Nitxan *et al.*, 2005; Roos-Jansåker *et al.* 2006; Strietzel *et al.*, 2007; Fransson *et al.*, 2008; Levin *et al.*, 2008; Aglietta *et al.*, 2011; Rinke *et al.*, 2010; Swierkot*et al.*, 2012).

Roos-Jansåker *et al.* (2006) observed in a group of 218 patients with 1057 implants, who were followed up for 9-14 years, that smoking was positively associated with peri-implantitis on a patient level ($p=0.02$). Fransson *et al.* (2008) observed that smokers were at an increased risk of peri-implantitis, resulting in probing pocket depths of ≥6 mm and suppuration on probing compared to non-smoking patients with comparable oral hygiene levels.

Aglietta *et al.* (2011) undertook a retrospective study examining the peri-implant bone levels of periodontally compromised and periodontally healthy smokers 10 years following delivery of the implant restoration. 10% (4/40) implants were lost over the 10 year period. Three of the four failures were amongst the periodontally compromised patients and all of the implant failures were after the fourth year of loading, however, differences were not statistically significant ($p>0.05$). 75% of the patients demonstrated bone loss of ≥3mm after 10 years, highlighting the detrimental effects of smoking on peri-implant bone levels. The authors commented that "*smoking synergistically enhanced the risk of implant failures and marginal bone loss*". However, some studies have not noted a significant association between smoking status and peri-implantitis (Aalam & Nowzari 2005; Roccuzzo *et al.*, 2010; 2012; Marrone *et al.*, 2012). Aalam & Nowzari (2005) noted in a retrospective study examining 198 implants in 74 patients, that there was no significant association between smoking status and peri-implantitis ($p>0.05$). Roccuzzo *et al.* (2012) observed in a 10-year prospective study of 112 partially dentate implant patients, a non-significant increase in peri-implant bone loss in patients with a history of periodontal disease compared to patients with no history of periodontal disease ($p>0.5$). Kolslander *et al.* (2009, 2011) observed in a study of 59 smoker patients and 50 non-smoking patients, that patients with a history of smoking were at an increased risk of dental implant loss (15.3 % and 2% respectively) ($p<0.05$). However, smokers were not observed to be at a significantly increased risk of peri-implantitis compared to non-smoking patients ($p>0.05$). Similarly, Marrone *et al.* (2013), in a follow up of 103 patients with dental implants for a mean time of 8.5 years, did not note a significant association between smoking status and peri-implantitis status ($p>0.5$).

Therefore, the question remains as to whether patients with a history of periodontal bone loss, or patients who smoke, are at a greater risk of implant loss and/or peri-implantitis.

3.2 Aims

The aims of this part of the present investigation were to investigate:

A. If a history of severe periodontitis was a risk indicator for peri-implantitis.
B. If a positive smoking status was a risk indicator for peri-implantitis.

3.3 Methods

3.3.1 Patient selection

Patients attending the Dublin Dental University Hospital and having one or more dental implants placed five years previously, were invited to attend an assessment appointment. The inclusion and exclusion criteria applied are described in Chapter 2 Section 2.1.2 and Section 2.1.3.

3.3.2 Inclusion criteria:

- Patients over the age of 18 years.
- Patients with dental implants that have been in place for five or more years.
- Provision of a signed Patient Informed Consent Form.

3.3.3 Exclusion Criteria:

A full medical history was taken and any patients reporting the following conditions were excluded from the study:

- Requirement for prophylactic medication.
- Pregnant or lactating females.
- Patients having taken antibiotics, antifungal or steroid medication, (which may affect the microbial composition), within two months of enrolment.
- Individuals under 18 years of age.
- Adults with learning disabilities and unable to provide consent.
- Patients with a terminal illness.

3.3.4 Ethical approval

This study had the approval of the Ethics Committee of the Faculty Research Ethics Group, Faculty of Health Sciences, Trinity College Dublin (Chapter 2, Section 2.1.1).

3.3.5 Clinical history and examination

Patients underwent a detailed dental and medical history and an oral examination including; a full mouth probing pocket depth measurement, measurement of periodontal attachment loss and bleeding on probing. A plastic periodontal probe with a pressure

sensitive indicator corresponding to 0.2 – 0.25N force was used for all periodontal and peri-implant probing (Click Probe, Kerr Hawe).

Peri-apical radiographs were undertaken utilising a Rinn holder (Dentsply UK) and employing the long cone paralleling technique, i.e. the peri-apical radiograph film, the implant and the collimator face were aligned as parallel as possible and the radiograph exposed for the recommended time interval dependant on the implant's anatomical location. Radiographs included both conventional and digital radiographs. Conventional radiographs were viewed on a light box in a darkened room and a photograph was taken at a shutter speed of 0.32 s on a Cannon 30 D camera with a Macro 250 mm lens (Canon (UK) Ltd.).

Similarly, where phosphor plates (Sidexis Dental Systems) were employed to capture the digital image of the radiograph, images were viewed in a darkened room and the settings adjusted to optimally view the peri-implant bone levels. Images were imported to Windows XP Paint Shop (Microsoft Corporation), cropped and pasted to Microsoft Office PowerPoint 2007 (Microsoft Corporation) and coded according to the patient's investigation number and the implant's anatomical location. All radiographs were examined by two examiners with regard to diagnosis of peri-implant bone loss of $\geq$2.5 mm and Kappa analysis revealed good agreement between examiners ($p<0.001$). Where discrepancies were present between examiners, a third examiner was employed. All examiners were blinded with regard to the identity of patients and the clinical parameters.

3.3.6 Definitions

Following the recommendations of the Peri-implantitis Working Group of the European Workshop on Periodontology (2011), implants demonstrating peri-implant sites with bleeding on probing were diagnosed as having peri-implant mucositis (Lang *et al.*, 2011). Following the recommendations of the Peri-implantitis Working Group of the European Workshop on Periodontology (2002), implants demonstrating each of the following criteria: bleeding or suppuration on probing; probing pocket depth of $\geq$6 mm and radiographic bone loss $\geq$2.5 mm, were classified as being affected by peri-implantitis (Berglundh *et al.*, 2002).

The lack of a clear definition of periodontitis consistently employed across studies limits the interpretation of available evidence for the relationship between periodontitis and peri-implantitis (Renvert & Persson, 2009). Following the recommendations of the European Workshop on Periodontology (2005), patients were classified as having

experienced severe periodontal disease if ≥30% sites demonstrated inter-proximal clinical attachment loss of ≥5 mm (Tonetti & Claffey, 2005).

Patients who were active smokers, or had ceased smoking within the 12 months prior to baseline examination, were classified as smokers. Patients who reported not smoking in the 12 months prior to examination were classified as non-smokers. On a patient level, patients with dental implants deemed positive for peri-implant mucositis were diagnosed as being peri-implant mucositis patients. Patients with implants deemed positive for peri-implantitis were diagnosed as peri-implantitis positive. It is important to note that where patients had any implants deemed positive for peri-implantitis, these patients were diagnosed as peri-implantitis positive, even in the presence of other healthy implants. Similarly, where patients had any peri-implant mucositis even in the presence of other healthy implants, a diagnosis of peri-implant mucositis was assigned, on a patient level. Where patients only had implants with no evidence of peri-implantitis or mucositis, a diagnosis of healthy implants was assigned on a patient level.

3.3.7 Statistical Analysis

The Fishers Exact Test, utilising the approximation of Katz *et al.* (1978) was employed to investigate therelationship of:

A. Smoking to diagnosis of peri-implantitis
B. Experience of severe periodontal disease to diagnosis of peri-implantitis

3.4 Results

Of the 51 patients meeting the inclusion criteria, 13 were male and 38 were female. Seven patients were edentulous and 44 were partially-dentate implant patients. Seventeen patients were classified as having a history of severe periodontal disease. Twelve patients were classified as smokers. Seven patients were both smokers and demonstrated a history of severe periodontal disease. Nineteen of the 51 patients, were positive for peri-implantitis or implant loss. Two patients experienced implant loss, both of which were positive for peri-implantitis of the remaining implants. The results are outlined in Tables 3.2 and 3.3. Smoking was not observed to be significantly associated with diagnosis of peri-implantitis ($p=0.1$). Experience of severe periodontal disease was significantly associated with diagnosis of peri-implantitis ($p=0.03$).

3.5 Discussion

In the present study, a demonstrated history of severe periodontal disease was observed to be significantly associated with a diagnosis of peri-implantitis (p=0.03). These findings are in line with the findings of Hardt *et al.* (2002), Karoussis *et al.* (2003) and Mengel *et al.* (2007) who also showed significant association between peri-implantitis and a history of severe periodontal disease, suggesting that there are common interacting mechanisms between the two disease processes. This likely relates both to the microflora present within the patient's mouth (Mombelli *et al.*, 1995) and genetic factors which determine how they respond to peri-implant and/or periodontal infections (Karoussis *et al.*, 2002). Conversely, Hultin *et al.* (2000*a*), Roccuzzo *et al.* (2012) and Marrone *et al.* (2013) did not yield the same result, instead noting an absence of any statistically significant association between peri-implantitis and a history of periodontal disease. This may be explained by the differing criteria being employed to define peri-implantitis and periodontal disease attributes, as it has previously been shown that in studying peri-implantitis, small changes in the diagnostic criteria employed result in large changes in the observed disease prevalence (Koldsland *et al.*, 2010; Koldsland *et al.*, 2011)

The distinguishing feature between peri-implant mucositis and peri-implantitis is peri-implant bone loss (Albrektsson & Isidor, 1994). It is recognised that a certain amount of peri-implant bone loss is to be expected as part of post-operative peri-implant bone remodelling (Lang *et al.*, 2011). However, the extent of bone loss required for a diagnosis of peri-implantitis, as opposed to post-operative peri-implant crestal bone remodelling, has varied considerably in the literature to date. The literature to date has been equivocal, which further hinders clarity in this area. Criteria employed in the literature to date include: progressive bone loss (Behneke *et al.*, 2002), continuous bone loss (Ekelund *et al.*, 2003), vertical bone loss (Ferreira *et al.*, 2006) and radiographic bone loss (Karoussis *et al.*, 2002). The studies cited above did not indicate the extent of bone loss required for a diagnosis of peri-implantitis. However, Roos-Jansaker *et al.* (2006) and Berglundh *et al.* (2002) utilised radiographic bone loss of ≥1.8mm and bone loss of ≥2.5 mm, respectively, following the first year in function.

The present study employed clear clinical criteria defining peri-implantitis, severe periodontal disease experience and current smoking status. The criteria defining peri-implantitis and severe periodontal disease were extracted from the recommendations of the Peri-implantitis Working Group of the European Workshop on Periodontology 2002 and 2005, respectively. A positive smoking status was attributed to any active, current

Table 3.2 Smoking status and peri-implantitis status of the patients.

	Peri-implantitis +	**Peri-implantitis -**
Smoking +	7	5
Smoking -	12	27

Table 3.3 History of severe periodontitis and peri-implantitis status of the patients.

	Peri-implantitis +	**Peri-implantitis -**
Severe Periodontitis History +	10	7
Severe Periodontitis History -	9	25

smoker or anyone who had ceased smoking within the 12 months prior to baseline examination. Furthermore, only subjects with implants present for five or more years were included in the present investigation. A limitation of the present study is its cross-sectional design, a randomised prospective study would give a higher level of evidence, as a selection bias may be present amongst those patients who chose to participate in the review appointment compared to those who declined. Experience of severe periodontal disease was observed to be significantly associated with a diagnosis of peri-implantitis ($p=0.03$)

The criteria employed in subdividing patients into those with periodontal disease experience and those without has varied from study to study. For example, Karoussis *et al.* (2003) divided patients into a) those whose implants were placed to replace a tooth or teeth which were lost due to periodontitis - they were considered "the periodontitis susceptible patient" and b) those who had implants placed to replace teeth that had been lost due to other reasons, these were classified as patients "without a history of chronic periodontitis". Mengel *et al.* (2007) did not present criteria that defined the level of periodontitis disease experience required for inclusion in either the generalised aggressive periodontitis patients or the periodontally healthy patient groups. Hardt *et al.* (2002) acknowledged the significant limitation of their study that was that the clinical parameters relating to the periodontal and peri-implant clinical examinations were not consistently available at follow up, and could not therefore be included in the analysis. The present study subdivided patients into those having experienced severe periodontal disease and those who had not, according to the recommendations of the European Workshop on Periodontology (2005), i.e. patients were classified as having experienced severe periodontal disease only if ≥30% sites demonstrated inter-proximal clinical attachment loss of ≥5 mm (Tonetti& Claffey, 2005). It may be suggested that this criteria represents a high threshold for periodontal disease experience and therefore had good specificity for patients with a strong susceptibility to periodontal disease, thereby adding strength to the present investigation.

The present study did not demonstrate an association between current smoking status and a diagnosis of peri-implantitis (Table 3.2). These findings were in agreement with those of Aalam & Nowzari (2005), Roccuzzo *et al.* (2010; 2012) and Marrone *et al.* (2013) who did not observe smokers to be at any increased risk of developing peri-implantitis, compared to non-smokers. However, many other studies have shown a positive association between smoking and the development of peri-implantitis (Hass *et*

al., 1996; Karoussis *et al.*, 2003; Nitxan *et al.*, 2005; Roos-Jansåker *et al.* 2006; Strietzel *et al.*, 2007; Fransson *et al.*, 2008; Levin *et al.*, 2008; Aglietta *et al.*, 2011; Rinke *et al.*, 2011; Swierkot *et al.*, 2012). Whilst the present study found no statistically significant relationship between smoking and peri-implantitis, 58% of the smokers were positive for peri-implant disease and only 31% of the non-smokers were positive for peri-implant disease. Whilst the present study meets the inclusion criteria employed by Renvert & Persson (2009), it may be argued that the failure to reach statistical significance is perhaps due to an inadequate number of participants being included in the present study. Therefore, the finding of smoking status not being observed to be significantly associated with peri-implantitis or implant loss should be interpreted in light of the current literature and the limitations of the present study.

3.6 Conclusions

In a population of 51 patients with dental implants in place for five or more years, a demonstrated susceptibility to periodontitis was noted to be positively associated with risk of peri-implantitis or implant loss ($p=0.03$). However, current smoking status was not observed to be significantly related to peri-implantitis status or implant loss ($p=0.1$).

Chapter 4

The clinical response of peri-implant infections to non-surgical treatment

4.1 Introduction

4.1.1 Peri-implant infections and non-surgical treatment

Peri-implant infections comprise both peri-implant mucositis and peri-implantitis. Peri-implant mucositis is defined as a reversible inflammatory change of the peri-implant soft-tissues without bone loss (Albrektsson & Issidor, 1994), whereas peri-implantitis is defined as an inflammatory process of the peri-implant tissues resulting in the loss of supporting bone (Albrektsson & Issidor, 1994). The goal of treatment of peri-implant infections is to control the infection and prevent disease progression (Heitz-Mayfield *et al.*, 2008). Limited evidence is available to support the efficacy of non-surgical mechanical treatment of peri-implant infections (Schwartz *et al.*, 2005; De Araujo *et al.*, 2006; Karring *et al.*, 2005; Renvert *et al.*, 2009). It may be suggested that to date there is a paucity of data investigating the efficacy of non-surgical treatment of peri-implant infections, in comparison to the volume and quality of the research on the response of periodontitis to non-surgical treatment. Non-surgical treatment of peri-implant infections comprises of oral hygiene instruction and full mouth debridement, with or without the adjunctive administration of systemic or local anti-microbial agents (Heitz-Mayfield *et al.*, 2008). Non-surgical mechanical debridement and oral hygiene instruction is the first line of treatment of gingivitis and periodontitis and has been observed to be effective in the treatment of gingivitis and in the treatment of the majority of periodontitis patients (Claffey *et al.*, 1994; Kaldahl *et al.*, 1996; Sanz *et al.*, 2012). However, the need for further research on the non-surgical treatment of peri-implant infections has been highlighted (Lang *et al.*, 1997; Roos Jansaker *et al.*, 2003; Renvert *et al.*, 2004; Renvert *et al.*, 2007; Renvert *et al.*, 2008; Heitz-Mayfield, 2008). Non-surgical mechanical treatment, especially when combined with adjunctive anti-microbial agents, has been observed to be effective in the treatment of peri-implant mucositis lesions (Ciancio *et al.*, 1995; Felo *et al.*, 1997; Strooker *et al.*, 1998), whereas several studies have observed limited or no improvements in peri-implant health following the non-surgical treatment of peri-implantitis lesions (Karring *et al.*, 2005; Renvert *et al.*, 2009; Persson *et al.*, 2010). It has been suggested that the difficulties in achieving healing following non-surgical mechanical treatment of implants affected with peri-implantitis, may be related to macro- and microscopic design features of the dental implant, as these act to limit non-surgical periodontal treatment by facilitating plaque accumulation and by impeding efficient debridement of the dental implant surfaces (Renvert *et al.*, 2006). Furthermore, several

important histopathological differences have been noted between peri-implantitis and periodontitis. Firstly, the peri-implant inflammatory pocket epithelium lesion extends deeper into the connective tissues directly contacting bone, in contrast to the periodontal inflammatory lesion which is usually separated from the alveolar bone by a connective tissue fibrous band. Secondly, the peri-implant pocket lesion involves a larger inflammatory infiltrate and a greater number of acute inflammatory cells than the lesions frequently observed in the periodontal tissues in cases of periodontitis (Berglundh *et al.*, 2011). Therefore, peri-implant lesions are often larger and extend deeper into the tissues and may be more difficult to treat non-surgically than periodontal lesions. Despite these difficulties, several studies have observed improvements in cases of peri-implantitis following non-surgical treatment, these include both improvements in bleeding on probing and probing pocket depths and have been noted in both animal (Mombelli & Lang 1992; Schwartz *et al.*, 2006) and human clinical studies (Schwartz *et al.*, 2005; De Araujo *et al.*, 2006).

The need for further investigations into the response of the peri-implant tissues to non-surgical treatment has been highlighted. It is recognised that in the treatment of peri-implantitis, successful elimination of the peri-implant infection is unpredictable. Van Steenberghe *et al.* (2012), publishing the conclusions of the Working Group of the Karolinska Institute of the Europerio Meeting 2012, concluded that *"no matter the treatment modality chosen, the majority of peri-implantitis lesions are not resolved by the treatment undertaken"*. In a systematic review of the literature, Renvert *et al.* (2008) concluded that from the available evidence, mechanical non-surgical treatment could be effective in the treatment of peri-implant mucositis, especially when combined with adjunctive anti-microbial agents. However, in peri-implantitis, non-surgical treatment was not found to be effective. Renvert*et al.* (2008) highlighted that there was limited available evidence on the response of peri-implant infections to non-surgical treatment.

4.2 Aim

To investigate the clinical effect of non-surgical mechanical treatment (i.e. oral hygiene instruction and mechanical debridement) in patients affected by peri-implant mucositis and/or peri-implantitis, nine months following treatment.

4.3 Methods

4.3.1 Patients

Patients attending the Dublin Dental University Hospital, and having one or more dental implant placed five years previously, were screened and those with one or more implant restorations, with a positive diagnosis of peri-implant mucositis or peri-implantitis, were asked to be enrolled in the present investigation. The inclusion and exclusion criteria applied are described in Chapter 2 Section 2.1.2 and Section 2.1.3. This study had the approval of the Ethics Committee of the Faculty Research Ethics Group, Faculty of Health Sciences, Trinity College Dublin (Chapter 2, Section 2.1.1).

Patients underwent a detailed dental and medical history and an oral examination including: a full mouth probing pocket depth measurement; measurement of periodontal attachment loss and bleeding on probing (Figure 4.1). A plastic periodontal probe with a pressure sensitive indicator corresponding to 0.2 – 0.25N force was used for all periodontal and peri-implant probing (Click Probe, Kerr Hawe).

Peri-apical radiographs were undertaken using a Rinn holder (Dentsply Rinn) and employing the long cone paralleling technique, i.e. the peri-apical radiograph film, the implant and the collimator face were aligned as parallel as possible and the radiograph exposed for the recommended time interval, dependant on the implant's anatomical location, as described in Chapter 2, Section 2.1.5. Radiographs included both conventional and digital radiographs. Conventional radiographs were viewed on a light box in a darkened room and a photograph was taken at a shutter speed of 0.32 s on a Cannon 30 D camera with a Macro 250 mm lens (Canon (UK) Ltd).

Similarly, where phosphor plates (Sidexis Dental Systems) were employed to capture the digital image of the radiograph, images were viewed in a darkened room and the settings adjusted to optimally view the peri-implant bone levels. Images were imported to Windows XP Paint Shop (Microsoft Corporation), cropped and pasted to Microsoft Office PowerPoint 2007 (Microsoft Corporation) and coded according to the patient's investigation number and the implant's anatomical location. All radiographs were examined independently by two examiners with regard to the diagnosis of peri-implant bone loss of $\geq$2.5 mm. Kappa analysis revealed good agreement between examiners ($p<0.001$). Where discrepancies were present between examiners, a third examiner was employed. All examiners were blinded with regard to the identity of patients and the clinical parameters.

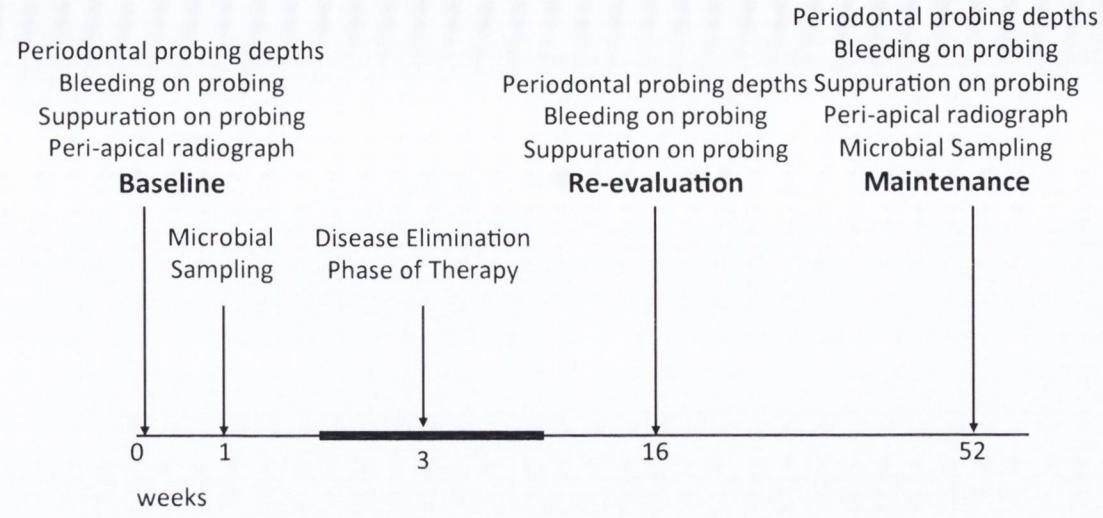

Figure 4.1 Sequence of visits.

Dental implants demonstrating peri-implant sites with bleeding on probing were diagnosed as being affected by peri-implant mucositis (Lang *et al.*, 2011). Implants demonstrating each of the following criteria: bleeding or suppuration on probing; probing pocket depth of ≥6 mm and radiographic bone loss ≥2.5 mm were classified as being affected by peri-implantitis (Berglundh *et al.*, 2002). Dental implants which did not meet the criteria of peri-implant bone loss of >2.5 mm and demonstrating at least one site with probing pocket depths of ≥6 mm but demonstrating bleeding or suppuration on probing were diagnosed peri-implant mucositis positive. Patients with implants not demonstrating bleeding or suppuration on probing were diagnosed as being healthy implants positive. On a patient level, patients with implants which were positive for peri-implant mucositis were diagnosed as peri-implant mucositis patients, whereas patients with implants positive for peri-implantitis were diagnosed as peri-implantitis positive. It is important to note that where patients had implants detected with peri-implantitis and peri-implant mucositis or peri-implant health, the diagnosis on the patient level was one of peri-implantitis. Similarly, where patients had both dental implants diagnosed as being healthy and others affected by peri-implant mucositis, a diagnosis of peri-implant mucositis was assigned on a patient level.

4.3.2 Treatment

Full mouth debridement was undertaken for each patient at separate visits to the baseline examination. Debridement was undertaken over one to four visits, according to the patient's ability to tolerate treatment and the difficulties of the instrumentation required (Figure 4.1). Local anaesthesia was employed when necessary. Debridement of implant fixture surfaces was undertaken employing titanium tipped curettes (Salvin Dental Specialties) as follows: gross supra-mucosal plaque and calculus deposits were removed firstly, if present. The curette tip was then placed in contact with the implant surface in the region of the most coronal threads. The curette tip was used to debride the implant surface in the region of the threads until it was deposit free, subsequently deeper threads were instrumented until the base of the pocket was reached. Following debridement, the implant surface was carefully checked using a plastic periodontal probe (Kerr Hawe) to locate any residual deposits. All residual deposits were removed and the implant surface repeatedly checked until a deposit free surface had been achieved. The natural teeth were debrided in a similar manner employing Gracey Curettes (Hu-Friedy).

Oral hygiene instruction was tailored to individual patient requirements. Patients were instructed to use either manual or battery driven toothbrushes according to their manual

dexterity and preference. Where patients had manual dexterity difficulties or if they stated a preference for an electric toothbrush, an electric tooth brush was recommended and patients were instructed in its safe and effective use. Where inter-proximal spaces were large enough to accommodate inter-proximal brushes (TePe) appropriate sizes were selected and the patients were instructed in the use of these. Where inter-proximal spaces were too small to accommodate inter-proximal brushes patients were instructed in the use of Super-floss (Oral B, Procter & Gamble)

4.3.3 Three and nine month examinations

Detailed periodontal examinations were undertaken three and nine months following completion of active treatment. Patients were also seen for an interim maintenance visit six months following completion of active treatment. At each of these visits, selective debridement was carried out at those sites that demonstrated bleeding on probing. Oral hygiene re-instruction was also given.

4.3.4 Statistical analysis

On a patient level, patients were divided into those affected by peri-implant mucositis (Peri-implant mucositis Group) and those affected by peri-implantitis (Peri-implantitis Group). For each patient, periodontal and peri-implant clinical data were collated both together and separately. Comparisons were made between the individual peri-implant and periodontal data and the combined peri-implant and periodontal data for each of the following clinical parameters: percentage of sites bleeding on probing (% sites BOP); percentage of sites demonstrating probing pocket depths of ≥6 mm (% sites ≥6 mm PPD); percentage of sites demonstrating suppuration on probing (% SOP). A formal model was employed comparing the clinical data within groups, and between groups, over the three time-points: baseline (T1); the three month examination (T2) and the nine month examination (T3). The within-patient clustering was controlled for by a patient-specific model using random effects. A logistic regression model that allowed for patients nesting within time was utilised.

Analysis was also undertaken on a unit level (i.e. tooth or implant) of probing pocket depth, bleeding on probing and suppuration on probing. In the unit level analysis for each patient, the tooth demonstrating the deepest probing pocket depth at baseline was included in the analysis, whereas all implants present were included in the analysis. Investigation for statistically significant changes was undertaken employing the unpaired t-test, as the data were normally distributed and comparable at two time points.

Comparisons were made between the deepest probing pocket depth of either the tooth or implant at baseline and at the nine months examination. Similarly, the presence or absence of bleeding on probing at a unit level was undertaken comparing the baseline (T1) and nine (T3) month data. Coding of the unit level bleeding on probing data was undertaken as follows: if any of the six points around the implant or tooth examined demonstrated bleeding on probing, the unit was recorded as positive for bleeding on probing. If no site bled on probing, the unit (either tooth or implant) was recorded as negative for bleeding on probing. Similarly, if suppuration on probing was detected at one or more sites, the unit was recorded as positive for suppuration on probing. If no suppuration on probing was detected, the unit was recorded as negative for suppuration on probing.

4.4 Results

The study was undertaken on an "intention to treat" protocol. Of the 47 patients who completed the course of non-surgical mechanical treatment and follow up, 40 patients attended the three month examination. Seven of the 42 patients who attended the nine month examination did not attend the three month examination. Five patients did not attend the nine month examination but attended the three month examination. Of the 47 patients included in the present investigation, 18 patients were diagnosed as having one or more implants affected by peri-implantitis (Peri-implantitis Group), whilst 35 patients were diagnosed as being affected by peri-implant mucositis (Peri-implant Mucositis Group).

Of the 47 patients included in the present investigation, 42 were partially dentate and five were fully-edentulous. Of the 42 partially-dentate patients, 41 had implant-supported crowns or bridges. One partially dentate patient had an implant-retained over-denture with a bar attachment on the mandibular arch opposing a natural maxillary dentition.

Five patients were fully-edentulous and were restored as follows: two had implant-retained over-dentures with bar attachments; one had an implant-retained over-denture with Locator™ attachments (ZEST Anchors LLC, California, USA); one had an implant-supported bridge on the mandibular arch and an implant-retained over-denture with a bar attachment on the maxillary arch; one fully-edentulous patient had implant-supported bridge-work in both the maxillary and the mandibular arches.

All of the implants reported in the present investigation employed an external hex connection between the implant fixture and the implant abutment. Seventeen patients were restored with Brånemark implants (Noble Biocare, Sweden); 21 had 3i implants (BIOMET 3i UK Ltd., UK) placed; one patient had both Brånemark and 3i implants placed, whilst four patients had ITI (Berne, Switzerland) implants. Two patients had dental implants placed in other countries and it was not possible to retrieve the implant types despite writing to the implant surgeons.

4.4.1 Bleeding on Probing

At each of the three time points, the Peri-implant mucositis Group was observed to demonstrate significantly lower percentages of sites bleeding on probing than the Peri-implantitis Group ($p \leq 0.05$) (Table 4.1). Furthermore, at each of the three time points, peri-implant sites demonstrated significantly higher percentages of sites bleeding on

Table 4.1 Mean percentage (and standard deviation) of sites demonstrating bleeding on probing for the peri-implant mucositis patients (peri-implant mucositits group) and those affected by peri-implantitis (peri-implantitis group).

	Site	% BOP (T1) Mean (SD)	% BOP (T2) Mean (SD)	%BOP (T3) Mean (SD)
Peri-implant mucositis	Periodontal	39.77 (26.12)	21.16 (22.95)	12.48 (15.56)
	Peri-implant	63.89 (32.45)	45.82 (34.34)	30.23 (29.48)
Peri-implantitis	Periodontal	47.04 (37.06)	40.33 (42.84)	23.08 (26.41)
	Peri-implant	69.76 (29.6)	70.23 (36.76)	35.59 (25.28)

Each group was analysed according to the percentage of sites demonstrating bleeding on probing at the three time-points (baseline (T1), the three month examination (T2) and the nine month examination (T3).

Abbreviations: BOP, bleeding on probing.

probing than the periodontal sites ($p \leq 0.05$). The percentage of sites bleeding on probing was observed to decrease significantly for the combined periodontal and peri-implant data at T3 compared to T1 ($p < 0.0001$).

For the Peri-implant mucositis Group, a highly significant improvement was noted in the percentage of peri-implant sites bleeding on probing when T3 was compared to T1 ($p < 0.0001$). A similar statistically significant reduction was observed in the Peri-implantitis Group in the percentage of peri-implant sites bleeding on probing between T3 and T1 ($p < 0.0001$). For the Peri-implant mucositis Group, the reduction in bleeding on probing was observed to be stepwise (i.e. of approximately equal magnitude between T1 and T2 and between T2 and T3), whereas for the Peri-implantitis Group, the reduction in bleeding on probing only became apparent at T3 and not at T1 or T2 (Table 4.1).

4.4.2 Percentage of sites with probing pocket depths ≥6 mm

The percentage of sites demonstrating probing pocket depths ≥6 mm at T1, T2 and T3 are presented in Table 4.2. For the Peri-implant mucositis Group, a reduction was noted in the percentage of sites with probing pocket depths ≥6 mm between T1 and T2 and between T2 and T3, however, differences were not significant ($p = 0.32$). For the peri-implantitis patients, the percentage of peri-implant sites with probing depths of ≥6 mm at T3 were significantly improved compared to T1 ($p = 0.02$).

A greater improvement was observed between T3 and T1 for the percentage of peri-implant sites with probing pocket depths of ≥6 mm, compared to the change in the percentage of periodontal sites with probing pocket depths of ≥6 mm ($p < 0.05$).

4.4.3 Suppuration on probing

The percentage of sites demonstrating suppuration on probing is outlined in Table 4.3. At the baseline examination, suppuration on probing was observed more frequently at peri-implant sites compared to periodontal sites. Also, following treatment, a trend towards a decrease in suppuration on probing was observed in peri-implant sites. However, it was not possible to undertake statistical analysis comparing the percentage of sites demonstrating suppuration on probing, as suppuration was observed on too few occasions for the analysis to be meaningful.

4.4.4 Unit level analysis

On a unit level, a highly statistically significant decrease was observed in the median peri-implant probing depths between T3 and T1 for those implants affected by peri-implantitis (i.e. the median peri-implant probing depth decreased from 6 mm (IQ range 6-7mm) to 5 mm (IQ range 4-6 mm) ($p<0.0001$). Following treatment, implants affected by peri-implant mucositis demonstrated a decrease in median peri-implant probing pocket depths at T3 compared to T1, with a reduction from 6 mm (IQ range 5-6 mm) at T1 to 4mm (IQ range 4-6 mm) at T3. The differences were statistically significant ($p<0.0001$).

A decrease in the deepest peri-implant and periodontal probing pocket depths was observed for both implants and natural teeth, between the time points T1 and T2 and between T2 and T3 as presented in Table 4.4. Employing a multi-level analysis of probing pocket depth as a function of tooth type (periodontal compared to peri-implant) and time (T2 and T3 compared to T1), the reduction in probing pocket depth was observed to be highly significant ($p<0.0001$). Furthermore, on a unit level, the periodontal sites demonstrated lower mean probing pocket depths than peri-implant sites by a mean value of 1.22mm at T1, 0.78mm at T2 and 1.14mm at T3. On a unit level, bleeding on probing was observed to decrease significantly for both periodontal and peri-implant sites over time ($p<0.0001$) (Table 4.5).

Table 4.2 Mean percentage of sites demonstrating probing pocket depths of ≥6 mm at baseline (T1), three months post-treatment (T2) and nine months post-treatment (T3) for the peri-implant mucositis and peri-implantitis groups.

	Site	Baseline % (T1)	Three month examination % (T2)	Nine month examination % (T3)
Peri-implant mucositis	Periodontal	2.92 (7.37)	1.98 (4.9)	1.37 (3.83)
	Peri-implant	22.38 (24.49)	14.97 (22.51)	10.35 (19.17)
Peri-implantitis	Periodontal	9.01 (17.41)	2.91 (5.57)	3.08 (4.75)
	Peri-implant	31.96 (14.72)	25.95 (20.84)	14.86 (14.55)

Table 4.3 Percentage of sites demonstrating suppuration on probing at T1, T2 and T3 for patients with peri-implant mucositis and peri-implantitis.

	Site	Baseline % (T1)	Three month examination % (T2)	Nine month examination % (T3)
Peri-implant mucositis	Periodontal	0.15 (0.65)	0.16 (0.47)	0.09 (0.44)
	Peri-implant	3.1 (6.73)	0 (0)	0 (0)
Peri-implantitis	Periodontal	0.08 (0.26)	0.39 (1.17)	0 (0)
	Peri-implant	0.46 (1.6)	1.85 (5.56)	0 (0)

Table 4.4 Multi-level analysis of probing pocket depth (PPD) as a function of unit type (periodontal or peri-implant) and time (T2 and T3 compared to T1).

	Estimate (mm)	t- value	p-value
Intercept	5.79	36.99	<0.0001
Baseline (T1) Periodontal compared to peri-implant PPD	-1.22	-9.09	<0.0001
Three month examination (T2)	-0.78	-5.13	<0.0001
Nine month examination (T3)	-1.14	-7.61	<0.0001

Table 4.5 Multi-level logistic analysis of bleeding on probing (BOP) as a function of tooth type (periodontal compared to peri-implant) and time (T2 and T3 compared to T1).

	Estimate (No. of occasions BOP)	z- value	p-value
Intercept	3.17	9.54	<0.0001
Baseline (T1) periodontal compared to peri-implant (BOP)	-1.95	-6.76	0.00276
Three month examination (T2)	-1.03	-2.99	<0.0001
Nine month examination (T3)	-1.85	-4.73	<0.0001

4.5 Discussion

The aim of the present study was to investigate the clinical effect of non-surgical mechanical treatment (i.e. oral hygiene instruction and mechanical debridement) on peri-implant infections. In the present study, improvements in periodontal and peri-implant clinical parameters were observed nine months following treatment.

For both the peri-implant mucositis and the peri-implantitis groups, significant reductions were observed on a patient level in the percentage of sites demonstrating bleeding on probing, nine months following the completion of the non-surgical treatment ($p<0.05$). Similarly, on a unit level, significant reductions were observed in peri-implant bleeding on probing for both the peri-implant mucositis and the peri-implantitis groups ($p<0.0001$). Reductions in peri-implant bleeding on probing following non-surgical treatment is in line with previous studies which similarly observed reductions in bleeding on probing in peri-implantitis (Mombelli *et al.*, 1992; Leonhardt *et al.*, 2003; Mombelli *et al.*, 2005; De araujoNobre *et al.*, 2006; Maximo *et al.*, 2009; Renvert*et al.*, 2009; Thone-Muhlig *et al.*, 2010; Sahm *et al.*, 2011; Machetti *et al.*, 2012) and peri-implant mucositis (Cianco *et al.*, 1995; Felo *et al.* 1997; Shenk *et al.*, 1997; Strooker *et al.*, 1998; Porras *et al.*, 2002; De Araujo Nobre*et al.*, 2006; Heitz-Mayfield *et al.*, 2011). Few studies have investigated the response of the peri-implant tissues to non-surgical, mechanical debridement alone (i.e. without the use of an adjunctive anti-microbial agent) in cases of peri-implant mucositis or peri-implantitis (Karring *et al.,* 2005; Renvert *et al.,* 2009; Thone-Muhling *et al.,* 2010).

Interestingly, in the current study, for the Peri-implant mucositis Group, the observed improvements at nine months (T3) in the percentage of peri-implant sites with probing pocket depths of ≥6 mm was not statistically significantly different to baseline (T1) ($p=0.32$). However, for the Peri-implantitis Group, a significant improvement was noted in the percentage of sites with probing pocket depths of ≥6 mm at nine months (T3) compared to baseline (T1) ($p=0.02$). On a unit (implant) level, significant reductions were noted in probing pocket depths for both peri-implantitis sites and peri-implant mucositis sites ($p<0.0001$) at nine months (T3) compared to baseline (T1).

The question arises as to why the peri-implant mucositis group failed to show a significant improvement in the percentage of sites with probing pocket depths of ≥6 mm on a patient level ($p=0.32$), whilst the peri-implantitis group did show a statistically significant improvement in the percentage of sites with probing pocket depths of ≥6 mm ($p=0.02$) at nine months (T3) compared to baseline (T1). Is it that clinically non-surgical

treatment of peri-implantitis is more effective than the non-surgical treatment of peri-implant mucositis? The failure of the improvements in the percentage of peri-implant sites with probing pocket depth ≥6 mm of the Peri-implant mucositits Group to reach statistical significance at nine months (T3) compared to baseline (T1), is in fact more likely a lack of statistical significance, rather than a lack of clinical significance. Clinically, improvements were frequently observed in the Peri-implant mucositis Group's peri-implant probing pocket depth, as can be seen by the reduction in their mean percentage of sites with probing pocket depths of ≥6 mm which more than halved over the treatment interval T1 to T3 (22.38% and 10.35%, respectively). Furthermore, on a unit (implant) level, statistically significant improvements were observed in peri-implant probing pocket depths of both the Peri-implant mucositis and the Peri-implantitis Groups between T3 and T1 ($p<0.0001$). Therefore, the lack of statistical significance in the change between T3 and T1 of the percentage of peri-implant probing pocket depths ≥6 mm for the Peri-implant mucositis Group, in contrast to the significant improvement observed in the Peri-implantitis Group, is most likely related to the differences between the two groups, firstly, in the baseline (T1) values of the mean percentage of sites with probing pocket depths of ≥6 mm, and secondly, in the magnitude of the standard deviations of the mean percentage of sites with probing pocket depths of ≥6 mm. The baseline mean percentage of sites with probing pocket depths of ≥6 mm was lower for the peri-implant mucositis group compared to that of the peri-implantitis group (22.38% and 31.96%, respectively). Therefore, there was a greater potential for reduction in the percentage of sites with probing pocket depths of ≥6 mm for the peri-implantitis group, than that of the peri-implant mucositis group. Furthermore, the range of values for the percentage of sites with probing pocket depths of ≥6 mm varied widely within the peri-implant mucositis group, in contrast to the range of values of the peri-implantitis group. This difference in variability of the range of values may in part be explained by the classification employed: The peri-implantitis group inclusion criteria required that all patients had to have at least one implant with a probing depth of ≥6 mm, demonstrated bleeding or suppuration on probing and demonstrated peri-implant bone loss of ≥2.5 mm (Berglundh *et al.,* 2002). All other patients with implants who demonstrated bleeding or suppuration on probing, but did not meet both the probing depth and bone loss additional criteria, were diagnosed as being affected by peri-implant mucositis. Therefore, the peri-implant probing depth range for the peri-implant mucositis group varied widely as patients could have had deep or shallow peri-implant probing depths and still meet the

criteria required for the diagnosis of peri-implant mucositis. Accordingly, the standard deviation of the peri-implant mucositis group's baseline (T1) mean percentage of peri-implant sites with probing pocket depths of ≥6 mm, was much higher compared to that of the peri-implantitis group (24.49% and 14.72%, respectively). This is important as the higher standard deviation, or the less uniform the data set is for a particular time point, the less likely it is that a clinically significant change will achieve statistical significance (Greenstein, 2003) Similarly, the lesser margin for change between two time points (as was the case for the mean value of the percentage of peri-implant sites with probing pocket depths of ≥6 mm between T1 and T3 in the peri-implant mucositis group, compared to that of the peri-implantitis group), the less likely it was that any observed clinical improvements would achieve statistical significance.

Few studies have observed improvements in peri-implant probing pocket depths in patients with peri-implantitis following non-surgical treatment (Mombelli *et al.,* 1992; Mombelli *et al.,* 2005; Schwartz *et al.,* 2005; De araujo*et al.,* 2006; Thone-Muhling *et al.,* 2010; Sahm *et al.,* 2011; Machtei *et al.,* 2012). Furthermore, Thone-Muhling *et al.* (2010) is the only other study which has observed reductions in probing pocket depths, whilst undertaking mechanical debridement without the use of adjunctive anti-microbial agents. In the present study, adjunctive antibiotics or antiseptics were not administered either systemically, or into the peri-implant sulcus professionally.

The findings of this investigation are in line with those of Schwartz *et al.* (2005) and De Araujo *et al.* (2006), who observed significant improvements in peri-implant clinical parameters following non-surgical debridement and oral hygiene instruction. The observed reduction in peri-implant inflammation, as reflected in the periodontal probing depth and bleeding on probing reduction, for both peri-implantitis and peri-implant mucositis patients, differs from that reported by Karring*et al.* (2005) and Renvert*et al.* (2009).

Karring *et al.* (2005) compared mechanical debridement (with carbon fibre curettes) to debridement with an air abrasive system, in 11 peri-implantitis patients following a split mouth design. Very limited improvements in bleeding on probing were noted and despite improved patient performed oral hygiene, neither treatment resulted in probing pocket depth reduction. This may in part be explained by the fact that the carbon fibre curettes are quite thick in cross-section and this may lead to instrumentation with carbon fibre curettes being more difficult in the more apical portions of the peri-implant pockets. Renvert *et al.* (2007) observed improvements in the treatment of peri-implant

infections non-surgically when undertaken in conjunction with the adjunctive administration of systemic antimicrobials or laser treatments. The authors hypothesised that difficulties encountered in effectively achieving the removal of the plaque and calculus biofilm in peri-implant pockets, meant that it was not possible to effectively resolve the inflammation in the periodontal tissues.

In the present study, non-surgical peri-implant treatment without the use of adjunctive anti-microbial agents was seen to result in improvements in the peri-implant parameters of probing pocket depth and bleeding or suppuration on probing. In the present study, thorough mechanical debridement of both the natural teeth and the dental implants was undertaken and debridement and oral hygiene instruction repeated at each visit. The repeated debridement and oral hygiene may help to explain the observed improvements in peri-implant parameters, as it is expected that with repeated mechanical debridement, further reductions in the sub-gingival microbial levels were achieved. Furthermore, with repeated oral hygiene, it is likely that patient performed oral hygiene practices improved in efficiency and patients were re-motivated at each visit.

Schwartz *et al.* (2005) observed improvements similar to the present investigation following utilisation of carbon fibre curettes and chlorhexidine digluconate (0.2%) in the treatment of 10 patients affected by peri-implantitis. The bleeding score was observed to decrease from 80% at baseline to 58% after six months ($p<.001$) and a mean change in clinical attachment level was observed to decrease from 6.2 +/-1.5 mm at baseline to 5.6 +/- 1.6 mm after six months ($p<0.001$).

Thone-Muhling *et al.* (2010) undertook a clinical randomised controlled trial in 13 patients, comparing the full mouth debridement (single visit) with or without adjunctive chlorhexidine 1% applied at the time of debridement and 0.2% rinse and spray for the 14 days following the treatment. Both groups demonstrated clinical improvements in probing pocket depths and bleeding on probing. However, no significant differences were observed clinically or microbiologically between the two groups, indicating that the chlorhexidine regimen did not result in a statistically significant difference.

However, Renvert *et al.*, (2006) undertook a clinical prospective randomised trial with a one year follow up of 32 patients with incipient peri-implant infections (bone loss limited to less than three threads of Brånemark implant in conjunction with a peri-implant probing pocket depth of at least 4 mm) comparing adjunctive minocycline microspheres to 1% chlorhexidine gel. Debridement was undertaken utilising specific scalers for implant treatment (Hawe Neo Oss, KerrHawe, USA) and a rubber cup with polishing

paste. Following mechanical treatment, 1ml of either chlorhexidine or minocycline was applied to the peri-implant sulcus in a double blind manner. Minocycline was seen to result in significant improvements in probing pocket depths and bleeding on probing ($p<0.05$), however, the chlorhexidine gel group only demonstrated significant improvements in bleeding on probing ($p<0.05$). These findings differ from the significant improvements observed in probing pocket depths for peri-implantitis patients in the present study on a patient ($p<0.05$) and unit ($p<0.0001$) level. Renvert *et al.* (2006) observed significant probing pocket depths only in those patients who received adjunctive minocycline ($p<0.05$) but not the chlorhexidine gel group ($p>0.05$). This may be explained at least in part by the fact that no re-instrumentation of the peri-implant or periodontal sites was undertaken by Renvert *et al.* (2006) in the 12 month observational interval (the only intervention which was permissible at the recall visits was oral hygiene re-instruction). In the current study, as topical anti-microbial agents were not applied at one time point, re-instrumentation was not precluded. Re-instrumentation of persistently deep sites is part of the routine maintenance care for patients with dental implant and was adopted in the present protocol.

Peri-implant suppuration on probing was noted to decrease following treatment for both the Peri-implant mucositis and the peri-implantitis groups, however suppuration on probing was observed on too few occasions to undertake statistical analysis, a difficulty that has been encountered in many other studies examining the response of the peri-implant (Karring *et al.*, 2005; Renvert *et al.*, 2006; Thone-Muhling *et al.*, 2010) and the periodontal tissues (Claffey *et al.*, 1994; Kaldahl *et al.*, 1996; Kalkwarf *et al.*, 1997) to non-surgical treatment.

Whilst on a patient level peri-implant sites demonstrated greater reductions in probing pocket depths and bleeding on probing than the periodontal sites ($p<0.05$), it is important to bear in mind that the peri-implant sites baseline bleeding on probing and probing pocket depths were greater and therefore had greater possibility for reductions than those of periodontal sites, as their baseline status was closer to health. Furthermore, peri-implant sites demonstrated a significantly higher percentage of sites bleeding on probing than the periodontal sites at each of the three time points ($p\leq0.05$). This finding may be explained in part by the inclusion criteria that required that all patients included in the study had to have at least one implant positive for peri-implantitis or peri-implant mucositis, and therefore all patients in the present study had at least one implant which, by definition, demonstrated bleeding or suppuration on probing (as this requirement is

included in both the criteria employed for the diagnosis of peri-implantitis and that of peri-implant mucositis). Also, it may be the case that the peri-implant tissues were more fragile and prone to bleeding on probing than the peri-implant tissues.

Also, it is interesting to note that the Peri-implantitis Group's reduction in bleeding on probing on a patient level only became apparent between the T2 and T3 examination, whereas the reduction observed in the bleeding on probing for the Peri-implant mucositis group took place more gradually. It may be the case that patients required repeated instrumentation and oral hygiene instruction to achieve the improvements observed in probing pocket depths and bleeding on probing. Alternatively, it may be that the peri-implant tissues require an extended time interval to demonstrate improvements in probing pocket depths. The duration of healing of the periodontal tissues with regards to probing pocket depth and bleeding or suppuration on probing, following non-surgical debridement, is well described (Claffey *et al.,* 1994). However, the duration of healing of the peri-implant tissues following non-surgical debridement has not yet been specifically investigated.

4.6 Conclusions

In conclusion, the present study observed that non-surgical mechanical treatment resulted in significant improvements in probing pocket depths and bleeding on probing in both patients with peri-implant mucositis and/or peri-implantitis. Therefore, non-surgical mechanical treatment may be recommended as a first line treatment for these patient groups.

Chapter 5

A comparison of the bacterial species recovered from sub-gingival sites by paper-point and curette sampling using checkerboard DNA-DNA hybridisation

5.1 Introduction

Sub-gingival microbial samples are taken clinically to provide diagnostic information on periodontal or peri-implant disease and for research purposes to further understand periodontal and peri-implant disease mechanisms (Loomer, 2004). The microbial species of the periodontal niche have been observed to reside within the highly ordered ecological system of the plaque biofilm on the tooth's root surface and in the fluid within the gingival crevicular fluid (GCF) in the periodontal pocket (Haffajee & Socransky, 2008). Similarly, in patients with dental implants, microbial species have been isolated from the plaque biofilm on the dental implant's surface and from the peri-implant crevicular fluid. The isolation of microorganisms from the sub-gingival microbial ecological niche is undertaken chiefly using paper-point sampling or curette sampling (Loomer, 2004).

Paper-points, routinely used for drying prepared root canals, are both fine and absorbent and are perhaps the most popular method of collecting sub-gingival microbial samples (Socransky, 1998). Curettes for sub-gingival root debridement are also often utilised for sub-gingival microbial sampling (Maximo *et al.*, 2009). However, there is no consensus yet as to whether the curette sampling technique or the paper-point sampling technique is more effective in recovering microbial species from subgingival/submucosal sites which best reflect the actual composition of the subgingival microflora (Baker *et al.*, 1991; Keil & Lang, 1991; Renvert *et al.*, 1992; Socransky, 1998; Gerber *et al.*, 2006; Jervoe-Storm *et al.*, 2007). It has been suggested that curette sampling collects plaque from the entire pocket, whereas the plaque adsorbed by paper-point sampling is derived mostly from the outer layer of the plaque biofilm and may contain more pathogenic microflora (Socransky, 1998). It has also been suggested that paper-points are less successful at collecting plaque in the deep portions of a periodontal pocket compared to areas near the gingival margin (Baker *et al.*, 1991). The relative merits of paper-point and curette sub-gingival sampling techniques have been explored in only a handful of clinical studies to date (Keil & Lang, 1991; Renvert *et al.*, 1992; Gerber *et al.*, 2006; Jervoe-Storm *et al.*, 2007).

Baker *et al.* (1991) undertook a laboratory investigation that examined the efficacy of paper-point sampling at recovering the microflora representative of a site with a non-homogenous distribution of microorganisms. These authors were concerned that paper-points may become saturated as they are placed into the periodontal pocket and therefore would not have the ability to absorb the inflammatory exudate fluid with the bacteria from the more apical regions of the periodontal pocket plaque biofilm. In order to

elucidate this, Baker *et al.* (1991) employed a model using multiple layers of liquid cultures of periodontopathogenic bacterial species. Varying sampling protocols were undertaken and the samples isolated were qualitatively and quantitatively compared. The investigators observed that at least 90% of the microorganisms recovered by paper-point sampling were from the layer of microorganisms in closest proximity to the paper-point, which was assumed to be the most superficial layer but this was not explained in the text. However, bacteria from the more apical regions of the biofilm were observed in the coronal portion of the paper-point, suggesting that capillary action may have played a role in the absorption of the bacteria within the paper-point. Baker *et al.* (1991) concluded that paper-points may yield samples which do not accurately reflect the microbial composition in the pocket from which the sample was taken.

A 10-1000 fold greater number of bacterial colony forming units (cfu) have been isolated from curette samples compared to single paper-point samples, however, increasing the number of paper-points employed was observed to result in a higher numbers of cfus recovered (Keil & Lang, 1991). Renvert *et al.* (1992) compared paper-point and curette sampling methods utilising culture techniques. Duplicate microbial samples were undertaken, each one week apart, in 12 patients with periodontitis, prior to and following treatment of their periodontal condition. Interestingly, spirochete spp. were recovered from paper-point samples in significantly higher numbers than from the curette samples, in samples taken both prior to and following periodontal treatment ($p<0.05$). Furthermore, paper-point samples were observed to result in significantly higher numbers of cfus being isolated, compared to curette samples, and this finding was observed irrespective of the sampling order (i.e. irrespective of whether the paper-point sampling or curette sampling was undertaken first).

However, in a study of 20 patients with chronic periodontitis for whom paper-point and curette samples were taken prior to and following treatment of their periodontal condition, Jervoe-Storm *et al.* (2007) observed that prior to treatment, curette sampling resulted in significantly greater numbers of species being detected compared to paper-point sampling techniques. Following treatment, Jervoe-Storm *et al.* (2007) reported that curette sampling was observed again to result in significantly greater numbers of species being detected compared to paper-point sampling. Jervoe-Storm *et al.* (2007) analysed paper-point and curette samples using real-time PCR techniques that examined the relative abundance of the periodontopathogens as a proportion of the total bacteria detected and termed this proportion "the total target pathogen proportion". Presumably,

the total target pathogen proportion was calculated as the sum of the concentration of the DNA of each of the target pathogens present, as a proportion of the total bacterial DNA detected. However, the authors did not provide details on how this total target pathogen proportion was calculated. Prior to treatment, curettes were observed to yield samples with somewhat higher target proportions than those of the corresponding paper-point samples (32% compared to 27%, respectively). Following treatment, paper-points were observed to yield samples with higher target proportions than those of the corresponding curette samples (24% compared to 19%, respectively). The authors concluded that overall, only minor differences were observed between curette and paper-point sampling techniques, and that a good agreement was observed between paper-point and curettes sampling techniques. It should be noted that real-time PCR usually cannot distinguish between living and dead microorganisms.

In another study by Gerber *et al.*, (2005), Proflow paper strips (Proflow Inc., Amityville, New York, USA) were placed in periodontal or peri-implant sites to absorb samples of the gingival crevicular fluid (GCF), from which the bacterial species present were investigated utilising checkerboard DNA-DNA hybridisation techniques. The GCF microbiological samples were then compared to curette microbiological samples taken from the periodontal or peri-implant pocket, where Gracey-designed steel curettes (Stalan GMBH, Ahrensburg, Germany) were applied to the root or implant surface to gain plaque samples for investigation (Gerber *et al.*, 2005). The total concentration of bacterial DNA present was calculated based on the summation of the bacterial DNA detected for each of the investigated species. In the samples taken from the peri-implant sites, GCF samples were observed to result in higher concentrations of total bacterial DNA being detected than in the samples gained utilising curettes ($p<0.001$). Furthermore, the authors noted that GCF samples resulted in significantly higher proportions of 28 of the 40 species investigated (p values varying between 0.005 and 0.001), compared to the corresponding curette sampling techniques. However, no definition or method of calculation of the proportional distribution of each bacterial species was given. Gerber *et al.* (2005) suggested that the increased efficiency of GCF sampling in yielding samples more representative of peri-implant sites may be due in part to the difficulties encountered in collecting deposits from the implant surface with curettes. Gerber *et al.* (2005) recommended that it may be beneficial to combine both GCF and curette sampling techniques, providing a pooled sample which would result in the most representative microbial sample for either periodontal or peri-implant sites.

From the available evidence, it is unclear as to whether paper-point or curette sampling differ in their efficiency in gaining sub-gingival/ submucosal microbial samples, especially from peri-implant sites. Therefore, further investigation is required regarding the relative merits of paper-point and curette sampling.

5.2 Study aim

The aim of this part of the present study was to semi-quantitatively compare the microbial species recovered from periodontal and peri-implant sites using paper-point and curette microbial sampling techniques, to increase understanding of the efficiency of these techniques in gaining samples representative of the microflora of the site from which they were taken.

5.3 Materials and Methods:

5.3.1 Patient selection

This study had the approval of the Faculty of Health Sciences Research Ethics Committee, Trinity College Dublin (see Chapter 2, Section 2.1.1 and Appendix 1).

Patients attending the Dublin Dental University Hospital and having had one or more dental implants placed five or more years previously, were invited to attend an assessment appointment as described in Chapter 2, Section 2.1.1. The patient inclusion and exclusion criteria are detailed in Chapter 2, Sections 2.2.2 and 2.2.3.

Patients underwent a detailed dental and medical history and an oral examination including: full mouth probing pocket depth measurement, measurement of periodontal attachment loss and bleeding on probing, as described in Chapter 2. Section 2.1.4 and radiographic examination undertaken as described in Chapter 2, Section 2.1.5.

5.3.2 Microbial Sampling

At least one week following the initial baseline examination, microbial sampling was undertaken as described in Chapter 2, Section 2.5. Both curette and paper-point sampling techniques were employed following careful tooth isolation. Paper-point sampling was undertaken first and curette sampling subsequently, as follows: paper-point samples were obtained using three size 35 sterile paper-points (Coltene Roeko) per site. Each of the three paper-points was individually inserted into the periodontal or peri-implant pocket until all three were present in the pocket together for 30 s. They were then removed and placed in a sterile 1.5 ml plastic tube (Sarstedt Ltd.) containing 1 ml of YEPD broth and sealed. A curette sample was also taken of the subgingival plaque deposits from each site. Curette samples from peri-implant sites were obtained using titanium tipped curettes (Salvin Dental Specialties) as follows: the curette tip was used to gain deposits from the implant fixture surface, initially from the coronal threads and subsequently the deeper threads. Following sampling, the tip of the curette was removed from the peri-implant pocket and rotated vigorously in a 1 ml sterile YEPD broth in a 1.5 ml tube in order to disperse the microorganisms. Curette samples were obtained from the periodontal sites using the same technique, but instead employing Gracey Curettes (Hu-Friedy). Sampling sites included the deepest site of each implant present, the deepest site of one periodontally healthy tooth (the contra-lateral unit to the implant restoration where

present, or the nearest unit to this) and one periodontally diseased tooth (the tooth with the deepest periodontal probing pocket depth) was sampled.

The present investigation is a methodological investigation as part of a larger investigation of the peri-implant and periodontal microflora. Therefore, the samples selected for analysis in the comparison of paper-point and curette sampling techniques were a subset of the larger collection of microbial samples. The selection of the sites whose samples were to be included in this methodological investigation comparing paper-point and curette sampling techniques was undertaken by a blinded third party not involved in the investigation, in a manner similar to throwing a dart at a list of numbers. Samples were taken from 40 sites in 27 patients. Gerber *et al.* (2005), employing a similar study design had observed statistically significant differences in a study group of 28 patients. Twenty-four periodontal sites in 23 patients were included as well as 16 peri-implant sites in 12 patients.

5.3.3 Microbial Analysis

Curette and paper-point samples were transferred to the microbiology laboratory in separate vials each containing 1 ml of YEPD broth. Following preparation of the samples (as outlined in Chapter 2, Section 2.2.6.1) samples were stored at -80°C until an adequate number had been collected for dispatch for analysis. Samples were transported on dry ice to the University of Bern Oral Microbiology Laboratory, Bern, Switzerland. Semi-quantitative estimates of 29 oral species (Chapter 2, Table 2.1) were determined in each sample using a modification of the checkerboard DNA-DNA hybridisation technique (Socransky *et al.*, 1994; Haffajee *et al.*, 1997*a*; Persson et al., 2007; Eick *et al.*, 2011) as described in Chapter 2, Section 2.2.6.1.

Additional to the checkerboard technique, real-time PCR was undertaken for four bacterial species including *A. actinomycetemcomitans, T. forsythia, P. gingivalis, P. intermedia* and *T. denticola* as described in Chapter 2, Section 2.2.6.2. Control reference strains used in real-time PCR experiments are shown in Chapter 2, Table 2.2. In the final analysis, only checkerboard DNA-DNA hybridisation results confirmed by real-time PCR for these species were considered valid and included in the analysis.

5.3.4 Data analysis

Data from checkerboard DNA-DNA hybridisation analysis of individual paper-point and curette samples were examined to investigate qualitative and quantitative differences between the microbial species detected using both sampling techniques. Descriptive

statistics were employed to compare the estimated bacterial cell density for individual bacterial species $x10^5$ cfu (based on DNA-DNA hybridisation) detected from all of the samples gained using both paper-point and curette sampling techniques under the following headings:

- Comparison of the number of occasions a greater number of bacterial species was detected from individual sites by either paper-point or curette sampling techniques.

- Comparison of the estimated number of bacteria cells of each of the investigated species or species groupings detected by both paper-point and curette sampling, by paper-point sampling alone or by curette sampling alone, from periodontal and peri-implant sites.

- Comparison of the estimated number of bacteria cells of each of the investigated species or species groupings detected under the following headings:

  Periodontal sites: a) paper-point samples b) curette samples

  Peri-implant sites: a) paper-point samples b) curette samples

- Comparison of the number of times paper-point and/or curette sampling from peri-implant and periodontal sites, detected each of the 18 bacterial species groupings investigated.

- Comparison of the frequency individual species or species groupings were detected by paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling (n=40) for:

  Both the periodontal and peri-implant sites.

  The periodontal sites

  The peri-implant sites.

The relative estimated bacterial cell density of DNA of individual species that were identified from individual sites utilising paper-point or curette sampling techniques were referred to as the percentage relative abundance (% relative abundance). The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species groupings, as a proportion of all of the species detected at an individual site. Comparisons were made of the percentage relative abundance of the

individual species groupings that were identified from individual sites utilising paper-point or curette sampling techniques employing descriptive statistics.

The estimated bacterial cell densities of *A. actinomycetemcomitans, P. gingivalis*, *T. forsythia*, *Treponema* spp. and staphylococcal spp. were compared between paper-point and curette samples utilising the Wilcoxn Rank Sum test.

5.4 Results

5.4.1 Patients

Paper-point and curette samples from 40 sites from 27 partially dentate implant patients were examined. Sixteen of the examined sites were peri-implant sites and 24 were periodontal sites. Curette and paper-point sampling techniques detected similar amounts of bacterial DNA as determined by checkerboard DNA-DNA analysis ($p<0.05$). Neither paper-point nor curette sampling was observed to routinely yield greater concentrations of bacterial DNA than each other: in 52.5% (21/40) of the sites examined, paper-point sampling yielded a greater amount of bacterial DNA compared to the corresponding curette sample.

In 55% (22/40) of the periodontal and peri-implant sites examined, paper-point sampling resulted in a greater number of bacterial species being identified than curette sampling when checkerboard analysis was undertaken (Tables 5.1 and 5.2). In contrast, in 30% (12/40) of the sites examined curette sampling resulted in detection of a greater number of bacterial species being detected than by paper-point sampling. In 15% (6/40) of sites, both paper-point and curette sampling resulted in the same number of bacterial species being detected.

For the periodontal sites, in 58.3% (14/24) of cases paper-point sampling resulted in the detection of a greater number of bacterial species compared to curette sampling. In 25% (6/24) of the periodontal sites, curette sampling resulted in the detection of a greater number of bacterial species than paper-point sampling and in 16.7% (4/24) of periodontal sites an equal number of bacterial species was detected using both paper-point sampling and curette sampling techniques (Tables 5.1 and 5.2).

Interestingly, in 12.5% (2/16) of the peri-implant sites tested, both paper-point and curette sampling detected the same number of bacterial species. In 50% (8/16) of the peri-implant sites, paper-point sampling detected a greater number of bacterial species than curette sampling, whilst in 37.5% (6/16) of the peri-implant sites, curette sampling detected a greater number of bacterial species than paper-point sampling (Table 5.1).

For each of the 18 species groupings investigated, neither curette nor paper-point sampling techniques were observed to yield a significantly greater estimated bacterial cell density (based on checkerboard DNA-DNA hybridisation), for either periodontal or peri-implant sites or both periodontal and peri-implant sites (Table 5.3) ($p>0.05$).

Table 5.1 Comparison of the number of occasions a greater number of bacterial species were detected from individual sites by either paper-point or curette sampling techniques.

	Number of sites where paper-points detected a greater number of bacterial species than curette sampling	Number of sites where curette samples detected a greater number of bacterial species than paper-point sampling	Number of sites where paper-points and curette samples detected equal numbers of bacterial species
Periodontal and peri-implant sites (n=40)	22	12	6
Periodontal sites (n=24)	14	6	4
Peri-implant sites (n=16)	8	6	2

Table 5.2 Number of bacterial species detected by paper-point and/or curette sampling from periodontal and peri-implant sites by checkerboard analysis.

Patient No.	Site	Peri-implant or periodontal	No. of bacterial species (n=18)		
			Both paper-point and curette sampling yielded same result	Detected by paper-point sampling alone	Detected by curette sampling alone
4	1	Periodontal	5	11	2
5	2	Periodontal	15	0	3
6	3	Periodontal	14	2	2
7	4	Periodontal	12	4	2
8	5	Periodontal	8	0	10
10	6	Periodontal	17	1	0
11	7	Periodontal	12	4	2
12	8	Periodontal	12	4	2
14	9	Periodontal	15	1	2
15	10	Periodontal	7	0	11
16	11	Periodontal	3	15	0
17	12	Periodontal	8	10	0
18	13	Periodontal	8	10	0
20	14	Periodontal	14	3	1
22	15	Periodontal	9	9	0
24	16	Periodontal	18	0	0
25	17	Periodontal	6	10	2
29	18	Periodontal	18	0	0
31	19	Periodontal	5	13	0
32	20	Periodontal	9	8	1
41	21	Periodontal	15	3	0
11	24	Periodontal	16	2	0
21	36	Periodontal	9	9	0
5	39	Periodontal	18	0	0
17	22	Peri-implant	5	0	13
22	23	Peri-implant	6	0	12
17	25	Peri-implant	5	13	0
25	26	Peri-implant	17	0	1
5	27	Peri-implant	14	4	0
5	28	Peri-implant	5	13	0
50	29	Peri-implant	18	0	0
35	30	Peri-implant	18	0	0
42	31	Peri-implant	15	0	3
42	32	Peri-implant	15	0	3
42	33	Peri-implant	11	7	0
17	34	Peri-implant	17	0	1
17	35	Peri-implant	10	8	0
46	37	Peri-implant	8	4	6
23	38	Peri-implant	15	2	1
39	40	Peri-implant	15	0	3

Table 5.3 Comparison of the means and standard deviations of the estimated bacterial cell density x10^5 cfu for each of the bacterial species groups investigated identified by paper-point and curette sampling from periodontal and peri-implant sites.

Bacterial species or species groupings	Paper-point mean (SD)	Curette mean (SD)	Periodontal paper-point mean (SD)	Periodontal curette mean (SD)	Implant paper-point mean (SD)	Implant curette mean (SD)
Actinomyces spp.	1.09 (1.24)	1.63 (2.08)	1.12 (1.19)	1.40 (2.2)	1.06 (1.29)	1.78 (2.03)
Aggregatibacter actinomycetemcomitans	1.01 (1.26)	0.55 (0.60)	0.90 (1.18)	0.6 (0.62)	1.09 (1.32)	0.5 (0.59)
Campylobacter spp.	0.48 (0.48)	0.58 (0.82)	0.43 (0.41)	0.69 (0.97)	0.51 (0.53)	0.51 (0.71)
Capnocytophaga spp.	1.30 (1.15)	1.51 (2.14)	1.3 (0.94)	1.6 (1.8)	1.3 (1.29)	1.45 (2.38)
Eikenella corrodens	0.82 (1.12)	0.84 (1.07)	1.2 (1.54)	0.95 (1.13)	0.57 (0.64)	0.77 (1.04)
Fusobacterium spp.	1.31 (1.13)	1.40 (2.02)	1.11 (0.95)	1.39 (2.04)	1.44 (1.23)	1.41 (2.05)
Lactobacterial spp.	1.51 (2.45)	2.03 (2.83)	2.61 (3.5)	2.15 (2.54)	0.78 (0.87)	1.95 (3.07)
Leptotrichia spp.	0.34 (0.41)	0.44 (0.74)	0.3 (0.26)	0.64 (1.08)	0.39 (0.49)	0.30 (0.35)
Neisseria spp.	0.57 (0.79)	0.52 (0.74)	0.65 (0.91)	0.66 (0.92)	0.51 (0.71)	0.43 (0.6)
Parvimoas micra	1.58 (1.31)	1.29 (1.43)	1.33 (1.3)	1.67 (1.65)	1.74 (1.31)	1.104 (1.24)
Porphymonas gingivalis	0.28 (0.82)	0.26 (0.47)	0.24 (0.22)	0.32 (0.45)	0.31 (1.06)	0.21 (0.49)
Prevotella spp.	0.56 (0.67)	0.53 (0.93)	0.67 (0.76)	0.80 (1 .32)	0.48 (0.60)	0.35 (0.5)
Selenomonas spp.	0.44 (0.62)	0.76 (1.32)	0.75 (0.82)	0.75 (1.27)	0.24 (0.32)	0.77 (1.37)
Oral streptococci	4.00 (6.95)	4.99 (7.72)	7.26 (10.00)	7.96 (10.09)	1.81 (2.07)	3.01 (4.95)
Tanerella forsythia	0.66 (1.16)	0.60 (1.52)	0.63 (0.75)	1.04 (1.68)	0.68 (1.38)	0.30 (0.42)
Treponema spp.	0.29 (0.28)	0.39 (0.79)	0.27 (0.19)	0.64 (1.22)	0.3 (0.33)	0.22 (0.21)
Veillonella spp.	1.73 (2.77)	2.34 (3.11)	1.46 (1.42)	1.62 (1.62)	1.91 (3.4)	2.82 (3.75)
Staphylococcal spp.	1.67 (2.16)	3.08 (6.13)	1.86 (2.30)	2.08 (2.59)	1.54 (2.03)	3.74 (7.63)
Estimated bacterial cell density x10^5 cfu for all of species identified	19.64 (18.55)	23.73 (24.87)	24.1 (23.87)	26.94 (23.47)	16.68 (13.74)	21.58 (26.03)

Abbreviations: SD, standard deviation from the mean; cfu, colony forming units.

No marked differences were observed between the bacterial species detected from peri-implant sites or periodontal sites (Tables 5.2, 5.3 and 5.4). Paper-point sampling recovered the following species more frequently than curette sampling: *Campylobacter* spp., *E. corrodens*, *L. acidophilus*, *Prevotella* spp. and *T. forsythia* (Table 5.4 and Figure 5.1).

No significant differences were observed in the estimated bacterial cell densities between the paper-point and curette samples for each of the following species: *A. actinomycetemcomitans* (p-value 0.66); *P. gingivalis* (p-value 0.65); *T. forsythia* (p-value 0.81); *Treponema* spp. (p-value 0.34) and staphylococcal spp. (p-value 0.45), when the Wilcoxn Rank Sum test was applied.

The number of occasions each of the 18 species groupings were detected from peri-implant and/or periodontal sites by each sampling technique (either curette or paperpiont) was examined in order to further investigate if qualitative differences were present in the samples gained utilising each technique (Table 5.4). *Actinomyces* spp. were detected in each of the 16 peri-implant sites investigated when the data from both paper-point and curette sampling techniques were combined (Figures 5.2 and 5.3, Tables 5.4, 5.5 and 5.6). However, paper-point sampling alone was only effective in identifying 88% (14/16) of the sites which harboured *Actionomyces* spp., whilst curette sampling only recovered *Actionomyces* spp. from 75% (12/16) of the peri-implant sites examined.

A similar pattern emerged for periodontal sites (Table 5.5). When both paper-point and curette sampling techniques were employed, *Actionmyces* spp. were detected in 91.7% (22/24) of sites. However, when only the paper-point sampling data was examined, 71% (17/24) of periodontal sites were identified as harbouring *Actionomyces* spp. Furthermore, when the curette sampling data alone was examined only in 75% (18/24) of the periodontal sites examined were *Actionomyces* spp. identified.

Out of the 40 sites investigated, *Actinomyces* spp. were detected in 23 sites using both paper-point and curette sampling, in eight sites by paper-point sampling alone, in seven sites by curette sampling alone and in two sites, it was not detected by either technique (Figure 5.1; Table 5.4). Similar findings were observed when the peri-implant and periodontal data for the detection of *P. gingivalis* were examined. When both paper-point and curette sampling techniques were employed, *P. gingivalis* was detected at 100% (16/16) of the peri-implant sites (Table 5.6). Paper-point sampling alone was effective in identifying *P. gingivalis* at 82% (13/16) of the peri-implant sites, whilst curettes sampling was effective in identifying *P. gingivalis* at 75% (12/16) of the peri-implant sites. *P.*

gingivalis was detected at 100% (24/24) of the periodontal sites examined when both paper-point and curette sampling techniques were employed (Tables 5.5 and 5.6).

However, paper-point sampling alone detected *P. gingivalis* at only 83% (20/24) of the periodontal sites, whilst curettes sampling was effective in only identifying *P. gingivalis* at 79% (19/24) of the periodontal sites examined (Table 5.5). *Tanerella forsythia* was detected at 93% (15/16) of the peri-implant sites when both techniques were employed. Paper-point sampling was successful in identifying *T. forsythia* at 87.5% (14/16) of the peri-implant sites, whilst curette sampling was effective in identifying *T. forsythia* at only 68.8% (11/16) of the peri-implant sites (Table 5.6). Of the periodontal sites examined, when both paper-point and curette sampling techniques were employed *T. forsythia* was detected at 95.8% (23/24) of the periodontal sites. Paper-point sampling was successful in identifying *T. forsythia* at 83% (20/24) of periodontal sites, whilst curette sampling resulted in *T. forsythia* being detected at only 58% (14/24) of periodontal sites (Table 5.5).

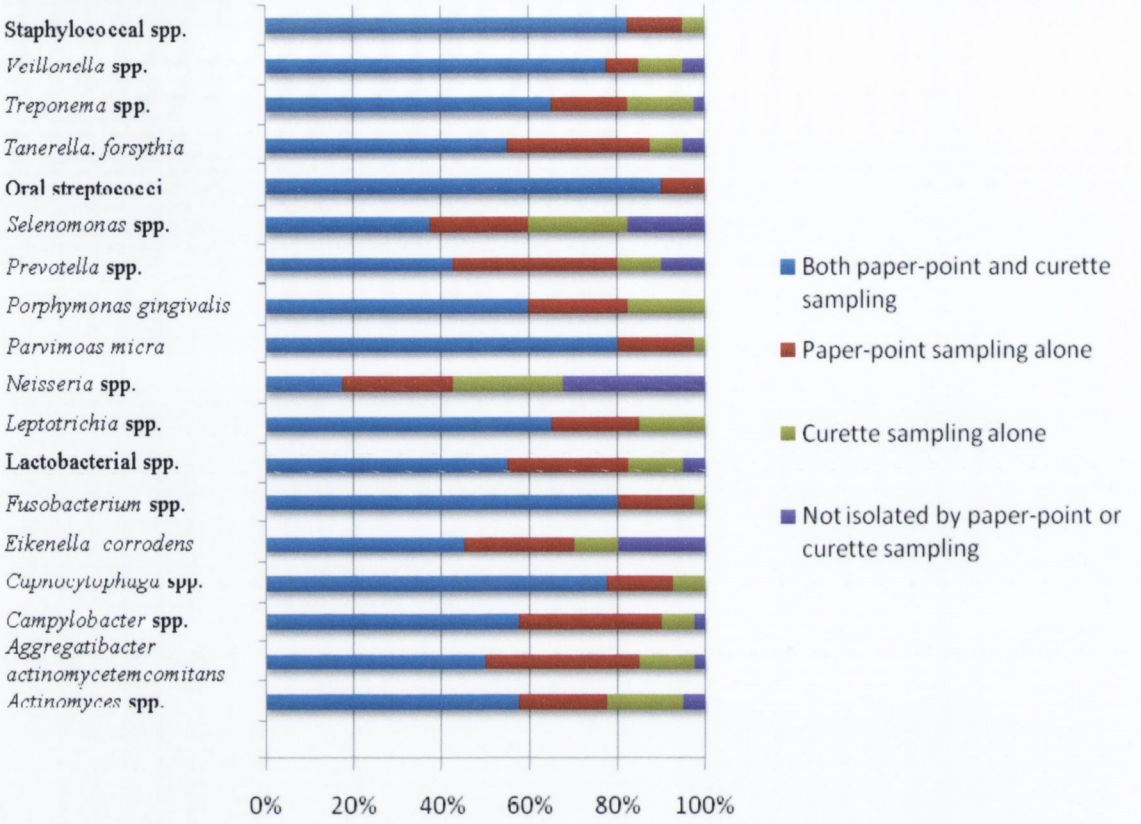

Figure 5.1. Comparison of the frequency individual bacterial species were detected by paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling for both periodontal and peri-implant sites (n=40). For example, at 57.5% (23/40) of sites both the paper-point sampling and curette sampling isolated *Actinomyces* spp.. For 20% (8/40) of sites the paper-point sampling isolated *Actinomyces* spp., whereas the curette sampling did not. However, in 17.5% (7/40) of sites the curette sampling isolated *Actinomyces* spp., whereas the paper-point sampling did not. In a further 5% (2/40) of sites *Actinomyces* spp. were not isolated from either the curette or the paper-point samples.

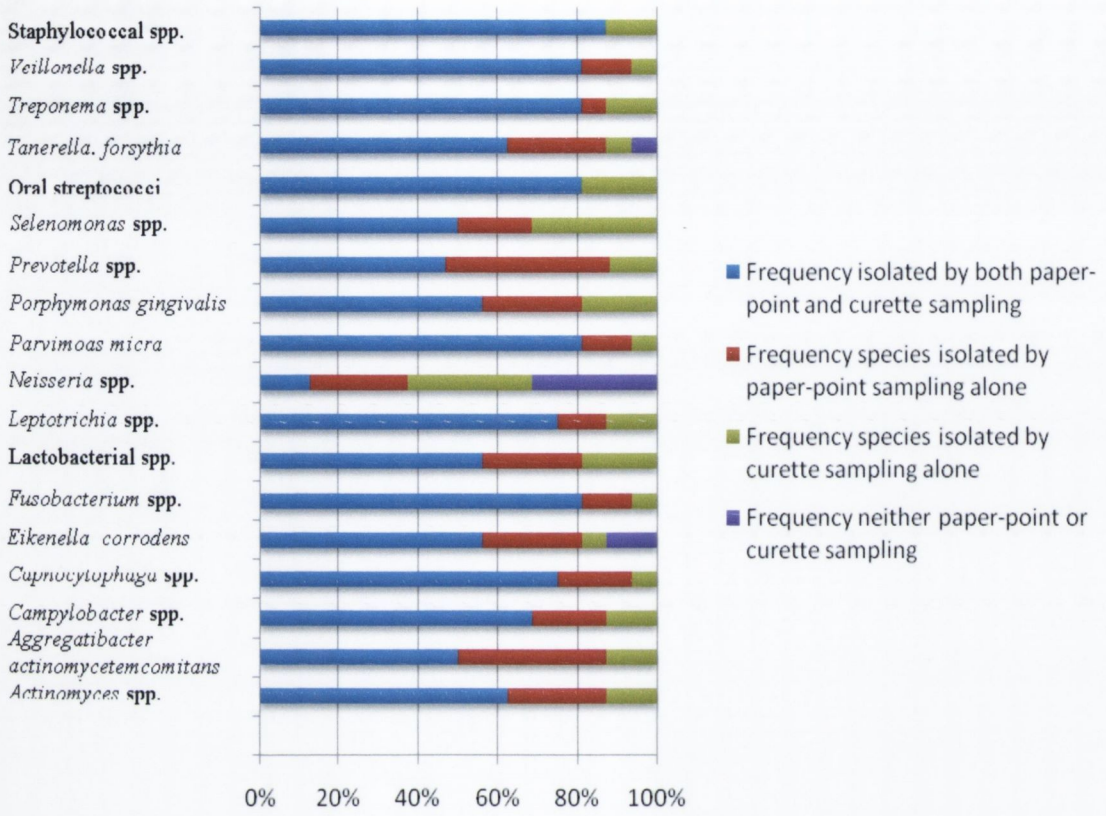

Figure 5.2 Comparison of the frequency individual species were detected from each of the peri-implant sites by either paper-point sampling alone, by curette sampling alone or by both of the paper-point and curette sampling techniques. For example, at 62.5% (10/16) of peri-implant sites both the paper-point sampling and curette sampling isolated *Actinomyces* spp. For 25% (4/16) of peri-implant sites the paper-point sampling isolated *Actinomyces* spp., whereas the curette sampling did not. However, in 12.5% (2/16) of peri-implant sites the curette sampling isolated *Actinomyces* spp., whereas the paper-point sampling did not. There were no peri-implant sites where either the paper-point or the curette sampling did not yield *Actinomyces* spp.

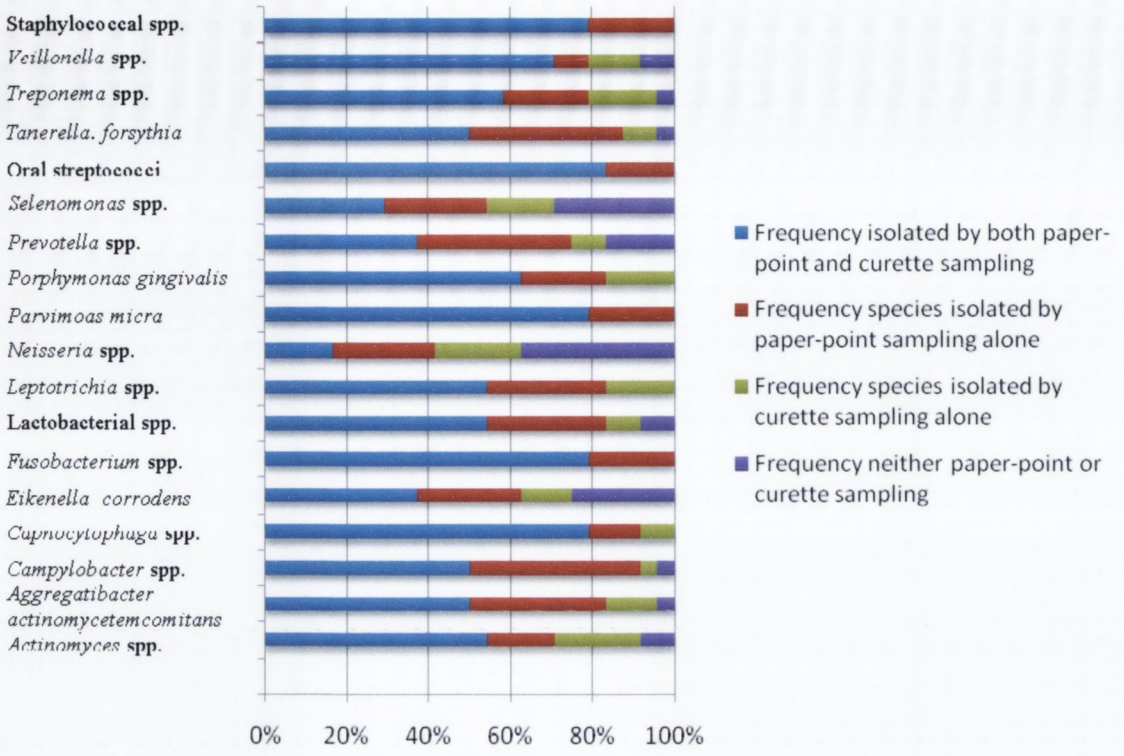

Figure 5.3. Comparison of the frequency individual species were detected from each of the periodontal sites by either paper-point sampling alone, by curette sampling alone or by both of the paper-point and curette sampling techniques. For example, at 50% of sites both the paper-point sampling and curette sampling isolated *Actinomyces* spp. For 15 % (4/26) of sites the paper-point sampling isolated *Actinomyces* spp., whereas the curette sampling did not. However, in 19.2% (5/26) of sites the curette sampling isolated *Actinomyces* spp., whereas the paper-point sampling did not. In a further 8% (2/26) of sites *Actinomyces* spp. were not isolated from either the curette or the paper-point samples.

Table 5.4 Comparison of the number of times paper-point and/or curette sampling detected each of the 18 bacterial species groupings investigated in either peri-implant or periodontal sites.

	No. of periodontal and peri-implant sites (n=40)			
	Both paper-point and curette sampling	**Paper-point sampling alone**	**Curette sampling alone**	**Not detected by paper-point or curette sampling**
Actinomyces spp.	23	8	7	2
Aggregatibacter actinomycetemcomitans	20	14	5	1
Campylobacter spp.	23	13	3	1
Capnocytophaga spp.	31	6	3	0
Eikenella corrodens	18	10	4	8
Fusobacterium spp.	32	7	1	0
Lactobacterial spp.	22	11	5	2
Leptotrichia spp.	26	8	6	0
Neisseria spp.	7	10	10	13
Parvimoas micra	32	7	1	0
Porphymonas gingivalis	24	9	7	0
Prevotella spp.	17	15	4	4
Selenomonas spp.	15	9	9	7
Oral streptococci	36	4	0	0
Tanerella forsythia	22	13	3	2
Treponema spp.	26	7	6	1
Veillonella spp.	31	3	4	2
Staphylococcal spp.	33	5	2	0

Table 5.5 Comparison of the frequency individual bacterial species were detected from each of the periodontal sites by either paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling.

	No. of periodontal sites (n=24)			
	Frequency detected by both paper-point and curette sampling	**Frequency species detected by paper-point sampling alone**	**Frequency species detected by curette sampling alone**	**Frequency neither paper-point or curette sampling detected**
Actinomyces spp.	13	4	5	2
Aggregatibacter actinomycetemcomitans	12	8	3	1
Campylobacter spp.	12	10	1	1
Capnocytophaga spp.	19	3	2	0
Eikenella corrodens	9	6	3	6
Fusobacterium spp.	19	5	0	0
Lactobacterial spp.	13	7	2	2
Leptotrichia spp.	13	7	4	0
Neisseria spp.	4	6	5	9
Parvimoas micra	19	5	0	0
Porphymonas gingivalis	15	5	4	0
Prevotella spp.	9	9	2	4
Selenomonas spp.	7	6	4	7
Oral streptococci	20	4	0	0
Tanerella forsythia	12	9	2	1
Treponema spp.	14	5	4	1
Veillonella spp.	17	2	3	2
Staphylococcal spp.	19	5	0	0

Table 5.6 Comparison of the frequency individual bacterial species were detected from each of the peri-implant sites by either paper-point sampling alone, by curette sampling alone or by both paper-point and curette sampling.

	No. of peri-implant sites (n=16)			
	Frequency detected by both paper-point and curette sampling	**Frequency species detected by paper-point sampling alone**	**Frequency species detected by curette sampling alone**	**Frequency neither paper-point or curette sampling detected**
Actinomyces spp.	10	4	2	0
Aggregatibacter actinomycetemcomitans	8	6	2	0
Campylobacter spp.	11	3	2	0
Capnocytophaga spp.	12	3	1	0
Eikenella corrodens	9	4	1	2
Fusobacterium spp.	13	2	1	0
Lactobacterial spp.	9	4	3	0
Leptotrichia spp.	12	2	2	0
Neisseria spp.	2	4	5	5
Parvimoas micra	13	2	1	0
Porphymonas gingivalis	9	4	3	0
Prevotella spp.	8	7	2	0
Selenomonas spp.	8	3	5	0
Oral streptococci	13	0	3	0
Tanerella forsythia	10	4	1	1
Treponema spp.	13	1	2	0
Veillonella spp.	13	2	1	0
Staphylococcal spp.	14	0	2	0

5.5 Discussion

The aim of this part of the present study was to semi-quantitatively compare the microbial species detected at periodontal and peri-implant sites using paper-point and curette sub-gingival sampling techniques. To the authors' knowledge, only one other study to date has compared the efficacy of paper-point and curette sampling techniques in isolating microbial species from both periodontal and peri-implant sites (Gerber *et al.,* 2005). The Gerber *et al.* (2005) study differed from the present investigation as they employed paper strips to take GCF samples from the periodontal or peri-implant sites. In the present investigation, paper-points (size 35) were employed to gain the GCF sample. Clinically, paper-points are firm and narrow and can be placed into the periodontal or peri-implant pocket and may access samples from deeper in the pocket than paper strips which are wider and less firm. Furthermore, in the Gerber *et al.* (2005) study, only sites with shallow probing depths were investigated, whereas in the present investigation sites with deeper probing depths were included.

In the present study, when viewing the number of bacterial species detected from individual sites when paper-point and curette sampling techniques were employed, only 15% (6/40) of individual sites detected the same number of species using paper-points and curettes (Tables 5.1 and 5.2, Figure 5.1). Only in 12.5% of sites did both paper-point and curette sampling result in the same species being detected, clearly indicating a disparity between sampling techniques, as different techniques consistently detected a different number of species to each other.

Paper-point sampling was observed to yield a greater number of species than curette sampling in 55% (22/40) of the sites. Paper-point sampling appeared to be more efficient in isolating *Campylobacter* spp., *E. corrodens*, *L.acidophilus*, *Prevotella* spp. and *T. forsythia* spp. than curette sampling. However, in 30% (12/40) of sites investigated curette sampling detected a greater number of species than paper-point sampling (Tables 5.1 and 5.2).

In the present investigation, neither paper-point nor curette sampling was observed to detect greater estimated bacterial cell densities. This is in contrast to the findings of Keil & Lang (1992) and Jervoe-Storm *et al.* (2007) who observed that curette sampling detected a greater number of species than paper-point sampling, and to those of Renvert *et al.* (1992) who observed paper-point sampling to result in higher numbers of cfus being detected than curette sampling. Furthermore, neither paper-point nor curette sampling were observed to be effective in yielding samples which are 100%

representative of the microbial profile of the peri-implant or periodontal sites examined. For example, *P. gingivalis* was identified as being present in 100% of the peri-implant and periodontal sites examined. However, paper-point sampling failed to isolate *P. gingivalis* from 18% (3/16) and 17% (4/24) of the peri-implant and periodontal sites respectively, and curette sampling failed to isolate *P. gingivalis* from 25% (4/16) and 21% (5/24) of the periodontal and peri-implant sites, respectively (Table 5.4). Therefore, for 18% of the peri-implant sites, a false negative result for the presence of *P. gingivalis* was obtained from paper-point samples, whilst for curette samples, a false negative result was observed for the presence of *P. gingivalis* for 25% of the peri-implant sites. A similar disparity between the data from paper-point and curette sampling techniques emerges when the data for the prevalence of *P. gingivalis* from the periodontal sites were examined. Furthermore, this lack of agreement between the data from paper-point and curette samples from individual sites can also be seen for many other species including *Actionomyces* spp. and *T. forsythia*. Therefore, markedly differing results may be obtained for the prevalence of microbial species detected from subgingival or submucosal sites depending on the individual sampling technique employed. The differing findings noted between curette and paper-point sampling techniques from individual sites in the present study are in agreement with the findings of Keil & Lang (1991), Renvert *et al.* (1992) and Gerber *et al.* (2005) who similarly noted a lack of agreement between curette and paper-point techniques.

The sensitivity of either paper-point or curette sampling alone in isolating the 29 microbial species investigated in the present study was limited. When paper-point and curette samples from individual sites were combined, a greater number of microbial species were identified as being present in the ecological niche sampled, for both the periodontal or peri-implant sites. Therefore, in microbial investigations of periodontal and peri-implant treatments, the sensitivity of the microbial data may not be optimal if either paper-point sampling alone or curette sampling alone are employed, as false negative results are to be anticipated. The sensitivity of the microbial data from either curette or paper-point sampling techniques of an individual site appears likely to be inferior to the data gained if both sampling techniques are employed. However, to the authors' knowledge, there are to date no clinical investigations of periodontal and peri-implant treatment which has employed both paper-point and curette sampling techniques.

5.6 Conclusions

In conclusion, marked qualitative and quantitative differences were noted between the microbial species yielded using either paper-point or curette sampling techniques. The observed differences in the microflora detected utilising paper-point and curette sampling techniques highlighted the limitations of each techniques in fully representing the sub-mucosal/ sub-gingival ecological niche from which sampling is undertaken. Paper-point sampling appeared to be somewhat superior to curette sampling in isolating the key periodontopathogens. However, curette sampling frequently yielded a greater number of species, including the key periodontopathogens, and also often yielded greater amounts of total DNA than paper-point sampling. The present study highlights the need for both paper-point and curette sampling to be undertaken, and the samples combined, in order that the microbial samples taken more fully represent the microflora of the site sampled, thereby facilitating the provision of more accurate microbial sampling.

Chapter 6

A semi-quantitative comparison of the microbiota around natural teeth and oral implants in patients with implants for five or more years determined by checkerboard analysis

6.1 Introduction

The microflora of peri-implant and periodontal sites share many common microbial species (Pontotiero *et al.*, 1994; Mombelli *et al.*, 1995). However, the newly exposed peri-implant site is a novel niche with perhaps uniquely different ecological characteristics than the periodontal site (Furst *et al.*, 2007). Therefore, differences may exist in how microorganisms colonise natural tooth surfaces and the associated subgingival environment, compared to the colonisation of titanium implants and their associated submucosal environment. Some studies reported differences between the peri-implant and periodontal microflora (Botero *et al.*, 2005; Furst *et al.*, 2007). However, a similar microflora has been identified in the periodontal and peri-implant sites of individual patients, therefore, periodontal and peri-implant infections are thought to be intimately related (Quirynen & Listgarten, 1990; Mombelli *et al.*, 1995; Mengel *et al.*, 1996; Leonhardt *et al.*, 2003; Botero *et al.*, 2005; Furst *et al.*, 2007). Furst *et al.* (2007) observed changes in the periodontal microflora of periodontal sites adjacent to newly placed dental implants when paper point samples, taken before and after implant placement, underwent checkerboard DNA-DNA hybridisation analysis. At the time of implant placement surgery, 26.2% of periodontal sites (of teeth adjacent to the newly placed dental implants), were positive for *S. aureus,* whereas 12 weeks following placement surgery, 39.1% of periodontal sites were positive for *S. aureus* and up to 15% of the 17 implant sites investigated in the 12 patients, were positive for *S. aureus* (Furst *et al.*, 2007).

An overview of the bacterial species identified and techniques employed in observational studies of the peri-implant and periodontal microflora are presented in Tables 6.1 and 6.2. A cause and effect relationship between dental plaque and peri-implant inflammation was first demonstrated in a proof of principle study by Pontoriero *et al.* (1994). This study follows the "experimental gingivitis" study of Loe *et al.* (1965), and has been termed the "experimental peri-implant mucositis" study. Following abstinence from oral home care methods (tooth brushing, use of interproximal cleaning aids and mouthwashes) and professional preventative measures (root debridement, scaling and polishing), inflammation was noted in the previously healthy peri-implant tissues and peri-implant mucositis diagnosed. When professional and home oral care measures were re-commenced, the inflammation of the peri-implant tissues resolved and peri-implant health was restored, clearly demonstrating the aetiological role of plaque deposits in the development of peri-implant mucositis. Furthermore, a higher microbial density has been

observed at peri-implant sites demonstrating peri-implant infections, compared to healthy implants in the same individual (Mombelli & Lang, 1992) and in individuals who only had implants with peri-implant health (Hultin *et al.*, 2002). Increased proportions of Gram-negative anaerobic and facultative anaerobic species have been isolated from implants demonstrating clinical signs of peri-implantitis compared to healthy implants (Rams *et al.*, 1983; Rams *et al.*, 1984; Sanz *et al.*, 1991; Leonhardt *et al.*, 1999; Mombelli *et al.*, 2002; Botero *et al.*, 2005), including *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* (Hultin *et al.*, 2000*b*; Luterbacher *et al.*, 2000; Hultin *et al.*, 2002; Mengel & Flores-de-Jacoby, 2005).

However, more recently, another study which employed checkerboard analysis, reported no qualitative or quantitative differences between the microflora of clinically healthy implants and those demonstrating signs of peri-implant infection (Renvert *et al.*, 2007).

Edentulous patients have been observed to harbour less *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* than partially dentate patients when paper-point samples were used to absorb saliva from the edentulous oral mucosa and the periodontal pockets of natural teeth (Divides *et al.*, 2006). It has been observed that the removal of all teeth prior to the commencement of implant treatment can result in *A. actinomycetemcomitans* no longer being isolated from the oral flora (Danser *et al.*, 1995). However, in the peri-implant microflora of 37 patients who had had their teeth extracted because of extensive periodontal disease and had implants placed for five or more years, a re-emergence of the traditional periodontopathogens including *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *A. actinomycetemcomitans* was observed, when peri-implant paper point samples were examined utilising culture techniques (Leonhardt *et al.*, 1999). *Aggregatibacter actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* have been more frequently detected in patients with a history of chronic periodontitis or aggressive periodontitis than in patients with no history of periodontal disease (Mengel & Flores-de-Jacoby, 2005). Furthermore, staphylococcal spp. and *Candida* spp. have been identified by culture techniques in samples from the peri-implant pockets of patients displaying signs of peri-implant infections (Leonhardt *et al.*, 1999).

It has been observed in many studies that microbial periodontal samples, taken using either paper-point or curette sampling techniques, may differ from each other depending on the sampling method employed (Baker *et al.*, 1991; Keil & Lang, 1991; Renvert *et al.*, 1992; Socransky, 1998; Gerber *et al.*, 2005). Controversy remains as to

Table 6.1 Microbial sampling technique and analysis methods employed for microbiological observational studies, comparing periodontal and peri-implant microflora.

		Method(s) of analysis			
Study	**Sampling method**	**Anaerobic culture**	**Checkerboard DNA-DNA hybridisation**	**Real-Time PCR**	**Dark-field microscopy**
Mombelli & Lang, 1992	Curette	+			
Hultin *et al.*, 2001	Paper-point		+		
Mombelli *et al.*, 1995	Paper-point	+			
Botero *et al.*, 2005	Paper-point	+			
Leonhardt *et al.*, 1999	Paper-point	+			
Renvert *et al.*, 2007	Paper-point		+		
Rutar *et al.*, 2007	Paper-point	+			
Van Winkelhoff & Wolf, 2000	Paper-point	+			
Quirynen *et al.*, 1996	Paper-point				+
Quirynen & Listgarten, 1990	Paper-point				+
Lee *et al.*, 1999	Paper-point		+		
Van Winkelhoff *et al.*, 2000	Paper-point				
Beikler *et al.*, 2004	Curette			+	
Cugini *et al.*, 2000	Curette		+		
Furst *et al.*, 2006	Paper-point		+		

Table 6.2 Microbial species identified in microbiological observational studies, comparing periodontal and peri-implant microflora.

Species	Peri-implant +	Periodontal +
A. naeslundii	Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007	Beikler *et al.*, 2004; Furst *et al.*, 2006; Rutar *et al.*, 2007
A.israelii	Furst *et al.*, 2006; Renvert *et al.*, 2007; Maximo *et al.*, 2009	Furst *et al.*, 2006
A. viscosus	Furst *et al.*, 2006; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Rutar *et al.*, 2007; Furst *et al.*, 2006
A. odontolyticus	Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Cugini *et al.*, 2000; Furst *et al.*, 2006; Rutar *et al.*, 2007
A. gerencseriae	Furst *et al.*, 2006; Maximo *et al.*, 2009;	Furst *et al.*, 2006
A. actinomycetemcomitans	Leonhardt *et al.*, 1999; Winkelhoff & Wolf., 2000; Hultin *et al.*, 2002; Botero *et al.*, 2005; Maximo *et al.*, 2009; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Rutar *et al.*, 2007; Furst *et al.*, 2006	Van Winkelhoff *et al.*, 2000; Beikler *et al.*, 2004; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007; Rutar *et al.*, 2007
C. gracilis	Lee *et al.*, 1999; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007; Maximo *et al.*, 2009;	Lee *et al.*, 1999; Cugini *et al.*, 2000; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007
C. rectus	Lee *et al.*, 1999; Hultin *et al.*, 2002; Botero *et al.*, 2005; Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Lee *et al.*, 1999; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007;
C. gingivalis	Furst *et al.*, 2006; Rutar *et al.*, 2007; Maximo *et al.*, 2009;	Cugini *et al.*, 2000; Furst *et al.*, 2006; Rutar *et al.*, 2007;
C. ochracea	Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009;	Cugini *et al.*, 2000; Furst *et al.*, 2006; Rutar *et al.*, 2007
E. corrodens	Hultin *et al.*, 2002; Botero *et al.*, 2005; Maximo *et al.*, 2009; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Furst *et al.*, 2006	Cugini *et al.*, 2000; Beikler *et al.*, 2004; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007
Eubacterium spp.	Botero *et al.*, 2005; Furst *et al.*, 2006; Renvert *et al.*, 2007; Maximo *et al.*, 2009	Cugini *et al.*, 2000; Furst *et al.*, 2006; Botero *et al.*, 2005
F. nucleatum spp. *nucleatum*	Lee *et al.*, 1999; Van Winkelhoff *et al.*, 2000; Hultin *et al.*, 2002; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007; Renvert *et al.*, 2007; Maximo *et al.*, 2009	Lee *et al.*, 1999; Van Winkelhoff *et al.*, 2000; Beikler *et al.*, 2004; Cugini *et al.*, 2000; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007
F. periodonticum	Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Cugini *et al.*, 2000; Furst *et al.*, 2006; Rutar *et al.*, 2007
L. acidophilus	Furst *et al.*, 2006; Renvert *et al.*, 2007	Furst *et al.*, 2006
L. buccalis	Furst *et al.*, 2006	Furst *et al.*, 2006
N. mucosa	Furst *et al.*, 2006; Maximo *et al.*, 2009	Furst *et al.*, 2006

Continued overleaf

Table 6.2 continued.

Species	Peri-implant +	Periodontal +
P. micra	Lee *et al.*, 1999; Van Winkelhoff *et al.*, 2000; Hultin *et al.*, 2002; Furst *et al.*, 2006; Maximo *et al.*, 2009	Lee *et al.*, 1999; Van Winkelhoff *et al.*, 2000; Cugini *et al.*, 2000; Furst *et al.*, 2006
P. gingivalis	Leonhardt *et al.*, 1999; Lee *et al.*,1999; Van Winkelhoff *et al.*, 2000; Hultin *et al.*, 2002; Botero *et al.*, 2005; Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Lee *et al.*, 1999; Van Winkelhoff *et al.*, 2000; Cugini *et al.*, 2000; Beikler *et al.*, 2004; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007
P. intermedia	Lee *et al.*, 1999; Winkelhoff *et al.*, 2000; Hultin *et al.*, 2002; Botero *et al.*, 2005; Van Furst *et al.*, 2006 Renvert *et al.*, 2007; Rutar *et al.*, 2007, Maximo *et al.*, 2009	Lee *et al.*, 1999 ;Van Winkelhoff *et al.*, 2000; Beikler *et al.*, 2004; Beikler *et al.*, 2004; Botero *et al.*, 2005; Furst *et al.*, 2006; Rutar *et al.*, 2007
P. nigrescens	Lee *et al.*, 1999; Hultin *et al.*, 2002; Botero *et al.*, 2005; Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Lee *et al.*,1999; Cugini *et al.*, 2000; Beikler *et al.*, 2004; Botero *et al.*,2005; Furst *et al.*, 2006; Rutar *et al.*,2007
P. melaninogenica	Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Furst *et al.*, 2006; Rutar *et al.*, 2007.
S. noxia	Lee *et al.*,1999; Hultin *et al.*, 2002; Furst *et al*, 2006; Rutar *et al.*, 2007; Maximo *et al.*, 2009.	Lee *et al.*,1999; Furst *et al*, 2006; Rutar *et al.*, 2007.
S. gordonii	Lee *et al.*,1999; Furst *et al.*, 2006; Maximo *et al.*, 2009	Lee *et al.*, 1999; Cugini *et al.*, 2000; Furst *et al.*, 2006
S. intermedia	Lee *et al.*, 1999; Hultin *et al.*, 2002; Furst *et al.*, 2006; Renvert *et al.*, 2007; Maximo *et al.*, 2009	Lee *et al.*, 1999; Cugini *et al.*, 2000; Furst *et al.*, 2006
S. oralis	Lee *et al.*, 1999; Furst *et al.*, 2006; Maximo *et al.*, 2009	Lee *et al.*, 1999; Cugini *et al.*, 2000; Furst *et al.*, 2006
S. mitis	Maximo *et al.*, 2009; Lee *et al.*, 1999; Furst *et al.*, 2006	Lee *et al.*, 1999; Cugini *et al.*, 2000; Furst *et al.*, 2006
S. aureus	Leonhardt *et al.*, 1999; Furst *et al.*, 2006	Furst *et al.*, 2006
T. forsythia	Leonhardt *et al.*, 1999; Lee *et al.*, 1999; Van Winkelhoff *et al.*, 2000; Hultin *et al.*, 2002; Furst *et al.*, 2006; Renvert *et al.*, 2007; Rutar *et al.*, 2007; Maximo *et al.*, 2009	Lee *et al.*, 1999; Cugini *et al.*, 2000; Van Winkelhoff *et al.*, 2000; Beikler *et al.*, 2004; Furst *et al.*, 2006; Rutar *et al.*, 2007;
T. denticola	Hultin *et al.*, 2002; Furst *et al.*, 2006; Maximo *et al.*, 2009	Cugini *et al.*, 2000; Beikler *et al.*, 2004; Furst *et al.*, 2006
T. socransky	Furst *et al.*, 2006; Maximo *et al.*, 2009; Renvert *et al.*, 2007	Furst *et al.*, 2006
V. parvula	Lee *et al.*, 1999; Furst *et al.*, 2006; Rutar *et al.*, 2007	Lee *et al.*, 1999; Beikler *et al.*, 2004; Furst *et al.*, 2006; Rutar *et al.*, 2007
Candida spp.	Leonhardt *et al.*, 1999	

which sampling technique yields the microbial sample that best represents the composition of the subgingival microflora of the site sampled. For example, Keil & Lang (1991) observed that curette sampling results in a higher numbers of colony forming units retrieved compared to paper-point sampling, whereas Renvert *et al.* (1992) noted, in an interventional study, that paper point sampling yielded significantly greater numbers of spirochete species than curette sampling, prior to treatment. Furthermore, other investigators have observed no significant differences in the species isolated when curette or paper-point sampling techniques were employed (Jervoe-Storm *et al.*, 2007). Therefore, a combination of sampling techniques has been recommended in order to yield samples which more fully represent the microflora of the site sampled (Gerber *et al.*, 2005). To date, no published study has compared the peri-implant and periodontal microflora utilising both paper point and curette sampling techniques. The extent of similarities and differences in peri-implant and periodontal microflora in health and disease remains incompletely understood.

The aims of this part of the present study were to compare qualitatively and semi-quantitatively, the composition of the subgingival microbial flora of periodontal and peri-implant sites in a cohort of patients, with implants in place for five years or longer, under the following headings:

a) Periodontal and peri-implant sites.
b) Peri-implant sites with a diagnosis of health, peri-implant mucositis and peri-implantitis.
c) Periodontal and peri-implant sites with probing depths of >5 mm and bleeding on probing compared to samples gained from those sites that do not meet these criteria.
d) Smokers and non-smokers.
e) Patients with a demonstrated susceptibility to periodontal disease and those with no demonstrated susceptibility to periodontal disease.

6.2 Materials and Methods

6.2.1 Patients

Patients attending the Dublin Dental University Hospital, and having one or more dental implants placed five years previously, were screened between October 2007 and September 2009. This study had the approval of the Ethics Committee of the Faculty Research Ethics Group-Faculty of Health Sciences, Trinity College Dublin (Chapter 2, Section 2.1.1 and Appendix 1). Those patients who met the inclusion criteria were asked to participate in the present investigation. Patient inclusion and exclusion criteria are described below.

6.2.1.1 Patient inclusion criteria

- Eighteen or more years of age.
- Dental implant placed five or more years previously
- Presence of one or more implant restorations with a positive diagnosis of peri-implant mucositis or peri-implantitis
- Patients signed the informed consent form approved by the Ethics Committee of the Faculty Research Ethics Group-Faculty of Health Sciences, Trinity College Dublin (see Chapter 2, section 2.1.1).

6.2.1.2 Patient exclusion criteria

A full medical history was taken and any patients reporting the following conditions were excluded from the study:

- Prophylactic medication requirement
- Pregnant or lactating women
- Patients having taken antibiotics, antifungal or steroid medication within two months prior to participation in the study
- Individuals under 18 years of age
- Adults with learning disabilities
- Adults who have a terminal illness

6.2.2 Dental and medical history and an oral examination

At the baseline consultation, a detailed dental and medical history and an oral examination was undertaken as described in Chapter 2, Section 2.1.4. This included a

detailed periodontal examination involving full mouth probing pocket depths measurement and measurement of recession and bleeding on probing.

6.2.3 Radiographs

Peri-apical radiographs were undertaken using a Rinn holder as described in Chapter 2, Section 2.1.5 and employing the long cone paralleling technique, i.e. the peri-apical radiograph film, the implant and the collimator face were aligned as parallel as possible and the radiograph exposed for the recommended time interval, dependant on the implant's anatomical location. Radiographs included both conventional and digital radiographs. Conventional radiographs were viewed on a light box in a darkened room and a photograph was taken at a shutter speed of 0.32 s on a Cannon 30 D camera with a Macro 250 mm lens (Canon UK Ltd., UK). Similarly, where phosphor plates (Sidexis Dental Systems, USA) were employed to capture the digital image of the radiograph, images were viewed in a darkened room and the settings adjusted to optimally view the peri-implant bone levels. Images were imported to Windows XP Paint Shop (Microsoft Corporation, USA) cropped and pasted to Microsoft Office PowerPoint 2007 (Microsoft Corporation, USA) and coded according to the patient's investigation number and the implant's anatomical location. All radiographs were examined independently by two experienced periodontist examiners with regard to the diagnosis of peri-implant bone loss of $\geq$2.5 mm. Kappa analysis revealed good agreement between examiners ($p<0.001$). Where discrepancies were present between examiners, a third experienced periodontist examiner was employed. All examiners were blinded with regard to the identity of patients and the clinical parameters.

6.2.4 Peri-implantitis patients, peri-implant mucositis patients and peri-implant healthy patients

Implants demonstrating each of the following criteria: bleeding or suppuration on probing; probing pocket depth of $\geq$ 6 mm and radiographic bone loss $\geq$ 2.5 mm, were classified as being affected by peri-implantitis (Berglundh *et al.*, 2002). Implants with bleeding or suppuration on probing but not meeting the criteria of peri-implant bone loss $\geq$ 2.5 mm and at least one site with probing pocket depth of $\geq$ 6 mm, were diagnosed as peri-implant mucositis positive. Patients with implants not demonstrating bleeding or suppurating on probing were diagnosed as having healthy implants.

6.2.5 Microbial sampling

Both curette and paper-point sampling techniques were employed following careful tooth isolation. Paper-point sampling was undertaken first and curette sampling subsequently as described in Chapter 2, Section 2.2.5. For each of 41 patients meeting the inclusion criteria, paper-point and curette samples were taken from the deepest site present of a) each implant present b) one periodontally diseased tooth (where present) and c) one periodontally healthy tooth (where present).

6.2.6 Semi-quantitative determination of microbial species by checkerboard DNA-DNA hybridisation

Following preparation of the samples (as outlined in Chapter 2, Section 2.2.6.1), a semi-quantitative determination of the density of 29 individual oral bacterial species present in each sample, was undertaken using the checkerboard DNA-DNA hybridisation technique (Socransky *et al.*, 1994; Haffajee *et al.*, 1997*a*; Persson *et al.*, 2007) as described in Chapter 2, Section 2.2.6.3. The probes and their source strains were compared as described by Ximenez-Fyvie *et al.* (2000). For all samples, checkerboard DNA-DNA hybridisation investigation was undertaken for the species shown in Chapter 2, Table 2.1. The bacterial species belonging to specific groupings were grouped together for data analysis (Chapter 2, Table 2.1).

As the checkerboard DNA-DNA hybridisation method employs whole genomic probes, cross reactivity from closely related species was possible, leading to concerns with this analysis approach. Therefore, real time PCR was undertaken for selected species. The selected species were *A. actinomycetemcomitans, T. forsythia*, *P. gingivalis,* and *T. denticola,* as these were four of the species most commonly associated with periodontal and peri-implant infections. In the analysis of the checkerboard data, only where the real-time PCR had confirmed the presence of each of these species, was the result of the checkerboard DNA-DNA hybridisation considered valid and included in the analysis. Furthermore, in order to mitigate any possible decreased specificity of the checkerboard DNA-DNA hybridisation technique, compared to culture or other molecular techniques, closely related species taxa were combined and used in data analysis to give a result for an individual species or a group of species, as outlined under the Species Groupings heading in Chapter 2, Table 2.1.

6.2.7 Real-Time PCR analysis

In addition to the checkerboard technique, real-time PCRs were undertaken for four bacterial species including *A. actinomycetemcomitans, T. forsythia*, *P. gingivalis,* and *T. denticola* (Chapter 2, Tables 2.2 and 2.3), as described by Eick *et al.,* (2011), and modified by using GoTaq qPCR Master mix (Promega Corporation, USA). In the final analysis, only results confirmed by Real-Time PCR for these species, were considered valid and included in the analysis.

DNA was extracted from 100 µl of each sample using the Chelex method (Yang *et al.*, 2008). Real-time PCR was carried out using a real-time analyser (7500 Real Time PCR System, Life Technologies, California, USA). Oligonucleotides were synthesised by Microsynth (Balgach, Switzerland) (Chapter 2, Table 2.3). The primers targeting 16S rDNA regions of *P. gingivalis*, *T. forsythia* and *T. denticola* were designed, as described by Ashimoto *et al.*, (1996) and those for *A. actinomycetemcomitans* were designed as described by Tran & Rudney (1999) (see Chapter 2, Table 2.3).

PCR amplifications were carried out in Real-Time PCR analyzer (7500 Real-Time PCR System, USA) in a reaction volume of 25 µl, consisting of: 2.5 µl template DNA and 22.5 µl of reaction mixture (containing 12.5 µl GoTaq PCR Master Mix (Promega), 0.5 µmol/L (1.25 µl) of each primer and 7.5 µl dH_2O). Negative and positive controls PCRs were included in each experiment. The positive controls consisted of 2.5 µl genomic DNA in concentrations equivalent to 10^2 to 10^7 cfu of the reference strain.

6.2.8 Comparison of periodontal and peri-implant microflora

The relative amounts of DNA of individual species that were identified from samples from individual sites utilising paper-point or curette sampling techniques, were referred to as the percentage relative abundance (% relative abundance). The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species/groupings as a proportion of all of the species detected at an individual site. Comparisons were made between:

a) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species/groupings investigated by the checkerboard DNA-DNA hybridisation technique for periodontal and peri-implant sites. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

b) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for peri-implant mucositis and peri-implantitis sites. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

c) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for periodontal healthy sites and periodontitis sites. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

d) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for patients who have a history of severe periodontal disease and those without a history of severe periodontal disease. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

e) The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species/groupings investigated by the checkerboard DNA-DNA hybridisation technique for patients who are current smokers and non-smokers. The Wilcoxon test was undertaken to compare values of the percentage relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

Descriptive statistics were employed to compare the values of the estimated bacterial cell density per individual species $x10^5$ (based on DNA-DNA hybridisation), detected from all of the samples gained using both paper-point and curette sampling techniques, from peri-implantitis sites compared to the peri-implant mucositis sites.

6.3 Results

6.3.1 Patients and samples

Forty-one patients, eight male and 33 female, contributed samples for this study. Eight patients were smokers (defined as patient who were currently smoking, or had stopped smoking in the previous year). Two patients stopped smoking following the baseline examination and one patient had stopped smoking in the year prior to baseline examination. The mean age was 44.85 years (range 28 to 62 years). Of the 41 patients included in the present investigation, 35 were affected by peri-implant mucositis and 12 by peri-implantitis. Six patients had both implants affected by peri-implant mucositis and implants affected by peri-implantitis present. Ninty-seven peri-implant sites were sampled, 71 of which were affected by peri-implant mucositis, 18 were affected by peri-implantitis and eight were healthy. In addition, 49 periodontal sites were sampled, 22 of which were from sites affected by periodontitis in 17 patients and 27 from healthy periodontal sites in 22 patients.

6.3.2 Semi-quantitative detection of microbial species

Of the 18 species/groupings examined, only the percentage relative abundance of *Treponema* spp. were noted to be significantly elevated in peri-implant sites compared to the periodontal microflora ($p<0.05$) (Table 6.3). No significant differences were noted in the microflora of peri-implant mucositis and peri-implantitis sites in relation to the percentage relative abundance of each of the 18 microbial species/groupings investigated (Table 6.4). The total of the mean values of the estimated bacterial cell density per individual species $x10^5$ (based on DNA-DNA hybridisation) detected from all of the samples investigated from peri-implantitis sites, was observed to be 33% greater than that of peri-implant mucositis sites.

On comparing the microflora of periodontal and peri-implant sites, with probing pocket depths of >5 mm and demonstrating bleeding on probing, to sites not meeting these criteria, a significantly greater percentage relative abundance of *T. forsythia* ($p= 0.0005$), *P. gingivalis* ($p<0.0001$) and *N. mucosa* ($p<0.05$) were noted when the Wilcoxon test was applied (Table 6.5).

Furthermore, *Treponema* spp. were observed to have a 60% higher percentage relative abundance within the peri-implant and periodontal microflora of patients with a history of severe periodontal disease, compared to patients who did not have a history of

severe periodontal disease ($p<0.05$). Also, the percentage relative abundance of *T. forsythia, P. gingivalis* and *A. actinomycetemcomitans* were elevated by 48.6%, 41.5% and 31.9% respectively, in the periodontal and peri-implant microflora of patients with a history of severe periodontal disease compared to those without a history of severe periodontal disease, but differences were not significant ($p>0.05$). Staphylococcal spp. (i.e. *S. aureus* and *S. epidermidis*) percentage relative abundance were elevated by 19.4% within the periodontal and peri-implant microflora of patients with a history of severe periodontal disease compared to those without, however, differences were not significant ($p>0.05$) (Table 6.6).

Streptococcal spp. were observed to have a 35.8% higher median percentage relative abundance within the periodontally healthy biofilm, compared to the percentage relative abundance of oral streptococci in the microflora of the periodontitis sites examined (Table 6.7). *Tanerella forsythia*, *Campylobacter* spp. and *E. corrodens* were observed to be elevated by 82%, 32.9% and 136.2% at periodontitis sites, compared to the periodontally healthy sites (Table 6.7). The median percentage relative abundance value of *A. actinomycetemcomitans* was 0% (with an interquartile range of 0-2), for the periodontal health sites, and 0% (with an interquartile range of 0-3), for the periodontitis sites. The median percentage relative abundance value of *P. gingivalis* was 0% (with an interquartile range of 0-2.75) for the periodontal health sites and 1% (with an interquartile range of 0-2.26) for the periodontitis sites compared to peri-implant sites. The median percentage relative abundance values of *T. forsythia, S. noxia, Campylobacter* spp. and *E. corrodens* for periodontally healthy sites were 54.9%, 73.6%, 75.25% and 42.3% of the periodontitis sites, respectively. However, differences were not significantly different ($P>0.05$). The median percentage relative abundance value of *P. gingivalis* in the periodontally healthy site was 0% (with an interquartile range of 0-2.26) and the median percentage relative abundance value of *P. gingivalis* in the periodontally healthy site was 1% (with an interquartile range of 0-2.75). Interestingly, *Prevotella* spp., *Treponema* spp. and staphylococcal spp. were observed to be elevated by 180.9%, 109.1% and 50.4% in the periodontal microflora of periodontally healthy sites examined, compared to those of the periodontitis sites (Table 6.7).

Current smoking status did not significantly influence the percentage relative abundance of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Treponema* spp. or staphylococcal spp. within the subgingival periodontal and peri-implant microflora for patients who were current smokers to those who were non-smokers ($p>0.05$) (Table 6.8).

Table 6.3 The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species/groupings investigated by checkerboard DNA-DNA hybridisation for periodontal and peri-implant sites.

	Percentage Relative Abundance		
Species groupings	**Periodontal median**	**Peri-implant median**	**p-value**
Actinomyces spp.	8.28	8.89	-
Aggregatibacter actinomycetemcomitans	0	0	0.16
Campylobacter spp.	1.78	1.72	-
Capnocytophaga spp.	4.97	4.26	-
Eikenella corrodens	3.00	3.04	-
Fusobacterium spp.	6.74	6.03	-
Lactobaccilus acidophilus	4.86	4.89	-
Leptotrichia buccalis	1.22	1.12	-
Neisseria mucosa	0.92	2.38	-
Parvimoas micra	7.44	8.55	-
Porphymonas gingivalis	0.85	1.2	0.33
Prevotella spp.	1.47	2.8	-
Selenomonas noxia	1.51	1.37	-
Streptococcal spp.	7.03	8.08	-
Tanerella forsythia	1.84	3.67	0.3
Treponema spp.	1.23	2.14	0.03
Veillonella parvula	7.84	8.23	-
Staphylococcal spp.	3.38	4.23	0.65

Table 6.4 The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings investigated by the checkerboard DNA-DNA hybridisation technique for peri-implant mucositis and peri-implantitis sites.

	Percentage relative abundance		
Species groupings	**Peri-implantitis median**	**Peri-implant mucositis median**	**p-value**
Actinomyces spp.	4.52 (0.90 to 0.08)	6.95 (2.45 to 10.57)	-
Aggregatibacter actinomycetemcomitans	1.5 (0 to 34.42)	1.51 (0 to 9.68)	0.72
Campylobacter spp.	1.61 (0.43 to 2.54)	1.65 (0.83 to 3.15)	-
Capnocytophaga spp.	3.58 (0.43 to 2.54)	4.0 (1.85 to 5.63)	-
Eikenella corrodens	2.81 (0.41 to 3.13)	2.39 (0.57 to 4.72)	-
Fusobacterium spp.	5.56 (3.31 to 9.7)	4.95 (3.65 to 7.98)	-
Lactobaccilus acidophilus	5.51 (2.59 to 6.35)	4.73 (2.76 to 6.84)	-
Leptotrichia buccalis	0.91 (0.58 to 1.71)	1.12 (0.62 to 2.22)	-
Neisseria mucosa	1.8 (0 to 3.2)	2.33 (0 to 5.00)	-
Parvimoas micra	9.01 (5.19 to 12.18)	8.34 (5.1 to 11.22)	-
Porpphymonas gingivalis	0.91 (0.41 to 4.98)	1.24 (0 to 5.98)	0.86
Prevotella spp.	2.72 (1.63 to 4.43)	2.25 (0 to 3.61)	-
Selenomonas noxia	1.24 (0 to 2.14)	1.22 (0 to 2.3)	-
Streptococcal spp.	6.6 (4.27 to 9.26)	7.7 (3.72 to 11.51)	-
Tanerella forsythia	4.59 (1.09 to 12.31)	3.00 (1.18 to 8.57)	0.74
Treponema spp.	2.07 (0.51 to 3.33)	1.99 (0.97 to 5.06)	0.7
Veillonella parvula	6.36 (3.56 to 10.02)	7.9 (4.65 to 11.43)	-
Staphylococcal spp.	4.09 (2.58 to 5.83)	4.3 (2.7 to 7.42)	0.77

Table 6.5 The median (inter-quartile range) value of the percentage relative abundance of each of the 18 species groupings (checkerboard DNA-DNA hybridisation) for sites with probing pocket depths (PPD) of >5 mm and demonstrating bleeding on probing (BOP), and sites not meeting these criteria.

	Percentage relative abundance		
Species groupings	**Sites with PPD ≥5 mm and BOP median**	**Sites without PPD ≥5 mm and BOP median**	**p-value**
Actinomyces spp.	4.89 (1.23 to 9.7)	6.36 (0.56 to 11.52)	-
Aggregatibacter actinomycetemcomitans	1.52 (0 to 12.47)	4.04 (0.27 to 20.02)	0.21
Campylobacter spp.	1.98 (0.92 to 3.15)	4.25 (0.79 to 7.27)	-
Capnocytophaga spp.	3.78 (2.05 to 4.97)	4.25 (1.79 to 7.27)	-
Eikenella corrodens	2.70 (1.25 to 3.89)	2.21 (0 to 3.37)	-
Fusobacterium spp.	6.17 (3.92 to 9.52)	5.7 (2.89 to 751)	-
Lactobaccilus acidophilus	4.94 (2.76 to 6.48)	4.07 (0.95 to 6.97)	-
Leptotrichia buccalis	1.03 (0.62 to 2.08)	1.15 (0.54 to 2.6)	-
Neisseria mucosa	2.39 (0.9 to 4.4)	0 (0 to 3.24)	-
Parvimoas micra	8.55 (4.98 to 13.75)	8.68 (4.21 to 13.62)	-
Porpphymonas gingivalis	2.23 (0.5 to 6.82)	0.59 (0 to 1.89)	<0.0001
Prevotella spp.	2.12 (0.8 to 3.8)	2.08 (0 to 3.89)	-
Selenomonas noxia	1.22 (0.17 to 2.17)	0.99 (0 to 1.98)	-
Streptococcal spp.	6.59 3.38 to 9.52)	7.4 (3.32 to 12.82)	-
Tanerella forsythia	6.13 (1.69 to 14.03)	1.60 (0 to 6.03)	0.0005
Treponema spp.	1.85 (0.91 to 3.63)	2.06 (0.68 to 4.89)	0.83
Veillonella parvula	6.78 (4.14 to 10.69)	7.53 (3.56 to 13.32)	-
Staphylococcal spp.	3.39 (2.37 to 6.09)	4.42 (2.72 to 7.59)	0.15

Table 6.6 The median (inter-quartile range) value of the percentage relative abundance[1] of *A. actinomycetemcomitans, P. gingivalis, T. forsythia, Treponema* spp., staphylococcal spp. at investigated by checkerboard DNA-DNA hybridisation for patients with and without a history of severe periodontal disease.

	Percentage relative abundance		
Species	**Periodontitis median**	**Non-periodontitis median**	**p-value**
Aggregatibacter actinomycetemcomitans	3.72 (2.01-6.42)	2.82 (0.92-4.43)	0.1
Porphymonas gingivalis	1.16 (0.45-5.62)	0.82 (0-2.43)	0.33
Tanerella forsythia	3.55 (1.64-8.82)	1.72 (0.25-14.01)	0.3
Treponema spp.	1.92 (1.33-3.76)	1.2 (0.35-2.44)	0.037
Staphylococcal spp.	4 (2.7-6.08)	3.35 (2.5-4.84)	0.75

[1]The Wilcoxon test was undertaken to compare values of the % relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

Table 6.7 The median (inter-quartile range) value of the percentage relative abundance[1] of each of the 18 species/groupings investigated by checkerboard DNA-DNA hybridisation for periodontal healthy sites and periodontitis sites.

	Percentage relative abundance	
Species groupings	**Periodontal health**	**Periodontitis**
Actinomyces spp.	5 (0-12)	4.93 (0.66-11.63)
Aggregatibacter actinomycetemcomitans	0 (0-2)	0 (0-3)
Campylobacter spp.	1.52 (0-3.14)	2.02 (0.9-3.67)
Capnocytophaga spp.	4.29 (0.56-10.65)	4.67 (3.3-7.31)
Eikenella corrodens	1.27 (0-2.79)	3 (2.33-3.56)
Fusobacterium spp.	7.18 (4.27-9.56)	7.1 (4.95-9.3)
Lactobaccilus acidophilus	4.05 (0-5.9)	5.2 (1.37-6.55)
Leptotrichia buccalis	1.36 (0.28-3.53)	1.04 (0.72-2.33)
Neisseria mucosa	0 (0-0)	0 (0-2.97)
Parvimoas micra	10.62 (4.23-20.7)	9.8 (4.55-14.56)
Porpphymonas gingivalis	0 (0-2.26)	1 (0-2.75)
Prevotella spp.	2.36 (0-6.85)	0.84 (0-1.9)
Selenomonas noxia	0 (0-1)	1.05 (0-1.65)
Streptococcal spp.	9.06 (5-14)	6.67 (1.92-9.65)
Tanerella forsythia	1.74 (0-6.85)	3.17 (0-17.9)
Treponema spp.	2.76 (1.32-6.41)	1.32 (0.6-3.6)
Veillonella parvula	7 (3-14)	7.45 (5.05-12.34)
Staphylococcal spp.	4.98 (3.57-9.48)	3.31 (3.06-4.79)

[1]The Wilcoxon test was undertaken to compare values of the % relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

Table 6.8. The median (inter-quartile range) value of the percentage relative abundance[1] of *A. actinomycetemcomitans, P. gingivalis, T. forsythia, Treponema* spp., staphylococcal spp. as investigated by checkerboard DNA-DNA hybridisation for patients who are current smokers and non-smokers.

	Percentage relative abundance		
	Smokers median	**Non-smokers median**	**p-value**
Aggregatibacter actinomycetemcomitans	2.43 (1.06-4.27)	3.72 (2.03-6.55)	0.19
Porphymonas gingivalis	0.73 (0.47-2.2)	1.01 (0-4.16)	1
Tanerella forsythia	2.09 (1.33-7.58)	3.46 (0.88-20.83)	0.79
Treponema spp.	1.77 (0.98-5.2)	1.69 (0.89-2.79)	0.8
Staphylococcal spp.	3.51 (2.94-5.15)	3.5 (2.51-6.25)	0.73

[1]The Wilcoxon test was undertaken to compare values of the % relative abundance for the following species: *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.

6.4 Discussion

6.4.1 Peri-implant compared to periodontal microflora

In the present investigation, only the percentage relative abundance of *Treponema* spp. was observed to be significantly elevated in peri-implant sites compared to periodontal sites ($p<0.05$) (Table 6.3). No other significant differences were noted comparing the microflora from periodontal and peri-implant sites. This finding is in accordance with the evidence to date (Quirynen and Listgarten, 1990; Mombelli *et al.*, 1995; Mengel *et al.*, 1996; Leonhardt *et al.*, 1999; Hultin *et al.*, 2000*b*; Botero *et al.*, 2005), that periodontal and peri-implant sites often share common microbial species. The findings of the present investigation support the theory that periodontal and peri-implant sites share a common flora and are intimately related. It is likely that periodontal sites may serve as a reservoir for microbial colonisation of titanium implants in the same mouth (Leonhardt *et al.*, 1999) and that translocation of microbial species from peri-implant sites to periodontal sites takes place (Furst *et al.*, 2007).

6.4.2 Peri-implant mucositis compared to peri-implantitis

No significant differences were noted in the percentage relative abundance of the bacterial species investigated, in sites affected by peri-implant mucositis and those affected by peri-implantitis. Other investigators previously noted similarities in the distribution of microbial species in peri-implantitis and peri-implant mucositis sites (Mombelli & Lang, 1992; Hultin *et al.*, 2002; Agerbaek *et al.*, 2006). However, in the present study, the total of the mean values of the estimated bacterial cell density per individual species (based on DNA-DNA hybridisation), detected from all of the samples investigated from peri-implantitis sites, was 33% greater than that of peri-implant mucositis sites. Hultin *et al.* (2002) similarly noted higher bacterial density around implants demonstrating peri-implant bone loss compared to those without. However, it is difficult to determine if the higher bacterial density is the initiating factor of the peri-implantitis or if it is a consequence of the peri-implantitis i.e. that the higher bacterial density results from the peri-implant bone loss and deeper pocketing, which is, by definition, observed at peri-implantitis sites.

6.4.3 Periodontal health and periodontitis

It is difficult to explain the elevated percentage relative abundance of *Prevotella* spp., *Treponema* spp. and staphylococcal spp. within the periodontal microflora of healthy sites compared to those affected by periodontitis (Table 6.7). Staphylococcal spp. are relatively common constituents of the normal oral flora and it may be the case that they are also common constituents of the normal periodontal flora. Furthermore, it is hypothesised that the presence of one or more dental implants in the patient's mouth, with their associated peri-implant microflora, may have influenced the composition of the patient's periodontal microflora (Furst *et al.,* 2002). Furthermore, the extent of the periodontitis disease severity was in general low and therefore, the differences in the periodontal microflora between the healthy and generally low to moderate periodontitis levels was not extensive.

6.4.4 Probing depths of >5 mm and bleeding on probing

Interestingly, percentage relative abundance levels of *T. forsythia* and *P. gingivalis* were observed to be significantly elevated from periodontal and peri-implant sites with probing depths of >5 mm and bleeding on probing ($p<0.01$), compared to those sites that do not meet these criteria (Table 6.5). Furthermore, the presence of *N. mucosa* was significantly elevated in periodontal and peri-implant sites with probing depths of >5 mm and bleeding on probing ($p<0.05$), compared to those sites that did not meet this criteria. The threshold probing pocket depth of >5 mm and bleeding on probing has been observed to be a key criterion in assessing the stability of a patients periodontal condition. Rams *et al.* (1983) and Rams *et al.* (1984) employed scanning electron microscopy and anaerobic culture to examine the morphology of the plaque samples from peri-implant sites and noted high levels of spirochetes, fusiform bacteria and cocoid rods in deep peri-implant sites demonstrating signs of bone loss, whereas implants with pockets not greater than 5 mm, yielded predominantly coccoid microbiota and very few rods. Almost half of the microorganisms cultivable from the deep sites were Gram-negative anaerobic rods, including *Fusobacterium spp.* and *P. intermedia*, thereby sharing many similarities with periodontal diseases. Claffey *et al.* (1995) observed that patients with one or more periodontal sites with probing pocket depths of ≥6 mm, with bleeding on probing at re-evaluation, were at an increased risk of periodontal disease progression with associated periodontal clinical attachment loss, compared to those patients that did not meet this criteria. Cho-Yan Lee *et al.* (2012) noted probing depths of >5 mm and bleeding on probing of periodontal sites to be risk indicators for increased peri-implant bone loss.

Agerbaek *et al.* (2006), employing checkerboard DNA-DNA hybridisation techniques, detected a higher bacterial density at peri-implant sites with probing pocket depths of ≥4 mm compared to those sites with probing pocket depths of ≤4 mm. Thus, the findings of the present study, that *T. forsythia*, *P. gingivalis* were highly significantly elevated ($p<0.01$) and *N. mucosa* levels significantly elevated ($p<0.05$) in periodontal and peri-implant sites with probing depths of >5 mm and bleeding on probing. These findings furthers our understanding of the microflora of deeper periodontal and peri-implant pockets.

6.4.5 Smokers compared to non-smokers

No significant differences were noted in the percentage relative abundance of the peri-implant or periodontal microflora of current smokers compared to those who were non-smokers ($p>0.05$) (Table 6.8). In the present study, eight patients were smokers (defined as patients who were currently smoking, or had stopped smoking in the previous year). However, two patients stopped smoking following the baseline examination and one patient had stopped smoking in the year prior to baseline examination. Therefore, the relatively small numbers of patients, included in the study, who were smokers, make the study inadequately powered to address the question of the comparison of the peri-implant and periodontal microflora in smokers compared to non-smokers. A much larger study would be required to address this question adequately, considering the permutations of ex-smokers, occasional smokers, number of years smoking and categories for patients who smoked different quantities of cigarettes per day. The 8th European Workshop on Periodontology (2012) recently recommended that, in order to study the role of smoking in peri-implantitis and to investigate the many confounding factors present, thousands or even tens of thousands of patients would be required to participate, likely in a worldwide study (Rocchietta & Nisand, 2012).

6.4.6 History of severe periodontal disease

Patients with a history of severe periodontal disease were observed to have higher levels of DNA for *Treponema* spp. at periodontal and peri-implant sites compared to patients who did not have a history of severe periodontal disease ($p=0.04$) (Table 6.6). The percentage relative abundance of *T. forsythia, P. gingivalis* and *A. actinomycetemcomitans* were noted to be elevated in patients with a history of severe periodontal disease compared to those with no history of severe periodontal disease, but differences were not significant ($p>0.05$). These findings are similar to those of Mengel *et al.* (2007) and

Mengel & Flores-de-Jacoby (2005) who similarly observed that *T. forsythia, P. gingivalis* and *A. actinomycetemcomitans* were more frequently isolated from the peri-implant microflora of patients with a history of periodontal disease compared to those without.

6.5 Conclusions

From the findings of the present investigation it may be concluded that:

a) *Treponema* spp. was observed to be significantly elevated in peri-implant sites compared to periodontal sites ($p<0.05$) No significant differences were noted in the microflora of peri-implant sites affected by peri-implant mucositis compared to those affected by peri-implantitis determined by checkerboard analysis for the species tested. However, the total of the mean values of the estimated bacterial cell density per individual species (based on DNA-DNA hybridisation), detected from all of the samples investigated from peri-implantitis sites, was 33% greater than that of peri-implant mucositis sites

b) *T. forsythia* and *P. gingivalis* percentage relative abundance levels were significantly elevated ($p<0.01$) and *N. mucosa* percentage relative abundance levels were elevated to a lesser extent ($p<0.05$) in periodontal and peri-implant sites demonstrating probing depths of >5 mm and bleeding on probing, compared to those that do not meet these criteria.

c) No significant differences were observed between the relative abundance of the 18 bacterial species groupings of current smokers compared to those who were non-smokers ($p>0.05$), however, this should be interpreted with caution as the present study was inadequately powered to address this issue adequately.

d) The percentage relative abundance of *Treponema* spp. were significantly elevated, from periodontal and peri-implant sites, in patients with a history of severe periodontal disease, compared to patients with no history of severe periodontal disease ($p<0.05$).

Chapter 7

Determination of semi-quantitative changes in the microbial flora around natural teeth and implants following non-surgical periodontal therapy

7.1 Introduction

The evidence in relation to the influence of non-surgical mechanical debridement and oral hygiene instruction on peri-implant infections is relatively scarce compared to the very significant volume of clinical and microbiological research that has been undertaken to enhance understanding of the response of periodontal infections to non-surgical periodontal treatment (Renvert *et al.*, 2006; Claffey *et al.*, 2008). From a microbiological perspective, the goal of the disease elimination phase of treatment of peri-implant infections is similar to that of the treatment of periodontal infections, i.e. a reduction in the numbers of those species associated with disease and the establishment of a local environment and micro-flora compatible with health (Mombelli *et al.*, 2005). Following mechanical debridement, adjunctive, topical antimicrobial agents have been applied locally in order to further shift the composition of the peri-implant microflora towards health. Topical agents investigated include tetracycline-containing fibres (Mombelli *et al.*, 1992), slow-release doxycycline (Buchter *et al.*, 2004), minocycline microspheres (Renvert *et al.*, 2004; Renvert *et al.*, 2006; Salvi *et al.*, 2007; Renvert *et al.*, 2008) and chlorhexidine gel (Persson *et al.*, 2007).

7.1.1 Observed effects of non-surgical mechanical treatment on peri-implant infections

The sampling methods and microbiological methods used in a selection of studies on the non-surgical treatment of peri-implant infections are shown in Table 7.1 Several studies have found that non-surgical treatment of peri-implantitis lesions resulted in limited or no improvements in peri-implant health (Karring *et al.*, 2005; Renvert *et al.*, 2009; Persson *et al.*, 2010). However, a reduction in bacterial load has been observed, following non-surgical debridement of implants affected by peri-implant infections, with or without the use of adjunctive antimicrobial agents, in animal infection models (Hayek *et al.*, 2005) and in human clinical studies (Mombelli & Lang, 1992; Strooker *et al.*, 1998; Mombelli *et al.*, 2001; Mombelli *et al.*, 2005; Persson *et al.*, 2006; Renvert *et al.*, 2009), but not in all studies (Renvert *et al.*, 2008). Similarly, in clinical investigations, following the non-surgical treatment of peri-implant infections, statistically significant reductions in density have been noted for specific microbial species, including: *P. gingivalis* (Strooker *et al.*, 1998; Persson *et al.*, 2006); *T. forsythia* (Strooker *et al.*, 1998; Mombelli *et al.*, 2005); *Fusobacterium* spp. (Mombelli & Lang, 1992; Strooker *et al.*, 1998; Mombelli *et al.*, 2001, 2005); *A. actinomycetemcomitans* (Strooker *et al.*, 1998; Persson *et al.*, 2006;

Table 7.1 Sampling methods and microbiological analytical techniques employed in studies on the non-surgical treatment of peri-implant infections.

Study	Curette	Paper-point	Dark-field microscopy	Culture	Checkerboard DNA-DNA hybridisation	Real-time PCR
Mombelli & Lang (1992)	+		+			
Mombelli *et al.* (2001)		+		+		
Renvert *et al.* (2008)		+		+	+	
Persson *et al.* (2006)	+				+	
Thone-Muhling *et al.* (2010)		+				+
Maximo *et al.* (2009)	+				+	
Heitz-Mayfield *et al.* (2011)		+			+	
Strooker *et al.* (1998)		+		+		
Luterbacher *et al.* (2000)		+				+
Renvert *et al.* (2009)		+			+	

Persson *et al.*, 2010); *Prevotella* spp. (Strooker *et al.*, 1998; Hayek *et al.*, 2005; Mombelli *et al.*, 2005); *Campylobacter* spp. (Strooker *et al.*, 1998; Mombelli *et al.*, 2005); *Lactobacillus* spp. (Persson *et al.*, 2010); streptococcal spp. (Persson *et al.*, 2010) and *Veillonella* spp. (Persson *et al.*, 2010) (Table 7.1). However, following extended observational intervals post non-surgical debridement of implants affected by peri-implant infections, an increase in the density of the microbial species associated with peri-implant infections has been observed (Persson *et al.*, 2006; Persson *et al.*, 2010).

7.1.2 Microbial sampling

It has been observed in many studies that microbial samples taken using either paper-point or curette sampling techniques, may differ from each other depending on the sampling method employed (Baker *et al.*, 1991; Keil & Lang, 1991; Renvert *et al.*, 1992; Socransky, 1998; Gerber *et al.*, 2005). Controversy remains as to which sampling technique yields the microbial sample that best represents the composition of the subgingival microflora of the site sampled. For example, Keil & Lang (1991) observed that curette sampling resulted in the retrieval of greater numbers of colony forming units compared to paper-point sampling, whereas Renvert *et al.* (1992), in an interventional study, noted that paper-point sampling yielded significantly greater numbers of spirochete species than curette sampling, prior to treatment. Furthermore, other investigators have observed no significant differences in the species isolated when curette or paper-point sampling techniques were employed (Jervoe-Storm *et al.*, 2007). Therefore, a combination of sampling techniques has been recommended in order to yield samples that more fully represent the microflora of the site sampled (Gerber *et al.*, 2005). No study to date has investigated the composition of the periodontal or peri-implant microflora with combined paper-point and curette sampling techniques.

7.2 Aim

The aim of this part of the present study was to investigate the influence of non-surgical mechanical treatment (i.e. oral hygiene instruction and mechanical debridement) on the peri-implant and periodontal microflora, nine months following the completion of active treatment, employing combined paper-point and curette sampling and using checkerboard DNA-DNA hybridisation techniques to compare semi-quantitatively the microflora before and after the clinical intervention.

7.3 Materials and Methods

7.3.1 Patients

Patients attending the Dublin Dental University Hospital, and having one or more dental implants placed five years previously, were screened between October 2007 and September 2009. This study had the approval of the Ethics Committee of the Faculty Research Ethics Group-Faculty of Health Sciences, Trinity College Dublin (see Chapter 2, Section 2.1.1 and Appendix 1). Those patients who met the inclusion criteria were asked to participate in the present investigation. Patient inclusion and exclusion criteria are described below.

7.3.1.1 Patient inclusion criteria

- Eighteen or more years of age.
- Dental implant placed five or more years previously.
- Presence of one or more implant restorations with a positive diagnosis of peri-implant mucositis or peri-implantitis.
- Signed the informed consent form approved by the Ethics Committee of the Faculty Research Ethics Group-Faculty of Health Sciences, Trinity College Dublin (see Chapter 2, Section 2.1.1 and Appendix 1).

7.3.1.2 Patient exclusion criteria

A full medical history was taken and any patients reporting the following conditions were excluded from the study:

- Prophylactic medication requirement.
- Pregnant or lactating women.
- Patients having taken antibiotics, antifungal or steroid medication within two months prior to participation in the study.
- Children under 18 years of age.
- Adults with learning disabilities.
- Adults who have a terminal illness.

7.3.2 Clinical examination and radiographs of peri-implant sites

At the baseline consultation, a detailed dental and medical history and an oral examination was undertaken, including a detailed periodontal examination (full mouth probing pocket depths measurement and measurement of recession and bleeding on

probing). The detailed periodontal examination was undertaken employing the manual pressure sensitive Click Probe (Kerr Hawe).

Intra-oral, peri-apical radiographs were taken of implant fixtures, utilising the long cone paralleling technique (as described in Section 2.2.2) to facilitate visualisation of the peri-implant bone levels. Two examiners independently examined the baseline examination radiographs. Where peri-implant bone loss >2.5 mm was noted, peri-implant bone loss of ≥2.5 mm was diagnosed and recorded for that implant. Following completion of the examination of the radiographs, the results from both examiners were compared. Kappa analysis revealed good agreement between examiners ($p<0.001$), with regard to diagnosis of peri-implant bone loss of ≥ 2.5 mm. Where discrepancies were present between examiners, a third examiner was employed and the majority decision rewarded.

7.3.3 Peri-implantitis, peri-implant mucositis and health implants

Implants demonstrating bleeding or suppuration on probing, probing pocket depth of ≥6 mm and radiographic bone loss ≥2.5 mm were classified as being affected by peri-implantitis (Berglundh *et al.*, 2002). Implants with bleeding or suppuration on probing but not meeting the criteria of peri-implant bone loss ≥2.5 mm and at least one site with probing pocket depth of ≥6 mm, were diagnosed as peri-implant mucositis-positive. Patients with implants not demonstrating bleeding or suppurating on probing were diagnosed as being healthy. For the purposes of this investigation, patients with implants positive for peri-implant mucositis were classified as "peri-implant mucositis patients", whereas patients with implants positive for peri-implantitis were classified as "peri-implantitis patients". It is important to note that where patients had implants affected by peri-implantitis, they were classified as peri-implantitis patients, even if they also had healthy implants or implants affected by peri-implant mucositis. Similarly, where patients had implants affected by peri-implantitis and healthy implants, they were classified as peri-implant mucositis patients.

7.3.4 Treatment

Full mouth debridement was undertaken for each patient (see Chapter 4, Figure 4.1). Debridement was undertaken over one to four visits, according to the patient's ability to tolerate treatment, the severity of the peroiodontal and peri-implant infections and the difficulties of the instrumentation required. Local anaesthetic was employed as necessary. Debridement of implant fixture surfaces was undertaken using titanium tipped curettes (Salvin Dental Specialties). If present, gross supra-mucosal plaque and calculus deposits

were removed firstly. The curette tip was then placed in contact with the implant surface in the region of the most coronal threads. The curette tip was utilised to debride the implant surface in the region of the threads until it was deposit free, subsequently deeper threads were instrumented until the base of the pocket was reached. Following debridement of all available surfaces, the implant surface was carefully checked utilising the Kerr Hawe Click Probe tip (Kerr Hawe) to locate any residual deposits. All residual deposits were removed and the implant surface repeatedly checked until a deposit free surface had been achieved. Natural teeth periodontal sites were debrided employing Gracey Curettes (Hu-Friedy), until no deposits were detectable on the root surface.

Oral hygiene instruction was tailored to individual patient requirements. Patients were instructed to use either manual or battery driven toothbrushes according to their preference and ability. Where inter-proximal spaces were large enough to accommodate inter-proximal brushes (Te Pe), appropriate sizes were selected and the patient instructed in the use of these. Where inter-proximal spaces were too small to accommodate inter-proximal brushes, patients were instructed in the use of super-floss (Oral B).

Re-evaluation was undertaken three months following completion of active treatment. Patients were seen again at six and nine months for maintenance care. Re-instrumentation and oral hygiene re-instruction was undertaken as required at recall visits (i.e. sites demonstrating bleeding on probing and/or the presence of plaque and/or calculus were re-instrumented and oral hygiene instruction repeated).

7.3.5 Microbial Sampling

Both curette and paper-point sampling techniques were employed following careful tooth isolation. Paper-point sampling was undertaken first and curette sampling subsequently as described in Chapter 2, Section 2.2.5.

7.3.6 Semi-quantitative determination of microbial species by checkerboard DNA-DNA hybridisation

Following preparation of the samples (as outlined in Chapter 2, Section 2.2.6.1), a semi-quantitative determination of the density of 29 individual oral microbial species present in each sample, was undertaken using the checkerboard DNA-DNA hybridisation technique (Socransky *et al.*, 1994; Haffajee *et al.*, 1997*a*; Persson et al. 2007) as described in Chapter 2, Section 2.2.6.3. The probes and their source strains were compared as described by Ximenez-Fyvie *et al.* (2000). For all samples, checkerboard DNA-DNA hybridisation investigation was undertaken for the species outlined in Chapter 2, Table

2.1. The bacterial species belonging to specific groupings were grouped together for data analysis (Chapter 2, Table 2.1).

7.3.7 Quantitative PCR detection of four bacterial species: Real-Time PCR analysis

Additional to the Checkerboard technique, Real-Time PCRs were undertaken for four bacterial species including: *A. Actinomycetemcomitans; T. forsythia*; *P. gingivalis* and *T. denticola* (Table 2.2) as described in Chapter 2, Section 2.6.2. In the final analysis, only checkerboard DNA-DNA hybridisation results confirmed by RT- PCR for these species were considered valid and included in the analysis.

7.3.8 Statistical analysis

The percentage relative abundance was calculated by computing the percentage of each of the 18 microbial species groupings, as a proportion of all of the species detected at an individual site.

The median bacterial cell density from the checkerboard DNA-DNA hybridisation analysis, was compared between baseline and nine months post-treatment for the 18 microbial species groupings investigated. Owing to the problems associated with multiple hypothesis testing, namely that with increasing numbers of hypotheses tested, the degrees of freedom decrease and there is an increased likelihood of encountering a Type I error (i.e. a particular hypothesis is not rejected when it should have been, also known as a false positive result), for a given p-value (Dudoit *et al.*, 2004). Therefore, only five of the 18 species groupings investigated were analysed using the Wilcoxon Rank Sum Test. These included: *A. actinomycetemcomitans, P. gingivalis, T. forsythia*, *Treponema* spp. and staphylococcal spp. For the remainder of the microbial species groupings examined, descriptive statistics were applied.

Relative abundance data was examined in order to investigate changes in the composition of the peri-implant and periodontal biofilm microbial communities following treatment, and help to overcome some of the limitations of differing plaque volumes being recovered on different sampling occasions (see Chapter 2, Section 2.7.3). Also, the changes in the periodontal and peri-implant sites relative abundance of *A. actinomycetemcomitans P. gingivalis, T. forsythia*, *Treponema* spp. and staphylococcal spp. between baseline and nine months following treatment were analysed utilising the Wilcoxon rank sum test with continuity correction. For the remainder of the microbial

species groupings examined, descriptive statistics were applied. Descriptive statistics were employed to examine:

a) The frequency of peri-implant sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 species groupings following treatment.
b) The frequency of periodontal sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 species following treatment.
c) The frequency of peri-implant mucositis demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 species following treatment.
d) The frequency of peri-implantitis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the percentage relative abundance of DNA isolated for each of the 18 bacterial species groupings following treatment.

7.4 Results

Twenty-eight patients provided microbial samples at baseline and at nine months following treatment. At baseline, of the 28 patients, eight patients had implants that were affected by peri-implantitis, 27 had implants that were affected by peri-implant mucositis and in seven patients, both implants affected by peri-implantitis and peri-implant mucositis were present.

Data from the checkerboard DNA-DNA hybridisation, undertaken at the Oral Microbiology Laboratory of the University of Bern, were compiled and underwent analysis comparing the periodontal and peri-implant microflora at baseline and at nine months following treatment.

A. actinomycetemcomitans and *P. gingivalis* demonstrated highly significant reductions in relative abundance nine months following treatment of peri-implant sites relative to baseline ($p<0.01$) (Table 7.3). However, no significant changes were observed for these species for periodontal sites ($p>0.05$) (Table 7.4).

The change in the estimated bacterial cell density for each of the investigated species groupings for peri-implant and periodontal sites is outlined in Table 7.2. No significant differences were observed between peri-implant and periodontal sites for the change in the estimated bacterial cell density for *A. actinomycetemcomitans P. gingivalis, T. forsythia*, *Treponema* spp. and Staphylococcal spp., comparing baseline to nine months following treatment ($p>0.05$).

To further elucidate the influence of non-surgical treatment on the percentage relative abundance of individual species groupings within biofilm communities of periodontal and peri-implant sites, a threshold of ≥1% change in relative abundance was applied. The numbers of peri-implant sites with a ≥1% increase in relative abundance, with no change in relative abundance or with a ≥1% decrease in relative abundance following treatment (i.e. at nine months), were examined for peri-implant (Table 7.1 and Table 7.5) and periodontal sites (Table 7.6 and Figure 7.2) for each of the 18 species groupings.

The change in the relative abundance following treatment of each of the 18 species groupings from peri-implant sites, is outlined in Figure 7.1 and Table 7.5. Greater than 50% of peri-implant sites demonstrated a decrease of ≥1% in the relative abundance of *L. acidophilus* (31/55) and *T. forsythia* (28/55), whilst for almost 50% of peri-implant sites, an increase in the relative abundance of oral streptococci (26/55), *Capnocytophaga*

spp. (26/55) and *E. corrodens* (26/55) of ≥1% was noted following treatment (Figure 7.1 and Table 7.5).

The change in the relative abundance following treatment of each of the 18 species groupings from periodontal sites, is outlined in Figure 7.2 and Table 7.6. At approximately 50% of periodontal sites, *T. forsythia* (11/25), *P. gingivalis* (13/25), *N. mucosa* (18/25), *S. noxia* (17/25) and *A. actinomycetemcomitans* (16/25) demonstrated a decrease of >1% in their relative abundance. Furthermore, approximately 50% of periodontal sites demonstrated an increase of >1% in the relative abundance of *V. parvula* (12/25) and oral streptococci (13/25) following treatment (Table 7.2 and Figure 7.2).

The change in the relative abundance, following treatment, of each of the 18 species groupings from peri-implant mucositis sites, is outlined in Figure 7.3 and Table 7.7. In approximately 50% of peri-implant mucositis sites, a decrease of >1% of the relative abundance of each of the following species groupings: *L. acidophilus* (21/40); *P. micra* (21/40); *P. gingivalis* (20/40); *T. forsythia* (20/40) and *Treponema* spp. (19/40) was noted following treatment. At the same time, approximately 50% of peri-implant mucositis sites demonstrated an increase of >1% in the relative abundance of *Capnocytophaga* spp., *E. corrodens* and oral streptococci spp. following treatment (Table 7.7 and Figure 7.3).

The change in the relative abundance following treatment of each of the 18 species groupings from peri-implantitis sites is outlined in Figure 7.4 and Table 7.8. Approximately 50% peri-implantitis sites demonstrated a decrease of >1% in the relative abundance of *L. acidophilus* (10/15), *E. corrodens* (9/15), *Fusobacterium* spp. (7/15) and *T. forsythia* (8/15), *Treponema* spp. (7/15) and staphylococcal spp. (7/15) following treatment. Whilst at approximately 50% of peri-implantitis sites, oral streptococci (8/15) and *V. parvula* (8/15) were observed to increase by >1% in relative abundance following treatment (Table 7.8 and Figure 7.4). However, compared to peri-implantitis sites, fewer peri-implant mucositis sites demonstrated an increase of ≥1% in the relative abundance of staphylococcal spp., being 40% (6/15) and 15.5% (7/40), respectively (Table 7.7; Figure 7.3). Furthermore, the relative abundance of *P. gingivalis* was observed to decrease by ≥1% at 50% (20/40) of peri-implant mucositis sites, but only at 26.7% (4/16) of peri-implantitis sites.

Overall changes in the percentage relative abundance for each of the 18 species groupings investigated between baseline and nine months following treatment at peri-implant sites are shown in Table 7.9. The percentage relative abundance of the 18 species

Table 7.2 Comparison of change in the microflora of periodontal and peri-implant sites following treatment, estimated bacterial cell density (inter-quartile range) x10^5 cfu.

Species groupings	Estimated bacterial cells (x10^5)		p-value
	Peri-implant median	Periodontal median	
Actinomyces spp.	-1.2 (-6.05 to 2.5)	0 (-8.43 to 4.68)	-
Aggregatibacter actinomycetemcomitans	-0.9 (-5.81 to 6.52)	0.11 (-4.91 to 2.94)	0.92
Campylobacter spp.	0 (0 to 1.3)	-0.02 (-4.91 to 2.94)	-
Capnocytophaga spp.	0.76 (-1.7 to 3.66)	0.02 (-1.47 to 0.4)	-
Eikenella corrodens	-0.46 (-2.07 to 1.36)	0 (-0.84 to 4.2)	-
Fusobacterium spp.	0.3 (-4.79 to 4.35)	-2 (-4.9 to 0.51)	-
Lactobacterial spp.	-1.4 (-3.5 to 0.84)	2.72 (-1.9 to 7.56)	-
Leptotrichia spp.	-0.13 (-0.73 to 0.75)	-0.43 (-2.02 to 0.45)	-
Neisseria spp.	0 (-2.72 to 1.95)	0 (0 to 0.94)	-
Parvimoas micra	0.17 (-5.44 to 4.18)	1.48 (-4.46 to 10.11)	-
Porphymonas gingivalis	-4.44 (-3.2 to 0.34)	0 (-1.66 to 0)	0.49
Prevotella spp.	-0.43 (-1.40 to 0.48)	0 (-2.29 to 0)	-
Selenomonas spp.	0.21 (-1.12 to 4.08)	0 (-0.13 to 0.23)	-
Oral streptococci	0.62 (-4.06 to 1)	5.24 (-2.32 to 9.97)	-
Tanerella forsythia	-0.9 (-4.42 to 5.3)	-0.23 (-6.54 to 0)	0.43
Treponema spp.	-0.92 (-4.41 to 8.58)	-0.43 (-6.21 to 5.82)	0.59
Veillonella spp.	0.58 (-3.08 to 6.27)	0.15 (-5.04 to 7.79)	-
Staphylococcal spp.	-0.29 (-2.89 to 2.98)	-0.28 (-1.86 to 2.7)	0.64

Note: 1. The Wilcoxon Rank Sum Test was applied to *A. actinomycetemcomitans, P. gingivalis, T. forsythia, Treponema* spp. and staphylococcal spp. (Oral streptococci spp. estimated bacterial cell density appear to likely be statistically significantly different between periodontal and peri-implant sites, however, differences were not investigated using the Wilcoxon Rank Sum test owing to concerns with multiple hypothesis testing as outlined in Section 7.5).

Table 7.3 The relative abundance[1] of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Treponema* spp., staphylococcal spp. at peri-implant sites at baseline and nine months following treatment, as determined by checkerboard DNA-DNA hybridisation analysis (n=55).

Species groupings	% Relative abundance Median (inter-quartile range)		p-value[1]
	Baseline	Nine months	
Aggregatibacter actinomycetemcomitans	3.5 (1.56-9.96)	1.66 (0-6.08)	0.004
Porphymonas gingivalis	1.25 (0-5.08)	0.46 (0-1.26)	0.009
Tanerella forsythia	3.42 (1.6-8.38)	2.88 (0.76-10.45)	0.81
Treponema spp.	4.79 (2.88-11.72)	7.27 (2.35-22.5)	0.41
Staphylococcal spp.	3.6 (2.4-6.58)	3.19 (1.46-7.48)	0.47

[1] The Wilcoxon Rank Sum test was applied.

Table 7.4 The relative abundance[1] of *A. actinomycetemcomitans, P. gingivalis, T. forsythia, Treponema* spp., staphylococcal spp. at periodontal sites at baseline and nine months following treatment as determined by checkerboard DNA-DNA hybridisation analysis (n=25).

Species groupings	% Relative abundance Median (inter-quartile range)		p-value[1]
	Baseline	Nine months	
A. actinomycetemcomitans	3.9 (2.19-.6.59)	2 (0-6.56)	0.26
P. gingivalis	0.56 (0-2.1	0.35 (0-0.77)	0.55
T. forsythia	1.56 (0-6.54)	0.75 (0-1.42)	0.44
Treponema spp.	4.23 (1.06-8.8)	1.99 (1.85-8.07)	0.83
Staphylococcal spp.	3.26 2.47-5.25)	3.2 (2-7.8)	0.94

[1] The Wilcoxon Rank Sum test was applied.

Table 7.5 Frequency of peri-implant sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species groupings following treatment (n=55).

Species groupings	No. of peri-implant sites (%)		
	≥1% increase in relative abundance	not ≥1% change in relative abundance	≥1% decrease in relative abundance
Actinomyces spp.	17 (30.9)	14 (25.5)	24 (43.6)
Veillonella spp.	23 (41.8)	11 (20)	21 (38.2)
Selenomonas spp.	7 (12.7)	33 (60)	15 (27.3)
Oral streptococci	26 (47.2)	7 (12.7)	22 (0.4)
Aggregatibacter actinomycetemcomitans	9 (16.3)	27 (49)	19 (34.5)
Campylobacter spp.	19 (34.5)	15 (27.2)	21 (38.1)
Capnocytophaga spp.	26 (47.2)	10 (18.2)	19 (34.5)
Eikenella corrodens	26 (47.2)	3 (5.5)	26 (47.2)
Fusobacterium spp.	22 (40)	11 (20)	22 (40)
Lactobacterial spp.	12 (21.8)	12 (21.8)	31 (56.4)
Leptotrichia spp.	10 (18.1)	33 (60)	12 (21.8)
Neisseria spp.	16 (29)	19 (34.5)	20 (36.4)
Parvimoas micra	18 (32.7)	11 (20)	26 (47.3)
Prevotella spp.	11 (20)	25 (45.5)	19 (34.6)
Porphymonas gingivalis	7 (12.7)	24 (43.6)	24 (43.6)
Tanerella forsythia	17 (30.9)	10 (18.2)	28 (50.9)
Treponema spp.	22 (40)	7 (12.7)	26 (47.3)
Staphylococcal spp.	13 (23.6)	19 (34.5)	23 (41.8)

Table 7.6 Frequency of periodontal sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=25).

Species groupings	No. of peri-implant sites (%)		
	≥1%increase in relative abundance	No change in relative abundance	≥1% decrease in relative abundance
Actinomyces spp.	9 (36)	9 (36)	7 (28)
Veillonella spp.	12 (48)	7 (28)	6 (24)
Selenomonas spp.	4 (16)	4 (16)	17 (68)
Oral streptococci	13 (52)	8 (32)	4 (16)
Aggregatibacter actinomycetemcomitans	5 (20)	4 (16)	16 (64)
Campylobacter spp.	4 (16)	9 (36)	12 (48)
Capnocytophaga spp.	7 (28)	14 (56)	4 (16)
Eikenella corrodens	8 (32)	5 (20)	12 (48)
Fusobacterium spp.	6 (24)	14 (56)	5 (20)
Lactobacterial spp.	12 (48)	9 (36)	4 (16)
Leptotrichia spp.	4 (16)	12 (48)	9 (36)
Neisseria spp.	6 (24)	1 (4)	18 (72)
Parvimoas micra	11 (44)	11 (44)	3 (12)
Prevotella spp.	4 (16)	9 (36)	12 (48)
Porphymonas gingivalis	2 (8)	10 (40)	13 (52)
Tanerella Forsythia	5 (20)	9 (36)	11 (44)
Treponema spp.	7 (28)	10 (40)	8 (32)
Staphylococcal spp.	8 (32)	10 (40)	7 (28)

[1]Detected by checkerboard analysis.

Table 7.5 Frequency of peri-implant sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance of DNA isolated for each of the 18 species groupings following treatment (n=55).

Species groupings	No. of peri-implant sites (%)		
	≥1% increase in relative abundance	not ≥1% change in relative abundance	≥1% decrease in relative abundance
Actinomyces spp.	17 (30.9)	14 (25.5)	24 (43.6)
Veillonella spp.	23 (41.8)	11 (20)	21 (38.2)
Selenomonas spp.	7 (12.7)	33 (60)	15 (27.3)
Oral streptococci	26 (47.2)	7 (12.7)	22 (0.4)
Aggregatibacter actinomycetemcomitans	9 (16.3)	27 (49)	19 (34.5)
Campylobacter spp.	19 (34.5)	15 (27.2)	21 (38.1)
Capnocytophaga spp.	26 (47.2)	10 (18.2)	19 (34.5)
Eikenella corrodens	26 (47.2)	3 (5.5)	26 (47.2)
Fusobacterium spp.	22 (40)	11 (20)	22 (40)
Lactobacterial spp.	12 (21.8)	12 (21.8)	31 (56.4)
Leptotrichia spp.	10 (18.1)	33 (60)	12 (21.8)
Neisseria spp.	16 (29)	19 (34.5)	20 (36.4)
Parvimoas micra	18 (32.7)	11 (20)	26 (47.3)
Prevotella spp.	11 (20)	25 (45.5)	19 (34.6)
Porphymonas gingivalis	7 (12.7)	24 (43.6)	24 (43.6)
Tanerella forsythia	17 (30.9)	10 (18.2)	28 (50.9)
Treponema spp.	22 (40)	7 (12.7)	26 (47.3)
Staphylococcal spp.	13 (23.6)	19 (34.5)	23 (41.8)

Table 7.6 Frequency of periodontal sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=25).

Species groupings	No. of peri-implant sites (%)		
	≥1%increase in relative abundance	No change in relative abundance	≥1% decrease in relative abundance
Actinomyces spp.	9 (36)	9 (36)	7 (28)
Veillonella spp.	12 (48)	7 (28)	6 (24)
Selenomonas spp.	4 (16)	4 (16)	17 (68)
Oral streptococci	13 (52)	8 (32)	4 (16)
Aggregatibacter actinomycetemcomitans	5 (20)	4 (16)	16 (64)
Campylobacter spp.	4 (16)	9 (36)	12 (48)
Capnocytophaga spp.	7 (28)	14 (56)	4 (16)
Eikenella corrodens	8 (32)	5 (20)	12 (48)
Fusobacterium spp.	6 (24)	14 (56)	5 (20)
Lactobacterial spp.	12 (48)	9 (36)	4 (16)
Leptotrichia spp.	4 (16)	12 (48)	9 (36)
Neisseria spp.	6 (24)	1 (4)	18 (72)
Parvimoas micra	11 (44)	11 (44)	3 (12)
Prevotella spp.	4 (16)	9 (36)	12 (48)
Porphymonas gingivalis	2 (8)	10 (40)	13 (52)
Tanerella Forsythia	5 (20)	9 (36)	11 (44)
Treponema spp.	7 (28)	10 (40)	8 (32)
Staphylococcal spp.	8 (32)	10 (40)	7 (28)

[1]Detected by checkerboard analysis.

Table 7.7 Frequency of peri-implant mucositis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=40).

Species groupings	No. of peri-implant sites (%)		
	≥1%increase in relative abundance	not ≥1% change in relative abundance	≥1% decrease in relative abundance
Actinomyces spp.	12 (30)	12 (30)	16 (40)
Veillonella spp.	15 (37.5)	9 (22.5)	16 (40)
Selenomonas spp.	4 (10)	26 (65)	10 (25)
Oral streptococci	18 (45)	6 (15)	16 (40)
Aggregatibacter actinomycetemcomitans	6 (15)	20 (50)	14 (35)
Campylobacter spp.	12 (30)	11 (27.5)	17 (42.5)
Capnocytophaga spp.	20 (50)	8 (20)	12 (30)
Eikenella corrodens	23 (57.5)	0 (0)	17 (42.5)
Fusobacterium spp.	17 (42.5)	8 (20)	15 (37.5)
Lactobacterial spp.	10 (25)	9 (22.5)	21 (52.5)
Leptotrichia spp.	8 (20)	21 (52.5)	11 (27.5)
Neisseria spp.	13 (30.2)	16 (37.2)	14 (32.6)
Parvimoas micra	12 (30)	7 (17.5)	21 (52.5)
Prevotella spp.	5 (12.5)	19 (47.5)	16 (40)
Porphymonas gingivalis	5 (12.5)	15 (37.5)	20 (50)
Tanerella forsythia	13 (32.5)	7 (17.5)	20 (50)
Treponema spp.	16 (40)	5 (12.5)	19 (47.5)
Staphylococcal spp.	7 (17.5)	17 (42.5)	16 (40)

[1]Detected by checkerboard analysis.

Table 7.8 Frequency of peri-implantitis sites demonstrating a ≥1% increase, no change or a ≥1% decrease in the relative abundance[1] of DNA isolated for each of the 18 species following treatment (n=15).

Species groupings	No. of peri-implant sites (%)		
	≥1% increase in relative abundance	not ≥1% change in relative abundance	≥1% decrease in relative abundance
Actinomyces spp.	5 (33.3)	2 (13.3)	8 (53.3)
Veillonella spp.	8 (53.3)	2 (13.3)	5 (33.3)
Selenomonas spp.	3 (20)	7 (46.7)	5 (33.3)
Oral streptococci	8 (53.3)	1 (6.7)	6 (40)
Aggregatibacter actinomycetemcomitans	3 (20)	7 (46.7)	5 (33.3)
Campylobacter spp.	7 (46.7)	4 (26.7)	4 (26.7)
Capnocytophaga spp.	6 (40)	2 (13.3)	7 (46.7)
Eikenella corrodens	3 (20)	3 (20)	9 (60)
Fusobacterium spp.	5 (33.3)	3 (20)	7 (46.7)
Lactobacterial spp.	2 (13.3)	3 (20)	10 (66.6)
Leptotrichia spp.	2 (13.3)	12 (80)	1 (6.7)
Neisseria spp.	3 (20)	6 (40)	6 (40)
Parvimoas micra	6 (40)	4 (26.7)	5 (33.3)
Prevotella spp.	6 (40)	6 (40)	3 (20)
Porphymonas gingivalis	2 (13.3)	9 (60)	4 (26.7)
Tanerella forsythia	4 (26.7)	3 (20)	8 (53.3)
Treponema spp.	6 (40)	2 (13.3)	7 (46.7)
Staphylococcal spp.	6 (40)	2 (13.3)	7 (46.7)

[1]Detected by checkerboard analysis.

Table 7.9 Changes in the percentage relative abundance for each of the 18 microbial species investigated[1] between baseline and nine months following treatment for sites demonstrating an increase, no change or decrease in probing depth.

Species groupings	% Change in relative abundance (median inter-quartile range)		
	Increase in probing depth	No change	Decrease in probing depth
Actinomyces spp.	3.37 (0.90 to 5.9)	2.74 (0.25 to 7.79)	2.3 (-2.86 to 8.07)
Aggregatibacter actinomycetemcomitans	0.44 (0 to 3.24)	0 (0 to 0.68)	0 (0 to 1.94)
Campylobacter spp.	-0.58 (-2.55 to 0.85)	0 (-1.08 to 1.05)	0.04 (-0.85 to 1.59)
Capnocytophaga spp.	-2.59 (-5.57 to -1.0)	-0.29 (-2.65 to 0.57)	-0.37 (-4.7 to 2.27)
Eikenella corrodens	-1.8(-3.4 to-1.56)	0.79 (0 to 1.87)	0.64 (-0.12 to 2.15)
Fusobacterium spp.	-6 (-9.37 to-1.17)	-1.94 (-5.44 to 3.17)	-0.08 (-2.24 to 8.2)
Lactobacterial spp.	2.65 (0.3 to 5.05)	0.59 (-6.67 to 3.37)	1.92 (-0.16 to 3.69)
Leptotrichia spp.	0.05 (-0.7 to 0.68)	0 (-0.71 to 0.49)	0.15 (-0.57 to 0.54)
Neisseria spp.	-1.23 (-5.73 to -0.09)	0.29 (0 to 1.09)	1.36 (-0.04 to 3.8)
Parvimonas micra	-0.84 (-3.47 to 0.72)	-0.29 (-1.27 to 3.35)	1.07 (-3.23 to 5.67)
Porphyromonas gingivalis	3.77 (-1.09 to 9.32)	0 (-0.11 to 2.21)	0.37 (error to 1.94)
Prevotella spp.	-0.89 (-1.83 to 0.56)	0.4 (0 to 1.55)	0.49 (-0.37 to 1.63)
Selenomonas spp.	-0.51 (-1.4 to 0.5)	0.42 (0 to 1.08)	0.26(-0.02 to 1.17)
Oral streptococci	7.37 (0.51 to 12.19)	-1.17 (-6.15 to 3.07)	-1.1 (-5.69 to 3.38)
Tanerella forsythia	1.33 (-3.39 to 11.26)	0 (-1.62 to 2.21)	1.69 (-0.72 to 4.98)
Treponema spp.	-0.81 (-5.11 to 15.11)	-3.43 (-8.62 to 0.43)	1.94 (-4.58 to 5.68)
Veillonella spp.	2.58 (-2.74 to 9.44)		0.05 (-3.04 to 6.76)
Staphylococcal spp.	1.31 (-0.31 to 3.57)	0.09 (-2.39 to 2.8)	0.23 (-2.89 to 2.81)

[1]Changes detected using checkerboard analysis.

groupings at peri-implant sites at nine months following treatment is shown in Table 7.10. The major changes between baseline and nine months are outlined below.

The change in the median relative abundance levels of *P. gingivalis* was observed to be 3.77% (-1.09% to 9.32%) at peri-implant sites that demonstrated increasing probing depths, 0% (-0.11% to 2.21%) at sites that demonstrated no change in probing depths, and 0.37% (0% to 1.94%) at sites that showed improvements in probing depths (Table 7.9). At baseline, the median relative abundance of *P. gingivalis* at peri-implant mucositis sites was 0.91% (0.41% to 4.98%) and 1.24% (0% to 5.98%) at peri-implantitis sites. Differences were not statistically significant when the Wilcoxon Rank Sum test was applied (p=0.86). Therefore, nine months following the completion of non-surgical periodontal treatment, the median relative abundance of *P. gingivalis* was observed to be 1.26% (0.54% to 1.38%), 0% (0% to 0.44%) and 0.45% (0% to 1.28%) at sites which demonstrated an increase, no change or a decrease in probing depths, respectively. The overall change in relative abundance for peri-implant sites, was not observed to change significantly from baseline to nine-months post-treatment (*P. gingivalis* from 0.56% (0% to 2%) to 0.35% (0% to 0.77%) (p=0.55). However for those sites that demonstrated increasing probing pocket depths, *P. gingivalis* was observed to increase ten fold compared to the change at peri-implant sites that demonstrated increasing probing pocket depths (Table 7.9).

Capnocytophaga spp. were observed to decrease by -2.59% (-5.57% to -1.0%), -0.29% (-2.65% to 0.57%) and -0.37% (-4.7% to 2.27%) at peri-implant sites that demonstrated an increase in probing pocket depths, no change in probing pocket depths or a decrease in probing pocket depths, respectively, over the observational interval (Table 7.9). The median percentage relative abundance of *Capnocytophaga* spp. were observed to be 3.58% (0.43% to 2.54%) at peri-implant mucositis sites and 4.0% (1.85% to 5.63%) at peri-implantitis sites. The median percentage relative abundance of *Capnocytophaga* spp. at peri-implant sites that demonstrated deepening probing depths nine months following completion of non-surgical treatment was observed to be 7.83% (6.95% to 9.54%), whilst for those sites which did not demonstrate a change of $\geq$1 mm in probing pocket depth, was observed to be 2.24% (0% to 5.93%) and 3.09% (0% to 6.99%) for those sites which demonstrated decreasing probing depths (Table 7.10). Thus, very marked differences were observed in the percentage relative abundance of *Capnocytophaga* spp. for sites with differing clinical outcomes at nine months post-treatment.

Oral streptococci are species that demonstrated binding sites for bacterial aggregation to other bacteria and the soft tissues.Therefore oral streptococci play a role in both the peri-implant biofilm in health and disease. Following treatment, the percentage relative abundance of oral streptococcal spp. was observed to increase by 7.37% (0.51% to 12.19%) at sites which demonstrated an increase in probing depth and decrease by -1.17% (-6.15% to 3.07%) at sites which demonstrated no change in probing depths and by -1.1% (-5.69% to 3.38%) at sites which demonstrated a decrease in probing depths (Table 7.9). At baseline, the median percentage relative abundance of oral streptococcal spp. was observed to be 6.6% (4.27% to 9.26%) at peri-implant mucositis sites and 7.7% (3.72% to 11.51%) at peri-implantitis sites.

Table 7.10 The percentage relative abundance at peri-implant sites at nine months following treatment for each of the 18 microbial species investigated[1] for sites demonstrating an increase, no change or decrease in probing depth.

Species groupings	% Relative abundance (median inter-quartile range)		
	Increase in probing depth	No change	Decrease in probing depth
Actinomyces spp.	5.01(0.22-10.42)	3.54(0-6.12)	3.29(0-8.6)
Aggregatibacter actinomycetemcomitans	0 (0-0)	0(0-3.04)	0(0-2.01)
Campylobacter spp.	2.31(2.16-3.72)	0.99(0-1.71)	1.16(0-2.04)
Capnocytophaga spp.	7.83(6.95-9.54)	2.24(0-5.93)	3.09(0-6.99)
Eikenella corrodens	3.82(3.4-4.36)	1.21(0-2.32)	1.5(0-3.41)
Fusobacterium spp.	12.31(10.14-15.96)	7.77(7.39-9.24)	7.1(1.54-12.38)
Lactobacterial spp.	3.52(1.58-4.58)	4.95(1.92-9.14)	2.91(0-4.94)
Leptotrichia spp.	1.39(1.18-1.71)	0.75(0.21-1.11)	0.99(0.29-1.74)
Neisseria spp.	3.73(0.82-6.25)	0(0-2.62)	0(0-3.37)
Parvimonas micra	8.55(5.69-9.94)	6.12(4.89-11.9)	4.91(1.99-8.75)
Porphyromonas gingivalis	1.26(0.54-1.38)	0(0-0.44)	0.45(0-1.28)
Prevotella spp.	2.56(1.36-3.63)	0.99(0-1.78)	1.43(0-3.16)
Selenomonas spp.	1.34(0.77-1.95)	0(0-0.95)	7.67(0-1.66)
Oral streptococci	8.37(4.92-12.04)	7.1(3.65-17.22)	6.26(2.31-9.62)
Tanerella forsythia	3.38(0.83-6.56)	1.02(0-10.52)	2.39(0.6-10.3)
Treponema spp.	7.46(4.14-7.54)	3.05(0.59-26.53)	4.63(1.87-15.9)
Veillonella spp.	6.75(1.49-9.5)	7.14(2.6-13.06)	5.92(2.29-9.09)
Staphylococcal spp.	3.05(2.87-4.73)	4.77(1.5-9.04)	2.94(0.84-5.97)

[1]Changes detected using checkerboard analysis.

7.5 Discussion

In the present investigation, a decrease in the sum of the bacterial DNA isolated for each of the 18 investigated species groupings from 71% of peri-implant sites was noted, nine months following treatment (i.e. debridement of the periodontal and peri-implant sites and the instigation of improved oral hygiene practices), indicating a reduction in bacterial density at peri-implant sites. This is in line with the findings of other investigators (Mombelli & Lang, 1992; Strooker *et al.*, 1998; Mombelli *et al.*, 2001; Mombelli *et al.*, 2005; Persson *et al.*, 2006 Renvert *et al.*, 2009), who similarly noted a reduction in bacterial density, following non-surgical treatment of peri-implant infections, with or without the use of adjunctive antimicrobial agents (for methodology employed by these investigators, see Table 7.1).

As the composition of the microflora of the peri-implant or periodontal biofilm has been observed to influence the peri-implant or periodontal health status (Mombelli *et al.*, 2002), the proportions of different species within the peri-implant and periodontal biofilm were of interest. The relative abundance measure was selected in an effort to overcome the problems presented by differing plaque/crevicular fluid volumes being recovered on different occasions of microbial sampling. A decrease was noted in the percentage relative abundance of bacterial species associated with periodontitis and peri-implant infections, in conjunction with an increase in more beneficial bacterial species, for both periodontal and peri-implant sites, following treatment. For example, highly significant reductions were observed in the percentage relative abundance of both *A. actinomycetemcomitans* and *P. gingivalis* ($p<0.01$) at peri-implant sites, following treatment (see Table 7.3). Almost 50% of peri-implant sites showed an increase of $\geq 1\%$ in the relative abundance of oral streptococci (26/55), *Capnocytophaga* spp. (26/55) and *E. corrodens* (26/55) following treatment (Figure 7.1 and Table 7.5). In the present investigation, as improvements in clinical parameters were observed, many of the changes observed in the relative abundance of the investigated bacterial species groupings at this >1% threshold, appear to fall in line with the expected changes in the peri-implant and periodontal microflora, confirming the appropriateness of this >1% threshold for change in relative abundance for future analysis.

The decrease in periodontopathogens, e.g. *P. gingivalis*, was more evident at peri-implant mucositis sites than at peri-implantitis sites (*P. gingivalis* relative abundance was observed to decrease by $\geq 1\%$ at 50% (20/40) peri-implant mucositis sites, but only at 26.7% (4/16) of peri-implantitis sites. Fewer peri-implant mucositis sites demonstrated an

increase of ≥1% in the relative abundance of Staphylococcal spp., however, compared to peri-implantitis sites, fewer peri-implant mucositis sites demonstrated an increase of ≥1% in the relative abundance of Staphylococcal spp., compared to peri-implantitis sites, being 40% (6/15) and 15.5% (7/40), respectively. These findings indicate that the level of severity of peri-implant infections may influences the likelihood of achieving successful outcomes for the treatment of peri-implant infections. This is in agreement with the previous literature in the treatment of peri-implant mucositis, as it has been observed that significant changes in clinical and microbiological parameters can be achieved with non-surgical treatment of these relatively shallow pockets (Ciancio *et al.*, 1995; Felo *et al.*, 1997; Strooker *et al.*, 1998, Heitz-Mayfield *et al.*, 2011). For example, in the non-surgical debridement of peri-implant mucositis lesions, a significant change in the total bacterial DNA concentration isolated from peri-implant sites after treatment was noted, if the baseline total DNA was equivalent to $>1.7 \times 10^5$ bacterial cells (employing checkerboard DNA-DNA hybridisation) (Heitz-Mayfield *et al.*, 2011). However, for peri-implantitis, the improvements in peri-implant microbial parameters following non-surgical treatment, was observed to be less than that of peri-implant mucositis lesions. This appears to relate largely to the difficulties of removing the biofilm from the dental implant surface, as it has previously been demonstrated that with surgical access, efficient debridement of dental implant surfaces can been predictably achieved (Maximo *et al.*, 2009), with resultant improvements in peri-implant microbial parameters, such as significant reductions in *P. gingivalis* and *T. forsythia* ($p<0.05$) Therefore, the question arises as to whether it is possible to achieve changes in the peri-implantitis microflora through non-surgical debridement alone? Renvert *et al.* (2009) noted significant reductions in the sum of the bacterial DNA identified at peri-implant sites immediately following non-surgical debridement of peri-implantitis lesions, using either hand instrumentation or ultrasonic instrumentation ($p<0.05$). However, one week following debridement, significant reductions were only noted in the sum of the bacterial DNA detected for the group of patients where hand instrumentation was undertaken ($p<0.05$). In the present study, changes in the peri-implant microflora were observed nine months following the non-surgical treatment of the peri-implant infections, in line with significant observed clinical improvements (see Chapter 5, Section 5.4). It is hypothesised that the repeated non-surgical debridement of periodontal and peri-implant sites, and the repeated oral hygiene instruction at each of the three monthly recall appointments, may have contributed to the sustained improvements in peri-implant microbial and clinical parameters observed. For

peri-implant sites, highly significant decreases were noted in the relative abundance of both *P. gingivalis* and *A. actinomycetemcomitans* following treatment ($p<0.01$). Strooker *et al.* (1998), Persson *et al.* (2006) and Persson *et al.* (2010) similarly observed significant reductions in *A. actinomycetemcomitans* and *P. gingivalis* following the non-surgical treatment of peri-implant infections, albeit with the adjunctive use of anti-microbial agents.

For periodontal sites, the observed decrease in periodontopathogens following treatment was not statistically significant ($p>0.05$). This lack of statistical significance may in part be explained by the severity of periodontal disease at presentation being generally mild. Therefore, as the baseline periodontal clinical parameters and the composition of the periodontal microflora at baseline were closer to health, there was less of a margin for improvement. Any improvements that took place were not observed to be statistically significant ($p>0.05$).

7.6 Conclusions

1) Following non-surgical debridement and improved oral hygiene measures, implants affected by peri-implant mucositis or peri-implantitis, demonstrated a decrease in many bacterial species grouping associated with periodontal and peri-implant infections (such as *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *Treponema* spp.), and an increase in species associated with periodontal and peri-implant health (such as oral streptococci, *V. parvula, Capnocytophaga* spp. and *E. corrodens*).

2) Improvements in clinical and microbiological parameters were less for implants affected by peri-implantitis compared to those affected by peri-implant mucositis.

Chapter 8

Enrichment of multilocus sequence typing clade 1 with oral *Candida albicans* isolates in patients with untreated periodontitis

8.1 Introduction

The periodontium is composed of the gingivae, periodontal ligament, root cementum and alveolar bone. In normal healthy gingivae, the free gingival margin and the tooth surface are in close proximity to each other, leaving very little space for microbial colonisation (Lindhe *et al.*, 2003). Periodontitis is an infection of the oral gingival tissue that is caused by a combination of microorganisms commonly found in dental plaque, such as streptococci, staphylococci, fusobacteria, *Porphyromonas* species, *Campylobacter* species, actinobacteria, and many others (Sardi *et al.*, 2011; Waltimo *et al.*, 2003). As periodontitis manifests itself, the gingival margin becomes enlarged, causing the gingival tissue to detach from the tooth resulting in the formation of periodontal pockets. This coincides with the spread of infection and an associated inflammatory response, leading to irreversible destruction of the periodontal ligament and alveolar bone. As the disease progresses, the depth of these periodontal pockets (probing pocket depth) increases, and bleeding and/or suppuration on probing of periodontal tissues also occur (Kinane *et al.*, 2003). A build up of dental plaque, development of dental caries or denture wearing are risk factors for the development of periodontitis, as are particular systemic conditions that affect host immune or inflammatory responses (McKaig *et al.*, 2000; Ryder *et al.*, 2000; Velegraki *et al.*, 1999). Treatment of periodontitis consists of mechanical cleaning of teeth and debridement of associated diseased tissue, followed by improved dental hygiene.

Candida spp. are commensal yeasts and opportunistic pathogens that reside on mucosal surfaces and can cause oropharyngeal infection, albeit usually in immunodeficient individuals, those with severe underlying diseases and upper denture wearers. In healthy oral carriers, *Candida* spp. typically reside on the buccal mucosa, tongue, palate and in the saliva. *Candida* spp. have been isolated from 40-60% of healthy mouths (Samaranayake *et al.*, 2009). It has been reported that they are rarely found in subgingival sites in patients with good oral health (Urzúa *et al.*, 2008) but may occur in the sub-gingival plaque of adults with severe periodontitis (Slots *et al.*, 1988). *Candida* spp. have frequently been isolated from periodontal pockets, however, their role, if any, in the aetiology of periodontitis remains to be elucidated (Reynaud *et al.*, 2001; Egan *et al.*, 2002; Waltimo *et al.*, 2003; Jarvensivu *et al.*, 2004;; Urzúa *et al.*, 2008; Sardi *et al.*, 2011). Several previous studies investigated the prevalence and possible role of *Candida* spp. in periodontitis, all of which identified *C. albicans* as the *Candida* spp. most frequently isolated from periodontal pockets (Cuesta *et al.*, 2010; Jewtuchowicz *et al.*,

2007; Melton *et al.*, 2010; Miranda *et al.*, 2009; Sardi *et al.*, 2011; Urzúa *et al.*, 2008). Pjetersson *et al.* (1987) described a case of a gingival abscess from which *Candida spp.* were cultured. Reynaud *et al.* (2001) reported the prevalence of yeasts in the examined periodontal pockets to be 15.6%. Song *et al.* (2005) suggested the prevalence of yeast species in periodontal pockets to range from 15 to 21%. Hannula *et al.* (2000) observed from the literature that yeasts are members of the subgingival microbial communities of approximately 20% of periodontitis patients. Listgarten *et al.* (1999) observed a prevalence of 14% for *Candida* spp. in samples of 993 sub-gingival microbial samples sent to a diagnostics microbiology laboratory. Samples included 196 samples that could be identified to be from sites affected by refractory periodontitis. Gonzalez *et al.* (1987), utilising scanning electron microscopy techniques, observed yeasts to be present in the gingival tissues of 26 out of 60 biopsies of periodontal tissue from 12 juvenile periodontitis patients (Gonzalez *et al.*, 1987).

Several of these studies also detected the presence of other *Candida* spp. including *Candida glabrata, Candida dubliniensis, Candida tropicalis, Candida guilliermondii* and *Candida parapsilosis* in the periodontal pockets of patients with periodontitis, however, it should be noted that two of these studies involved patients who also had insulin-dependent diabetes, who were prone to *Candida* colonisation and infection (Melton *et al.*, 2010; Sardi *et al.*, 2011; Willis *et al.*, 2000). Urzúa *et al.* (2008) reported that patients with chronic periodontitis had a significantly higher level of colonisation with *Candida* at subgingival sites than periodontally healthy individuals. However, it is currently difficult to attribute a definitive role for *Candida* spp. in periodontitis, as studies reporting the relative abundance of *Candida* in periodontal pockets are mostly lacking. Furthermore, questions remain with regard to the optimal sub-gingival sampling technique. Clinical investigations comparing paper-point and curette sampling have noted significant differences between samples gained from the same site employing these sampling techniques (Gerber *et al.*, 2006; Renvert *et al.*, 1992). To date, there have been no investigations combining both paper-point and curette sampling techniques in the investigation of the isolation of yeast species from periodontal sites.

Candida spp. possesses virulence factors that may further implicate them in the pathogenesis of periodontal disease. Proteinases of *C. albicans* can focally induce the destruction of host protein networks. *Candida* spp. are tolerant to most host innate immune systems. *Candida* spp. may have a role in the infrastructure of periodontal microbial plaque and its adherence to the periodontal tissues. *Candida* spp. are typically

found on the outer layer of the plaque biofilm and thus may have a role in the evasion of microorganisms within the plaque biofilm from host immune systems (Jarvensivu *et al.*, 2004).

Leonhardt *et al.* (1999) observed in a controlled study of the microbial species present around failing implants, that *C. albicans* was detected in 10% of the diseased dentate patients' peri-implant pockets, and none of the diseased edentulous patients' peri-implant pockets, healthy dentate or edentulous patients' peri-implant pockets. The authors questioned the role of yeasts in periodontitis/ peri-implantitis, stating: *"Due to the preference of yeasts for an aerobic environment, their etiological role in periodontitis could be questioned. Similarly, the significance of yeasts in peri-implant lesions could be questioned. Also, in the present study, yeasts were only sporadically present in the peri-implantitis lesions and preferentially in the partially edentulous cases."* Reynaud *et al.* (2001) commented that: *"It seems reasonable to consider that yeasts, especially Candida, are frequently occurring microorganisms in the periodontal microbial flora. The presence of yeasts in periodontal pockets does not seem to be especially associated with refractory periodontitis."* Leonhardt *et al.* (1999) suggested that the presence of yeasts in periodontal pockets may be transient and should be evaluated in a longitudinal study. Also, Leonhardt *et al.* (1999) highlighted a need to investigate the association between periodontal yeasts and other periodontal pathogens.

An increased incidence of *Candida* spp. has been identified in periodontal sites of immunocompromised patients compared with healthy controls. Chomicz *et al.* (2001) identified *C. albicans* strains from swabs from "different sites of periodontium, dental plaques and dental pockets" at a prevalence of 53.2% in 15 insulin treated diabetic patients, compared to 13.3% in 15 healthy controls. Yuan *et al.* (2001) suggested than *C. albicans* may play an important role in periodontitis affecting both non-insulin dependent diabetes mellitus patients and those without the condition. *Candida* spp. have been observed to be present in the periodontal pockets of both smokers and non-smokers, exhibiting signs of early onset periodontitis. However, deep sites in smokers demonstrated a statistically significantly greater prevalence of *Candida* spp. than deep sites in non-smokers (Kamma *et al.*, 1999). Patients taking oral contraceptives have been observed to have a statistically significantly elevated carriage of *Candida* spp. within their periodontal pockets, than non-oral contraceptive users (Brusca *et al.*, 2010). It has been suggested that gingivitis and periodontitis affecting patients with HIV do not differ from gingivitis and periodontitis affecting immunocompetent patients (Reichart, 2003).

Considerable diversity of yeast species have been isolated from the oral cavity of periodontitis patients. Ito *et al.* (2004) identified 39 *C. albicans* isolates, nine *C. tropicalis* and two *C. glabrata* species from periodontally healthy individuals, whilst 30 *C. albicans*, three *C. tropicalis* and two *C. glabrata* species were isolated from periodontitis patients. It is unclear as to the sample method employed or the prevalence of these species. Kutsyk & Pavliuk (2003) calculated that of 68 *Candida* strains isolated from the periodontal pockets of patients with generalised periodontitis, 72.1% (+/- 5.43%) were *C. albicans*.

Candida spp. did not constitute greater than 10% of the total viable count of 973 subgingival samples from 535 periodontal patients (Dahlen & Wikstrom, 1995). *Candida* spp. have been observed to colonise polytetrafluorethelene (PTFE) membranes employed in regenerative periodontal surgical procedures (Tempro & Nalbandian, 1993; Grevstad & Leknes, 1993). *Candida* spp. have been observed to make up a small proportion of the microorganisms in patients with aggressive periodontitis associated with Fanconi's anaemia constituting (0.3% of the total microorganisms). Slots *et al.* (1991) concluded that "*C. albicans in human periodontal pockets appears to follow a selectivity pattern similar to that of other oral surfaces.*"

A high prevalence of yeasts within root filled teeth with chronic apical periodontitis has been observed (Peciuliene *et al.*, 2001). Yeast species, enteric bacteria and enterococci are also isolated from the intra-coronal access of root canal treated teeth at a prevalence of up to 22% (Ferrari *et al.*, 2005). Yeasts have been isolated from infected root canals of teeth with apical periodontitis at a prevalence of approximately 15 to 20%, either in pure cultures or in association with bacteria (Waltimo *et al.*, 2003). It has been observed that there is a 13.8 times greater chance of detecting *Candida* spp. within the root canals of patients where yeast isolates are detected within their saliva (Egan *et al.*, 2002).

Several previous studies investigated the genetic relatedness of *C. albicans* isolates recovered from the periodontal pockets, gingival sulci and oral mucosae of patients with periodontitis, using molecular typing techniques such as random amplified polymorphic DNA (RAPD) fingerprinting, electrophoretic karyotyping and ABC genotyping, the latter based on the presence or absence of an intron in 25S rDNA (Barros *et al.*, 2008; Pizzo *et al.*, 2002; Sardi *et al.*, 2011; Song *et al.*, 2005). Pizzo *et al.* (2002) used electrophoretic karyotyping to show that some *C. albicans* genotypes are unique to subgingival isolates, suggesting some adaptation to this environment. In contrast, Barros *et al.* (2008) identified a genetically homogenous population of *C. albicans* strains in the

oral cavities of patients with periodontitis using RAPD. However, RAPD can be affected by a multitude of variables, its reproducibility is poor and its resolving power is significantly lower than targeted, species-specific DNA comparison methods such as multilocus sequence typing (MLST) (Tyler *et al.*, 1997). Another study revealed that 66/128 (51.6%) of subgingival *C. albicans* isolates recovered from a group of 11 diabetic patients with periodontitis, belonged to ABC genotype B and the remaining were ABC genotype A. However, it is important to note that diabetics are prone to oral candidiasis. Furthermore, the discriminatory power of ABC genotyping is poor as only three types can be discriminated using this technique (Sardi *et al.*, 2011).

In the past decade, advances in DNA sequencing technologies have enabled the informative and detailed population structure analyses of *Candida* spp. to be undertaken, particularly following the establishment of species-specific MLST schemes. The application of MLST to yeast species enables direct comparison of concatenated DNA sequence data and resulting sequence types (STs) from distinct groups of isolates, for example, those recovered from distinct anatomical sites, geographical locations or patient cohorts.

Previous MLST-based population biology analyses have been carried out on both *C. albicans* and *C. dubliniensis*. The population structure of *C. albicans* consists of 17 different MLST clades to which 97 % of *C. albicans* isolates can be assigned (Odds *et al.*, 2008). These isolates can also be distinguished by ABC genotyping, and the proportions of A, B or C genotypes can differ significantly between MLST clades. The population structure of *C. albicans* can also be analysed using an algorithm that is based upon related sequence types (BURST). This is based on the allelic profiles defined by MLST, and enables isolates to be grouped into clonal complexes (CCs), as well as predicting putative founding genotypes. The *C. dubliniensis*-specific MLST technique was developed by researchers at this laboratory and has shown that the population of *C. dubliniensis* is composed of three closely related clades that can be further distinguished on the basis of DNA sequence of the internal transcribed spacer (ITS) region of the rDNA operon.

The main purpose of the present study was to investigate the prevalence and relative abundance of *Candida* spp. in periodontal pockets, healthy subgingival sites and in oral rinse samples of patients with untreated periodontitis. Isolates of the two most prevalent species recovered, *C. albicans* and *C. dubliniensis*, were subject to MLST analysis. *Candida albicans* isolates recovered from periodontitis patients were compared

with oral carriage isolates recovered by oral rinse sampling from healthy individuals without periodontitis. To our knowledge, this type of analysis has not been reported for isolates recovered from periodontitis patients. This was undertaken in order to determine whether particular clonal groups were associated with periodontitis.

8.2 Materials and Methods

8.2.1 Patients included

Twenty-one patients with untreated periodontitis attending the Dublin Dental University Hospital and meeting specific inclusion criteria (see below) were included in the study (Table 8.1). Of these, 9/21 (42.9%) were male and 12/21 (57.1%) were female. During assessment, patients were provided with a patient information sheet approved by the Ethics Committee of the Faculty of Health Sciences, University of Dublin, Trinity College Dublin, Ireland. A detailed medical and dental history of each patient was taken followed by an oral examination, including a periodontal examination. A dentist experienced in treating periodontitis carried out all of the clinical examinations between November 2007 and September 2009. The percentage of periodontal pockets with depths greater than 4 mm and 6 mm was recorded, as was the presence of bleeding on probing (BOP) and suppuration on probing (SOP), all as described previously using standard procedures (Nyman *et al.*, 2003). At a follow-up appointment at least one week after their initial appointment, informed consent was obtained and microbial sampling for *Candida* spp. was carried out as described below. The average age of the patients with periodontitis was 42.9 years (range 27-61 years). In total, 4/21 patients with periodontitis were smokers and three patients wore partial dentures (Table 8.1).

Fifty healthy subjects were also included in the present study. This group consisted of 21 males and 29 females with an age range of 26-73 years with mean age of 49.7 years. These healthy subjects were attending the Accident and Emergency Department of the Dublin Dental University Hospital and were sampled by oral rinse as described below.

All molecular and population analyses of *Candida* isolates was undertaken by Dr. Brenda McManus and Professor David Coleman from the Dublin Dental University Hospital, Division of Oral Biosciences, Microbiology research Unit.

8.2.2 Inclusion and exclusion criteria

All patients with periodontitis included in the present study met the following inclusion criteria: they were 18 years of age or older; had at least one periodontal site demonstrating greater than 6 mm probing depth and bleeding on probing (BOP) and two further sites demonstrating greater than 4 mm probing depths and BOP; a minimum of four teeth per quadrant and had given informed consent. Periodontitis patients and healthy

subjects were excluded from the study if they met any of the following criteria: pregnancy or lactation; diabetes or asthma; steroid treatment during the last year; antibiotic or anti-fungal treatment in the previous two months.

8.2.3 Sampling of the oral cavity, periodontal pockets and gingival sites for *Candida* spp.

All sampling of the oral cavity, periodontal pockets and gingival sites was carried out at the Dublin Dental University Hospital. In order to obtain a quantitative estimate of the *Candida* burden in the oral cavity, patients were requested to rinse their mouths with 10 ml of sterile distilled water provided in a 50 ml plastic sample container for 30 s and then to return the washings to the same container. Rinse samples were transported to the microbiology laboratory for processing within an hour. Upon receipt, a 1 ml aliquot was transferred to a sterile 1.5 ml Eppendorf Safe-lock microfuge tube (Eppendorf) and vortexed for 1 min, after which time a 0.1 ml sample was plated in duplicate onto CHROMagar Candida medium (CHROMagar, Paris, France) and incubated at 37°C for 48 h in a static incubator (Gallenkamp, Leicester, UK).

Subgingival sites selected for sampling included one healthy subgingival site with an absence of BOP and the two deepest periodontitis sites observed during clinical examination that displayed BOP. No healthy sites were available for sampling for two patients with periodontitis (Table 8.1: patients 2 and 11). Sampling sites were isolated and supra-gingival plaque removed with cotton wool pellets. Samples were obtained using three no. 35 sterile paper-points per site (Steri-cell, Coltene Whaledent, Altstätten, Switzerland). The paper-points were then immediately introduced into a sterile 1.5 ml plastic tube (Sarstedt) containing 1 ml of yeast peptone dextrose (YEPD) broth and sealed. A curette sample was also subsequently taken of the subgingival plaque deposits from each site. A sterile disposable curette (Swede Dental AB, Örebro, Sweden) was inserted into each site, to the depth of the pocket in the case of periodontal sites, applied against the root surface, and moved coronally. Following sampling, the tip of the curette was removed and rotated vigorously in a 1 ml sterile YEPD broth in a 1.5 ml tube in order to disperse the microorganisms (Renvert *et al.*, 1992). Subgingival paper-point and curette samples were transferred to the microbiology laboratory in YEPD transport medium, stored at 4°C and processed within 24 h.

Prior to culture, subgingival paper-point and curette samples contained in YEPD were vortexed at maximum speed using a Heidolph vortex (Heidolph) for 30 s, centrifuged at 2600 x *g* for 3 min and the pellet resuspended in 1 ml YEPD by vortexing

Table 8.1 Clinical characteristics of patients with periodontitis

Pt.	Age	Sex	Partial denture wearer Y/N	Smoker Y/N	% Periodontal pockets with BOP	% Pocket depth		Healthy and Periodontitis sites sampled in patients with periodontitis			
						> 4 mm deep	> 6 mm deep	Sites	Probing depth (mm)	BOP Y/N	SOP Y/N
1	27	M	N	Y	83.3	55	27.7	HS	2	N	N
								PS 1	5	Y	N
								PS 2	5	Y	N
2[a]	40	F	Y	N	93	93.5	91	PS 1	7	Y	N
								PS 2	7	Y	N
3	47	F	N	N	13	87	20	HS	2	N	N
								PS 1	8	Y	N
								PS 2	7	Y	N
4	61	M	N	N	45.6	25.3	9.3	HS	2	N	N
								PS 1	6	Y	N
								PS 2	6	Y	N
5	36	M	N	N	69.6	35.1	5.3	HS	2	N	N
								PS 1	4	Y	N
								PS 2	6	Y	N
6	45	F	N	N	61	44	21.5	HS	2	N	N
								PS 1	10	Y	N
								PS 2	7	Y	N
7	35	M	N	N	63	50	20	HS	2	N	N
								PS 1	6	Y	N
								PS 2	7	Y	N
8	55	M	N	Y	85.8	49.9	15.4	HS	2	N	N
								PS 1	6	Y	N
								PS 2	6	Y	N
9	28	F	N	N	96.5	45.3	2.7	HS	2	N	N
								PS 1	7	Y	N
								PS 2	7	Y	N
10	37	F	N	N	76.5	39.2	5.8	HS	2	N	N
								PS 1	5	Y	N
								PS 2	5	Y	N
11[a]	45	M	Y	Y	100	77.7	21.1	PS 1	7	Y	Y
								PS 2	8	Y	N
12	49	F	N	N	79.8	60.4	25.6	HS	3	N	N
								PS 1	9	Y	N
								PS 2	5	Y	N
13	45	M	N	N	56.8	28.1	12.6	HS	2	N	N
								PS 1	10	Y	Y
								PS 2	5	Y	N
14	45	M	No	No	10.5	11.5	33.3	HS	3	N	N
								PS 1	6	Y	N
								PS 2	6	Y	N
15	56	F	No	No	39.8	15.2	7.2	HS	2	N	N
								PS 1	6	Y	N
								PS 2	10	Y	N
16	36	F	No	No	61.3	8.6	1.3	HS	2	N	N
								PS 1	4	Y	N
								PS 2	5	Y	N
17	31	F	No	No	75	70	4	HS	2	N	N
								PS 1	8	Y	N
								PS 2	7	Y	N
18	36	F	No	No	68.8	25.8	3.2	HS	2	N	N
								PS 1	5	Y	N
								PS 2	4	N	N
19	30	M	No	No	87.5	39.5	7.8	HS	2	N	N
								PS 1	4	Y	N
								PS 2	6	Y	N
20	61	F	Yes	Yes	42.5	29.6	2.7	HS	3	N	N
								PS 1	4	Y	N
								PS 2	5	Y	N
21	56	F	No	No	73.1	60.1	21.2	HS	2	N	N
								PS 1	7	Y	N
								PS 2	7	Y	N

Abbreviations: Pt, Patient; Y/N, Yes/No; BOP, Presence of bleeding on probing of the periodontal pocket; HS, Healthy Site; PS 1, Periodontal site 1; PS 2 Periodontal site 2; SOP, Presence of suppuration on probing of the periodontal pocket. [a] No healthy sites were available for sampling in this patient.

for 30 s. Following this, 0.1 ml was plated in duplicate on CHROMagar Candida agar medium and incubated at 37°C for 48 h in a static incubator. The detection limits of all of these sampling procedures were 10 cfu/ml of sample.

8.2.4 Identification of *Candida* isolates

Following incubation, CHROMagar Candida plates were examined and the relative abundance of different coloured colonies was counted and recorded. Selected isolates of each colony colour and morphology were purified by subculture for further analysis. Isolates were initially presumptively identified on the basis of colony colour and morphology; *C. albicans* isolates were distinguished from *C. dubliniensis* isolates on the basis of the darker blue-green and smaller colony size of *C. dubliniensis* in contrast to the larger, paler green colonies formed by *C. albicans* upon primary isolation on this medium (Jewtuchowicz *et al.*, 2008). Definitive identification was undertaken by determining *Candida* isolate substrate assimilation profiles using the API ID 32C yeast identification system (bioMérieux, Marcy l'Etoile, France) as described previously (Lindhe *et al.*, 2003). Confirmation of *C. albicans* and *C. dubliniensis* identification was also carried out by PCR experiments using species-specific *ACT1*-specific primers (Donnelly *et al.*, 1999). Several single colony isolates of each colour present on primary CHROMagar Candida plates were identified for each clinical sample. Following identification, isolates were stored on plastic beads in Microbank cryogenic vials (Pro-lab Diagnostics, Cheshire, UK) at -80°C.

8.2.5 Routine Candida culture

Candida isolates were routinely cultured on YEPD agar or in YEPD broth at 37°C. Liquid cultures were grown overnight at 37°C in an orbital incubator (Gallenkamp) with shaking at 200 rpm.

8.2.6 Nucleic acid isolation

Analytical grade or molecular biology-grade chemicals were purchased from Sigma-Aldrich or Fisher Scientific Ltd. (Loughborough, United Kingdom). GoTaq DNA polymerase was purchased from the Promega Corporation. Extraction of DNA from *Candida* isolates for PCRs was performed as described by McManus *et al.*, (2011).

8.2.7 ABC genotyping of *C. albicans*

Template DNA extracted from selected *C. albicans* isolates were assigned to genotypes A, B or C based on differential PCR amplification of the internal transcribed spacer region of the 25S rRNA gene as previously described (McCullough *et al.*, 1999).

8.2.8 Genotyping of *C. dubliniensis*

Template DNA was tested in separate PCR amplification experiments with each of the primer pairs G1F/G1R, G2F/G2R, G3F/G3R, and G4F/G4R to identify the ITS genotype of each *C. dubliniensis* isolate as described previously (Gee *et al.*, 2002).

8.2.9 Multilocus sequence typing (MLST)

Candida albicans isolates selected for MLST were recovered by curette, paper-point and oral rinses and were chosen where possible, to represent different periodontal pockets. These isolates were subjected to MLST analysis as previously described (McManus *et al.*, 2011). Selected *C. dubliniensis* isolates were subjected to MLST using the optimised scheme described by McManus et al. (2008). All DNA sequencing reactions were undertaken commercially by Source BioScience LifeSciences (Dublin, Republic of Ireland) using an ABI 3730*xl* DNA analyser. Sequence analysis was performed by examination of chromatogram files using the ABI prism Seqscape software, version 2.6 (Applied Biosystems, Foster City, California, USA).

Identification of genotypes, allelic profiles and STs was achieved using the consensus *C. albicans* MLST website (http://calbicans.mlst.net/) or using a local *C. dubliniensis* MLST database (McManus *et al.*, 2008, McManus *et al.*, 2009), respectively. Clade and CC allocation was carried out using previously identified reference STs (Odds *et al.*, 2007) by UPGMA dendrograms and by eBURST, respectively, as described previously (Odds *et al.*, 2007). Phylogenetic analyses were carried out using MEGA software program version 5 (Kumar *et al.*, 2004).

For comparative genetic analysis of oral *C. albicans* isolates recovered from patients with periodontitis and those recovered from healthy individuals, *C. albicans* isolates recovered from oral rinse samples of 50 healthy volunteers attending the Accident and Emergency Department at the Dublin Dental University Hospital were also investigated.

8.2.10 Statistical analyses

Fisher's exact tests or Student *t* tests were carried out on datasets in the current study using GraphPadQuickCalcs (http://www.graphpad.com/quickcalcs/index.cfm).

8.3 RESULTS

8.3.1 Clinical characteristics of patients with periodontitis

The average percentage of periodontal sites exhibiting BOP was 65.8 ± 24.6%. In total, 45.1 ± 23.7 % of periodontal pockets had a depth of > 4 mm and 17.1 ± 19.4 % of periodontal pockets had a depth of > 6 mm (Table 8.1).

8.3.2 *Candida* spp. and cell densities recovered from patients with periodontitis

Candida spp. were recovered from either 10/21 oral rinses, and/or periodontal sites of 10/21 (47.6%) patients with periodontitis. *Candida albicans* isolates were recovered from 10/21 (47.6%) and *C. dubliniensis* isolates were recovered from 5/21 (23.8%) patients with periodontitis (Table 8.2). All of the *C. dubliniensis* isolates recovered were co-isolated with *C. albicans. Candida kefyr* was isolated with *C. albicans* from one subgingival sample taken from patient 2. *Candida parapsilosis* was isolated from one subgingival site in patient 11, and was the only yeast species isolated from this site (Table 8.2), however, a *C. albicans* isolate was also recovered from another site by paper-point in this patient. In the ten patients from whom *Candida* spp. were isolated from oral rinse sample, *Candida* spp. were also isolated from their subgingival sites. Interestingly, in nine of these ten patients from whom *Candida* spp. were recovered by oral rinse, *Candida* spp. were also recovered at higher densities from periodontal sites in each patient. In two patients (patients 2 and 11), *Candida* spp. isolated from the subgingival niche included species not isolated from the oral rinse sample, reflecting site-specific colonisation and the differing ecological niche of the subgingival site compared to the oropharynx in general. Interestingly, *C. albicans* isolates were recovered from a healthy site in patient 16 using both curette and paper-point sampling, this was the only periodontitis patient in the study from whom *Candida* spp. were recovered from a healthy site (Table 8.2). *Candida* spp. density counts ranged from 10 cfu/curette sample to 30,000 cfu/curette sample (average 3,528 ± 8,743 cfu/curette sample), from 10 cfu/paper-point sample to 40,000 cfu/ paper-point sample (average 3,910 ± 5,466 cfu/paper-point sample) and from 10 cfu/ml to 8000 cfu/ml (average 1,536 ± 2,384 cfu/ml) for oral rinse samples. The average *Candida* density counts recovered from periodontal pockets (3,719 cfu/sample) was 2.4-fold higher than the average *Candida* density counts recovered from oral rinse samples (1,536 cfu/ml) of the same periodontitis patients.

Table 8.2 *Candida* species and cell density recovered from patients with periodontitis

Patient	Oral rinse (CFU/ml)	Paperpoint samples of periodontal sites (CFU/sample)			Curette samples of periodontal sites (CFU/sample)		
		Healthy subgingival site	Periodontitis site 1	Periodontitis site 2	Healthy subgingival site	Periodontitis site 1	Periodontitis site 2
1	*C. albicans* (80) *C. dubliniensis* (10)	NCI (0)	*C. albicans* (1,240) *C. dubliniensis* (170)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
2	*C. dubliniensis* (8,000)	No healthy sites available	*C. albicans* (8,000)	*C. albicans* (160)	No healthy sites available	*C. albicans* (30,000) *C. kefyr* (1,200)	*C. albicans* (10)
3	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
4	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
5	*C. albicans* (310) *C. dubliniensis* (80)	NCI (0)	NCI (0)	*C. albicans* (20,000)	NCI (0)	*C. albicans* (10)	*C. albicans* (680)
6	*C. albicans* (80) *C. dubliniensis* (110)	NCI (0)	Not tested	Not tested	NCI (0)	*C. albicans* (1,300)	*C. albicans* (1,000)
7	*C. albicans* (2,660)	Not tested	Not tested	Not tested	NCI (0)	*C. albicans* (280)	*C. albicans* (230)
8	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
9	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
10	*C. albicans* (550)	NCI (0)	*C. albicans* (283)	*C. albicans* (1,090)	NCI (0)	*C. albicans* (1,240)	Not tested
11	*C. albicans* (730)	No healthy sites available	*C. albicans* (10)	*C. parapsilosis* (4,600)	No healthy sites available	NCI (0)	NCI (0)
12	*C. albicans* (1,210)	NCI (0)	*C. albicans* (10)	*C. albicans* (4,520) *C. dubliniensis* (150)	NCI (0)	NCI (0)	*C. albicans* (570) *C. dubliniensis* (570)
13	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
14	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
15	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
16	*C. albicans* (780)	*C. albicans* (40,000)	*C. albicans* (2,680)	NCI (0)	*C. albicans* (20,000)	*C. albicans* (2,970)	*C. albicans* (1,090)
17	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
18	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
19	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
20	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
21	*C. albicans* (760)	NCI (0)	*C. albicans* (1,900)	*C. albicans* (6,020)	NCI (0)	*C. albicans* (710)	*C. albicans* (490)

Abbreviations: CFU; Colony forming units, NCI; No *Candida* isolated

8.3.3 *Candida* spp. and cell densities recovered from healthy volunteers

Oral rinse sampling of 50 healthy volunteers was also carried out. This was included in the present study for comparison of oral *Candida* carriage rates in periodontally healthy individuals with periodontitis patients. *Candida* spp. were recovered from 16/50 (32%) of these patients. *Candida albicans* was recovered from all of these 16 individuals, and one also yielded *C. glabrata*. *Candida* cell densities ranged from 20 cfu/ml to 1,080 cfu/ml (average 279 ± 317 cfu/ml) for oral rinse samples (individual patient data not shown). Average counts from oral rinse samples of *Candida*-positive healthy carriers (279 cfu/ml) were 5.5-fold lower ($p=0.05$; two-tailed unpaired Students *t* test) than average counts from oral rinse samples of periodontitis patients (1,536 cfu/ml).

8.3.4 MLST analysis and ABC genotyping of *C. albicans* isolates from periodontitis patients and healthy oral carriers

Thirty-one *C. albicans* isolates recovered from periodontitis patients were subject to MLST and yielded 19 distinct sequence types (STs). Two of the MLST alleles (*SYA*-178 and *VPS13*-242) and 13 of the STs recovered from patients with periodontitis have not been identified previously. More than one ST was identified in five of these patients; isolates belonging to least two different STs were identified in patients 2, 5, 7, 16 and 21 (Table 8.3). Five isolates recovered from patient 16 using different sampling methods (paper-point, oral rinse and two curette samples) yielded four distinct STs. All five isolates recovered from patient 10 were identified as ST 444 despite being recovered from different sites and using different sampling methods (Table 8.3). The majority (21/31) of *C. albicans* isolates recovered from patients with periodontitis belonged to MLST clade 1, the most predominant MLST clade in the *C. albicans* population structure (Odds *et al.*, 2007). Similarly, 26/31 of the *C. albicans* isolates were found to belong to *C. albicans* ABC genotype A and five to genotype B (Table 8.3). *C. albicans* isolates belonging to MLST clade 1 and ABC genotype A were recovered from 6/9 and 7/9 periodontitis patients, respectively.

The 16 oral *C. albicans* isolates from healthy carriers were also subject to MLST. Unique STs were identified for each of these 16 isolates; four belonged to MLST clade 1, five to MLST clade 2, four to MLST clade 4, one to MLST clade 5, one to MLST clade 11 and one to MLST clade 15 (Table 8.3). The majority of these isolates (13/16) belonged to ABC genotype A and the remaining three isolates were genotype B (Table 8.3).

8.3.5 Genetic comparison of oral *C. albicans* isolates recovered from periodontitis patients and from healthy individuals

To test the genetic relatedness of the *C. albicans* populations recovered from the two subject groups, a neighbour-joining tree based on *p*-distance was constructed using the concatenated MLST sequences. Only unique STs were included in the analysis, any duplicate STs were omitted in order to reduce bias. In several patients, isolates recovered yielded distinct STs that were very closely related to each other. The concatenated MLST sequences for these STs differed by loss of heterozygosity in three nucleotide sites or less, suggesting microvariation in persisting STs in these periodontal pockets. Microvariation of persisting STs was observed in periodontitis patients 2, 5, 7, 16 and 21.

The majority of the STs identified in isolates recovered from patients with periodontitis (21/31) belonged to clade 1, these clade 1 STs were recovered from 6/9 (71.4%) patients from whom recovered *C. albicans* isolates were subject to MLST. In contrast, only 4/16 (25%) of the STs identified in isolates recovered from 16 healthy *Candida* carriers belonged in this clade (Table 8.3 and Figure. 8.1). Surprisingly, one of the STs (ST 1673) recovered from a healthy volunteer separated from the rest of the clade 1 isolates when analysed by neighbour-joining analysis. In order to investigate this further, a separate neighbour-joining tree was constructed containing additional clade 1 STs (data not shown). In this tree, the outlying ST clustered neatly into clade 1, so this outlier is most likely due to the limited number of STs included in the analysis.

Nearest-neighbour analysis of the STs in this tree indicated that the distribution of STs recovered from patients with periodontitis and those recovered from healthy individuals differed significantly ($p=0.04$; Fisher's two-tailed exact testing) as indicated by the differing clade distribution (Table 8.3 and Figure 8.1).

8.3.6 MLST analysis of *C. dubliniensis* isolates recovered from patients with periodontitis

A *C. dubliniensis* isolate recovered from patient 1 and two *C. dubliniensis* isolates recovered from patient 12 were examined using the *C. dubliniensis*-specific MLST scheme. One of these isolates was recovered from patient 12 using curette sampling and was identified as ST 7, and the other isolate was recovered from this patient by oral rinse and identified as ST 50. The third isolate examined was recovered by curette sampling of patient 1 and was also identified as ST 7. The ITS genotypes of these three isolates were also examined and revealed to be ITS genotype 1, the most commonly identified

Table 8.3 MLST STs, allelic profiles and ABC genotypes of oral *C. albicans* isolates recovered from periodontitis patients and healthy carriers

Patient	Isolate	Sample (Site)[a]	ST	*AAT*	*ACC*	*ADP*	*PMI*	*SYA*	*VPS*	*ZWF*	CC	MLST clade	ABC genotype
Periodontitis													
P1	RM36	PP (1)	69	2	5	5	2	2	6	5	1	1	A
P2	RM3	C (2)	2046	2	3	2	65	2	6	5	1	1	A
P2	RM6	PP (2)	2046	2	3	2	65	2	6	5	1	1	A
P2	RM8	C (1)	2046	2	3	2	65	2	6	5	1	1	A
P2	RM15	PP (1)	2047	2	3	2	31	2	27	5	S	1	A
P2	RM9	C (1)	2070	2	3	2	2	2	6	20	1	1	A
P2	RM10	C (1)	73	2	3	2	2	2	6	5	1	1	A
P2	RM14	PP (3)	804	2	3	2	2	2	20	5	1	1	A
P5	RM1	C (1)	2039	60	7	40	1	7	11	15	12	11	A
P5	RM2	C (1)	2040	60	7	21	1	7	114	15	12	11	A
P5	RM4	PP (1)	2071	60	7	21	1	7	11	15	12	11	A
P5	RM5	PP (1)	2071	60	7	21	1	7	11	15	12	11	A
P5	RM16	ORS	2072	60	7	21	1	6	126	15	S	11	A
P7	RM41	C (1)	360	8	14	8	4	7	3	35	2	4	B
P7	RM43	C (2)	2073	8	7	8	4	7	3	35	2	4	B
P7	RM49	ORS	2073	8	7	8	4	7	3	35	2	4	B
P10	RM28	PP (2)	444	2	5	5	4	2	6	5	1	1	A
P10	RM34	C (1)	444	2	5	5	4	2	6	5	1	1	A
P10	RM27	C (1)	444	2	5	5	4	2	6	5	1	1	A
P10	RM25	PP (2)	444	2	5	5	4	2	6	5	1	1	A
P10	RM29	PP (2)	444	2	5	5	4	2	6	5	1	1	A
P11	RM38	ORS	73	2	3	2	2	2	6	5	1	1	A
P12	RM21	PP (1)	2038	2	2	5	2	2	242	5	1	1	A
P12	RM23	PP (1)	2038	2	2	5	2	2	242	5	1	1	A
P16	RM17	C (1)	2042	8	3	5	2	2	5	5	1	1	A
P16	RM18	PP (1)	2043	8	3	5	2	2	6	5	1	1	A
P16	RM26	C (1)	171	8	3	6	2	2	6	5	1	1	A
P16	RM19	ORS	444	2	5	5	4	2	6	5	1	1	A
P16	RM20	ORS	444	2	5	5	4	2	6	5	1	1	A
P21	RM11	ORS	2044	28	7	38	65	106	122	15	41	8	B
P21	RM12	C (1)	2045	28	7	38	2	106	122	15	41	8	B
Healthy Carriage													
HV100	Ca100	ORS	1428	2	23	5	102	2	20	20	S	1	A
HV102	Ca102	ORS	1660	3	5	5	2	2	76	5	1	1	A
HV107	Ca107	ORS	1664	4	5	6	2	2	106	5	S	1	A
HV103	Ca103	ORS	1661	40	24	41	21	4	76	27	S	2	A
HV106	Ca106	ORS	1663	4	5	4	4	139	26	4	2	2	A
HV109	Ca109	ORS	1666	4	2	14	4	139	41	67	S	2	A
HV112	Ca112	ORS	1669	36	2	6	4	49	41	4	S	2	A
HV105	Ca105	ORS	659	11	26	6	4	34	60	119	9	4	B
HV104	Ca104	ORS	1662	3	26	6	4	34	60	55	S	4	B
HV110	Ca110	ORS	1667	8	14	8	4	56	10	8	3	4	A
HV111	Ca111	ORS	1668	14	14	30	4	56	3	8	S	4	B
HV108	Ca108	ORS	1665	5	27	37	4	34	105	12	S	11	A
HV114	Ca114	ORS	1671	4	19	6	4	61	15	201	58	15	A
HV115	Ca115	ORS	1672	4	60	6	4	4	41	4	S	2	A
HV116	Ca116	ORS	1673	2	3	10	2	2	94	2	S	1	A
HV117	Ca117	ORS	1674	13	3	6	34	62	8	47	S	5	A

[a] Sampling methods and periodontal sites from which each isolate was recovered is indicated by abbreviation (see below) and by site number in parenthesis.

Abbreviations: HV, Healthy Volunteer; C; curette, PP; paper-point, ORS, oral rinse sample; ST; sequence type; CC, clonal complex

genotype (data not shown). These data showed that these isolates belonged to MLST clade C1, the most densely populated clade in the population structure of *C. dubliniensis*.

8.4 Discussion

The purpose of the present study was to investigate the prevalence, cell density and relative abundance of *Candida* spp. in periodontal pockets, healthy gingival sites and the oral cavity of patients with untreated periodontitis. Isolates of the most prevalent species identified, *C. albicans,* were subject to MLST analysis and compared with the corresponding data from *C. albicans* isolates recovered from healthy oral *Candida* carriers without periodontitis in order to determine if there was any association of specific clonal groups with the disease. This type of in-depth population analysis has not been undertaken previously for *C. albicans* isolates recovered from patients with untreated periodontitis.

Although the group of patients with untreated periodontitis in the current study is relatively small (n=21), sampling was undertaken at an average of three separate sites per patient and using three different sampling methods including curettes, paper-points and oral rinse sampling. To date, there have been no investigations comparing paper point and curette sampling techniques in the investigation of the isolation of yeast species from periodontal sites. For this reason, we have used both sampling techniques in the present study. This comprehensive sampling regimen was carried out in order to get an accurate representation of the *Candida* spp. prevalent in the periodontal pockets of patients with periodontitis.

The chromogenic medium CHROMagar Candida was used to culture *Candida* from clinical samples in order to enhance detection of separate *Candida* spp. Several different *Candida* spp. commonly isolated from humans exhibit characteristic colony colours on this medium, enabling the detection of mixed species in individual clinical specimens. *Candida* isolates were subsequently definitively identified by substrate assimilation profile analysis and by PCR. Several previous studies that reported on *Candida* spp. from periodontitis patients used conventional mycological media such as Sabouraud's agar for primary isolation of *Candida* spp. (Egan *et al.*, 2002; Reynaud *et al.*, 2001; Song *et al.*, 2005). However, colonies of several *Candida* spp. can appear indistinguishable following primary culture on such media resulting in failure to detect mixed species from clinical specimens (Coleman *et al.*, 1993).

Isolates of the two most prevalent species, *C. albicans* and *C. dubliniensis* were further characterised by ABC or ITS genotyping as well as by MLST. To our knowledge, this is the first study to examine *C. albicans* and *C. dubliniensis* isolates recovered from periodontal pockets by MLST, which is now considered the gold standard in molecular

typing and population analysis of *Candida* spp. Previous studies on subgingival *Candida* isolates relied on less reproducible, subjective techniques such as RAPD and electrophoretic karyotyping (Barros *et al.*, 2008; Pizzo *et al.*, 2002; Song *et al.*, 2005). The advantage of MLST is that it relies on species-specific DNA sequence-based comparison of isolates and the data can be stored electronically in databases. Data from new isolates can be gradually added to the existing database and can be compared directly with isolates recovered from other study groups.

Candida albicans was the most commonly *Candida* spp. identified in this group of patients with periodontitis, which is in agreement with several previous studies (Egan *et al.*, 2002; Jarvensivu *et al.*, 2004; Barros *et al.*, 2008; Jewtuchowicz *et al.*, 2008; Urzúa *et al.*, 2008; Sardi *et al.*, 2011). This species was most commonly recovered from 10/21 (47.6%) periodontitis patients by both oral rinse sampling and by sampling periodontal pockets using both paper-points and curettes. Similarly, *C. albicans* was the species most frequently isolated from the oral rinse sampling of periodontally healthy subjects, being recovered from 16/50 (32%) subjects; only one of these healthy patients harboured a non-*C. albicans* species, *C. glabrata,* which was co-isolated with *C. albicans*. The average *Candida* density counts recovered from periodontal pockets were 2.4-fold higher than the average *Candida* density counts recovered from oral rinse of the same periodontitis patients. Furthermore, average counts from oral rinse samples of *Candida*-positive healthy carriers were 5.5-fold lower than average counts from oral rinse samples of periodontitis patients.

In patients 2 and 11, *Candida* spp. recovered from periodontal pockets were not recovered by oral rinse sampling of the same patient, or by sampling of an alternative periodontal pocket (Table 8.2) indicating site-specific colonisation. Urzúa *et al.* (2008) previously observed that the oral mucosae of 94-100% of patients with periodontitis were colonised with *Candida* spp., whereas only 44% of these patients showed *Candida* colonisation of periodontal pockets. In the present study, the oral mucosa of 9/21 (42.9%) patients with periodontitis were colonised by *Candida*, and 10/21 (47.6%) of these patients showed *Candida* colonisation of periodontal pockets, which demonstrates that *Candida* spp. are not a definitive cause of periodontitis. However, it is likely that *Candida* may exacerbate the condition, especially in periodontal pockets harbouring high *Candida* densities. Furthermore, the results of our study showed that individual periodontal sites are specifically colonised by distinct *Candida* spp. that are not found on the oral mucosa or in other periodontal pockets, suggesting some specific enrichment at these sites with

particular isolates or species. These results suggest that oral rinse sampling do not accurately reflect *Candida* populations present in periodontal pockets. Overall, these findings suggest *Candida* proliferation in at least some periodontal pockets, and that the *Candida* counts detected were not just due to contamination by saliva. Nineteen of the 21 periodontitis patients had healthy subgingival sites available for sampling for *Candida*, but only one of these sites yielded *Candida* isolates. The healthy site sampled from patient 16 yielded very high counts of *C. albicans* (20,000 cfu/curette sample and 40,000 cfu/paper-point sample), however, only 780 *C. albicans* cfu/ml were recovered by oral rinse sampling of this patient (Table 8.2). The probing depth (2 mm) and lack of bleeding and suppuration on probing at this healthy site (Table 8.1) and lack of local inflammation suggested that the tooth and the site were healthy. It is difficult to explain this anomalous finding.

MLST analysis of *C. albicans* isolates from periodontitis patients revealed that 13/19 (68.4%) of the STs identified were previously unidentified and that the majority of these isolates belonged to clade 1, the most predominant MLST clade in the population structure of *C. albicans*. These STs were recovered from 6/9 (67.7%) patients from whom recovered *C. albicans* isolates were subject to MLST, suggesting an enrichment of this clade with isolates recovered from periodontitis patients. There did not appear to be a considerable difference in STs identified in isolates recovered from oral rinse and periodontal pockets, as for patient 10, separate isolates recovered by rinse, curette and paper-point were all identified as ST444. Interestingly, two *C. albicans* isolates recovered from patient 16 were also identified as belonging to this ST (Table 8.3). Examination of the *C. albicans* MLST database (http://calbicans.mlst.net/) revealed that isolates recovered from blood, the oesophagus and the oral cavity of patients in Hong Kong, Brazil, the Netherlands and Ireland have been identified as ST444. The presence of this ST in more than one patient may reflect a high prevalence of this particular ST in the population at large.

Two isolates recovered from patient 21 by curette and oral rinse were identified as ST 2045 and ST 2044, respectively, but these isolates differed only by one site of heterozygosity in one allele. In contrast to the STs associated with periodontitis, only 4/16 (25%) STs recovered from the 16 separate healthy subjects belonged to MLST clade 1, whereas the rest of the isolates belonged to other clades (Table 8.2). Nearest-neighbour analysis and Fisher's exact testing supported the significant difference in clade distribution between oral *Candida* carriers and patients with periodontitis (Figure 8.1).

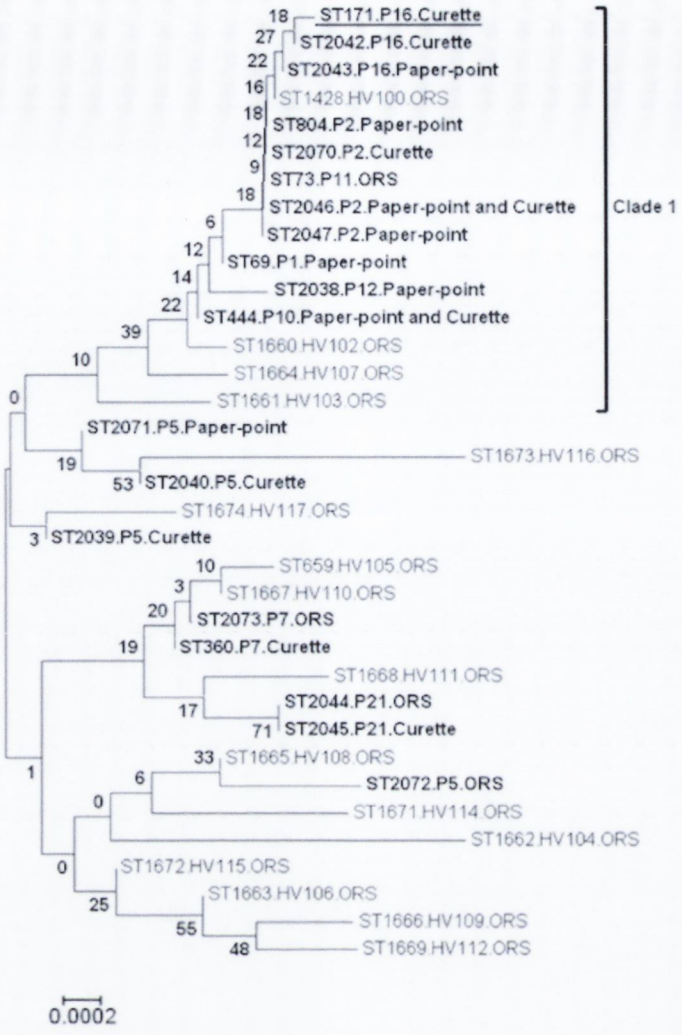

Figure 8.1 Neighbour-joining tree based on the polymorphic nucleotides in 19 of the unique STs identified in 31 *C. albicans* isolates recovered from nine different patients with periodontitis, and 16 unique STs identified from oral rinse sampling of 16 periodontally healthy patients. Sequence types identified amongst isolates recovered from patients with periodontitis are indicated in bold typeface and the patients and sampling methods from which these STs were recovered are indicated alongside each ST. The ST identified in the isolate recovered from the healthy site of patient 16 (P16) is indicated in bold and underlined typeface. Sequence types identified amongst isolates recovered from periodontally healthy patients are indicated in normal typeface and the patients and sampling methods from which these STs were recovered are indicated alongside each ST. The scale indicates *p*-distance. Eleven distinct STs recovered from 6/9 separate periodontitis patients belonged to clade 1, whereas only 4/16 STs recovered from healthy subjects belonged to this clade. Nearest-neighbor analysis of the STs in this tree indicated that the distribution of STs recovered from patients with periodontitis and those recovered from healthy individuals differed significantly (p=0.04; Fisher's two-tailed exact testing).

Abbreviations: P; Periodontitis patient, OC; periodontally healthy oral *Candida* carrier, ORS; Oral rinse sample.

Previous studies have reported that the *C. albicans* MLST clade 1 is enriched with isolates recovered from superficial infections, as well as with ABC genotype A isolates (Odds *et al.*, 2007). The current study shows that clade 1 is also enriched with isolates recovered from periodontitis patients. Previous to the current study, neither the *C. albicans* nor *C. dubliniensis* MLST databases contained any ST information on isolates recovered from periodontal pockets, or on isolates recovered using curettes or paper-point sampling. This information has now been entered into the respective *C. albicans* and *C. dubliniensis* MLST databases for use in future analyses.

Interestingly, *C. dubliniensis* isolates were co-recovered with *C. albicans* from 5/21 (23.8%) of patients with periodontitis in the current study. The prevalence of *C. dubliniensis* in this group of 21 patients with periodontitis (23.8%) is higher than the 3.5% is usually reported in the oral cavities of healthy individuals (Ponton *et al.*, 2000). Indeed, no *C. dubliniensis* isolates were recovered by oral rinse sampling of the 50 periodontally healthy patients included in the present study. Three of the isolates recovered from two of the patients with periodontitis were subject to MLST, which showed that these isolates were typical representatives of the isolates typically recovered from the human oral cavity, all three of these isolates belonged to ITS genotype 1 and MLST clade C1. This clade is the most heavily populated and least divergent clade, accounting for 66 % of the 94 isolates examined by staff at this laboratory to date (McManus *et al.*, 2008; McManus *et al.*, 2009).

8.5 Conclusions

This study is the first to investigate *Candida* isolates recovered from periodontitis patients by MLST, which is highly reproducible and reliable in determining the genetic relatedness between isolates of the same species. The current study used three different sampling methods to examine the *Candida* spp. and densities present in the periodontal pockets and from the oral mucosa of periodontitis patients, comparing them with that from the oral mucosa of healthy subjects attending the same hospital. It is possible that the differences in *Candida* density between periodontal pockets and oral rinse samples, and the differential population distribution between isolates recovered from patients with periodontitis and those recovered from periodontally healthy individuals may reflect some selection of adapted isolates of *Candida* spp. to this more anaerobic environment, or to other pathogens and their products present in the plaque-biofilm associated with periodontitis. It is not possible to say definitively that the presence and/or abundance of

Candida in the periodontal pockets contributes to the progression of periodontitis, or if it is simply a marker for the worsening of the condition. However, given the array of virulence factors (such as secreted aspartyl proteinases) that are expressed by *C. albicans,* and the high cell densities often observed at periodontitis sites, it is likely that these organisms contribute significantly to the destruction of periodontal tissues. The novel findings of this study highlight the need for further investigation of this patient group. Such a study should examine a larger number of periodontitis patients on a longitudinal basis, and should also involve the application of MLST to recovered *Candida* isolates, analysing multiple isolates recovered from the each periodontal site, and examining multiple periodontal sites.

Chapter 9

General Discussion

9.1 Peri-implant infections

Peri-implant infections have been reported to affect approximately 10% of dental implants and 20% of patients within 5-10 years after implant placement (Mombelli *et al.*, 2012). Peri-implant infections are difficult to treat and may result in the loss of integration of dental implants (Van Steenberghe *et al.*, 2012; Pjetursson *et al.*, 2012). Several studies reported an increased prevalence of peri-implantitis and loss of integration of dental implants in patients with a history of periodontal disease compared to patients without a history of periodontal disease (Hardt *et al.*, 2002; Karoussis *et al.*, 2003; Cho-Yan Lee *et al.*, 2012; Renvert *et al.*, 2012). However, other studies did not report a significantly increased risk of peri-implantitis in patients with a history of periodontal disease compared to those without (Broccard *et al.*, 2000; Hultin *et al.*, 2000*a*; Baelum & Ellegaard, 2004; Rosenberg *et al.*, 2004; De Boever *et al.*, 2009). Furthermore, peri-implantitis has been observed to affect patients with no demonstrated susceptibility to periodontal disease (Lang & Berglundh, 2011). In light of these conflicting reports, the present study was undertaken to further elucidate the aetiology of peri-implant infections in relation to clinical risk indicators and the oral bacterial species associated with peri-implantitis. In the present investigation, checkerboard DNA-DNA hybridisation analysis was utilised to determine semi-quantitatively the distribution of a range of bacterial species in subgingival samples from implant and natural tooth sites. Many of these species have previously been associated with periodontal disease.

9.2 Checkerboard DNA-DNA hybridisation analysis: strengths and weaknesses

Checkerboard DNA-DNA hybridisation was undertaken to semi-quantitatively investigate the prevalence and density of a panel of 18 bacterial species groupings (Chapter 2, Table 2.1) in multiple sub-gingival plaque samples from diseased and healthy implants and natural teeth. Many of these bacterial species contribute to the pathogenesis of periodontal disease. Checkerboard DNA-DNA hybridisation involves lysing bacteria present in clinical samples, denaturing the chromosomal DNA released, fixing the DNA to nylon membranes and subsequently hybridising the DNA with a selection of probes consisting of whole genome DNAs labelled with chemoflourescent tags from a panel of reference bacterial species (Socransky *et al.*, 1994; Persson *et al.*, 2006; Renvert *et al.*, 2009). This approach permits the semi-quantitative estimation of the cell density of

particular bacterial species present in clinical samples by indirect comparison with known standards. The strength of the checkerboard DNA-DNA hybridisation technique lies in its ability to be used for the simultaneous detection of multiple bacterial species in clinical samples (Haffajee *et al.*, 2009). Furthermore, checkerboard DNA-DNA hybridisation facilitates the processing of relatively large numbers of samples comparatively quickly. This technique also facilitates the undertaking of investigations that otherwise may not be possible, due to limited resources in terms of equipment, consumables and labour costs. Checkerboard DNA-DNA hybridisation has been used successfully to further understanding of shifts in subgingival bacterial species profiles according to health or disease status and in understanding responses to treatment (Socransky *et al.*, 2004).

Concerns have been raised concerning the specificity of checkerboard DNA-DNA hybridisation (Socransky *et al.*, 2004; Chan *et al.* 2004; Gellen *et al.*, 2007). Because checkerboard analysis utilises whole cell genomic DNA probes, cross-reactivity between probes generated from particular bacterial species and DNA from different species of bacteria present in clinical samples cannot be excluded, especially with closely related taxa due to presence of shared genes, closely related genes and/or mobile genetic elements and extrachromosomal elements such as plasmids. Furthermore, due to competition between the DNA from different species for probe binding sites, checkerboard DNA-DNA hybridisation probes may not detect all cells of their respective target species which are present in clinical samples, and the sensitivity of this microbial analytical approach has been questioned (Socransky *et al.*, 2004). Finally, little is known about the prevalence and persistence of DNA from dead microorganisms in subgingival sites. Checkerboard DNA-DNA hybridisation cannot distinguish between viable and dead microorganisms and little is known about the persistence of DNA from dead microorganisms or about the persistence of dead microbial cells at various anatomical locations. Both of these phenomena are likely to affect the interpretation of checkerboard DNA-DNA hybridisation data. Finally, the preparation of clinical sample DNA for checkerboard analysis assumes that different species of bacteria present in a clinical sample are lysed at the same efficiency.

Socransky and co-workers undertook an investigation of the specificity and sensitivity of the checkerboard DNA-DNA hybridisation technique (Socransky *et al.*, 2004). Checkerboard DNA-DNA hybridisation was employed to evaluate 8,887 sub-gingival plaque samples from 79 periodontally healthy and 272 chronic periodontitis subjects in addition to 8,126 samples from 166 subjects prior to and following periodontal

therapy. In order to investigate the sensitivity of the checkerboard technique, cultures of each of the probe reference strains were adjusted to cell densities of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 cfu/ml. In order to investigate the specicifity of the technique, four investigators trained in the use of the checkerboard technique each prepared their own DNA probes (Socransky *et al.*, 2004). Purified DNA samples from 10^6 cfu of each of 80 bacterial species commonly found in the oral cavity were deposited on hybridisation membranes. Whole genomic probes were prepared for 40 species and adjusted in concentration to detect 10^4 cfu of each of the investigated species and checkerboard DNA-DNA hybridisation undertaken. Probes to certain species showed essentially no cross-reactions to other species at a level >10% of the target probe signal. These included probes to *A. actinomycetemcomtans, A. odontolyticus, T. forsythensis, C. rectus, C. sputigena, E. nodatum, E. saburreum, F. nucleatum ss vincentii, L. buccalis, N. mucosa, P. gingivalis, P. micross, S. noxia, S. oralis, T. denticola, T. socranskii* and *V. parvula*. Socransky *et al.* (2004) observed that *F. nucleatum ss vincentii* exhibited weak cross-reactions with *F. nucleatum ss nucleatum* and *F. nucleatum ss polymorphum* as well as a weak reaction to *Campylobacter rectus. Streptococcus intermedius* exhibited cross-reactions to *S. anginosus* and *S. constellatus.* These findings indicated that the checkerboard DNA-DNA hybridisation technique exhibited a high measure of sensitivity and specificity. It is important to note that many of the species included in the investigation of *Socransky et al.* (2004) are species that were included in the panel of investigated species in the present study.

In the present study, in order to mitigate any possible limitations of the specificity of the checkerboard DNA-DNA hybridisation technique, closely related species were grouped together by the investigators (Dr. Sigrun Eick) at the Oral Microbiology Laboratory of the University of Bern and, of the 29 species investigated, 18 species groupings were employed in the analysis (Chapter 2, Table 2.1). Also, RT-PCR was employed to determine the presence or absence of four key pathogens most commonly associated with periodontal and peri-implant infections including *A. actinomycetemcomitans, T. forsythia, P. gingivalis* and *T. denticola* (Chapter 2, Table 2.2). For these species, only when RT-PCR results had confirmed the detection of the species concerned, were the results of the checkerboard considered valid and included in the analysis. These measures were adapted in order to help to increase the reliability of the checkerboard DNA-DNA hybridisation analysis system employed.

9.3 Comparison of periodontal and peri-implant microflora

9.3.1 Periodontal and peri-implant microflora

Checkerboard DNA-DNA hybridisation analysis was undertaken to compare the periodontal and peri-implant microflora. *Treponema* spp. were the only investigated species which differed significantly between periodontal and peri-implant sites examined, being significantly elevated in peri-implant sites (p=0.03) (Chapter 6, Table 6.3). These findings are in agreement with previous studies that reported an increase in the prevalence and density of *Treponema* spp. in the peri-implant microflora of patients affected by peri-implantitis, compared to patients with healthy implants (Hultin *et al.*, 2002). Furthermore, it has recently been reported that *Treponema* spp. formed part of a group of seven bacterial species, comprising of *T. forsythia*, *P. gingivalis*, *T. socranskii*, *S. aureus*, *S. anaerobius*, *S. intermedius*, and *S. mitis*, which were commonly detected at peri-implantitis sites using checkerboard DNA-DNA hybridisation techniques (Persson & Renvert, 2013).

In the present study, *Treponema* spp. were detected at statistically significantly higher levels in the samples from peri-implant sites, compared to those taken from periodontal sites (Chapter 6, Tables 6.3). Kumae *et al.* (2012) also identified higher densities of *Treponema* spp. associated with peri-implantitis compared to periodontitis using 16S pyrosequencing. This suggests that factors in the local environment of the periodontal pocket or the peri-implant sites may have favoured the growth of individual species compared to other oral ecological niches. Site specific colonisation was also observed in the investigation of the distribution of *Candida* spp. within the periodontal pocket and oral rinse samples from patients with untreated periodontal disease and healthy controls (Chapter 8, Table 8.2). The distribution of *C. albicans* STs between both groups was significantly different (p=0.009) with an enrichment of *C. albicans* Clade 1 isolates in periodontal pockets relative to oral carriage isolates from healthy controls (Chapter 8). Furthermore, it was observed that in one patient *C. parapsilosis* was recovered from periodontal samples but not from the oral wash sample in the same patient (Chapter 8, Table 8.2). Site-specific colonisation is likely related to local factors within the periodontal or peri-implant sulcus that influence microbial colonisation and growth. Factors which may influence microbial growth at implant sites include the titanium surface corrosion ions, the macroscopic or microscopic surface topography (Meyer *et al.*, 2006), the other species present within the subgingival or submucosal ecological niche

(Mombelli, 2002), the local pH or the oxygen tension within portions of the plaque biofilm (Haffajee *et al.*, 2006).

However, for 17 of the 18 bacterial species groupings examined, species groupings were observed to be similar between periodontal and peri-implant sites sampled (Chapter 6, Table 6.3), highlighting that the mouth may be considered as a single ecosystem and that the translocation of microorganisms from one ecological niche to another may take place. This finding is in accordance with the evidence to date that periodontal and peri-implant sites often share common microbial species (Quirynen & Listgarten, 1990; Mombelli *et al.*, 1995; Mengel *et al.*, 1996; Leonhardt *et al.*, 1999; Hultin *et al.*, 2000*b*; Botero *et al.*, 2005). Botero *et al.* (2005) observed a significant correlation between the sub-gingival microflora from dental implants and those of teeth adjacent to the implant for Gram-negative enteric rods (p=0.02) and *P. gingivalis* (p= 0.04). This phenomenon is reflected in the findings presented in Chapter 8, Section 8.3.2 where it was observed that where *C. albicans* was recovered particular from oral rinse samples in individual individual patients, it was highly likely that *C. albicans* would also be recovered from paper-point or curette samples taken from untreated periodontal pockets in the same patient (p=0.0001). Furthermore, in the MLST nearest-neighbour analysis of the STs in the *C. albicans* tree indicated that the distribution of STs recovered from patients with periodontitis and those recovered from healthy individuals differed significantly (p=0.04; Fisher's two-tailed exact testing) as indicated by the differing clade distribution (Table 8.3 and Figure 8.1). *Candida dubliniensis* was detected on three occasions in the 21 periodontitis patients (twice from periodontal pocket samples and once from oral rinse samples). All three *C. dubliniensis* isolates were identified in the MLST nearest-neighbour analysis of the STs in the *C. dubliniensis* tree as being from Clade 1, the most densely populated clade in the population structure of *C. dubliniensis*.

Biomaterial, anatomical and host defence differences between periodontal and peri-implant sites are likely to present somewhat differing ecological conditions for periodontal and peri-implant microbial biofilms. However, many similarities are present between periodontal and peri-implant disease processes, namely that, periodontal and peri-implant infections both require the presence of plaque as an initiating and stimulating factor (Pontoriero *et al.*, 1994; Zitzmann *et al.*, 2001). Therefore, maintenance care is important in preventing peri-implant mucositis and peri-implantitis (Rinke *et al.*, 2011). Similar to periodontitis, patient susceptibility to peri-implant infections may be in part accounted for by genetic factors. A significant increase in the prevalence of allele 2 for

the *AL-1RN* gene has been observed between peri-implantitis patients and controls (56.5% vs. 33.3% respectively) (OR 2.6) (Laine *et al.*, 2006). The findings of the present investigation would support the theory that periodontal and peri-implant sites share a common flora and are intimately related. Therefore, it is important to ensure that the periodontal microflora is predominantly one associated with health.

9.3.2 Periodontal health and periodontitis

The observed percentage relative abundance of *Prevotella* spp., *Treponema* spp. and staphylococcal spp. (i.e. *S. aureus* and/or *S. epidermidis*) was elevated within the periodontal microflora of healthy sites compared to those affected by periodontitis (Chapter 6, Table 6.7). It may be hypothesised that the presence of one or more dental implants in the patient's mouth, with the associated peri-implant microflora, may have influenced the composition of the patients' periodontal microflora.

9.3.3 Peri-implant mucositis and peri-implantitis

In the present study the sum of the estimated bacterial cell density (based on DNA-DNA hybridisation) of each species grouping detected from all of the samples was observed to be 33% greater from peri-implantitis sites than for peri-implant mucositis sites. Hultin *et al.* (2002) similarly noted a higher bacterial density around implants demonstrating peri-implant bone loss compared to those without. However, it is difficult to determine if the higher bacterial density is the initiating factor for the peri-implantitis or if it is a consequence of the peri-implantitis. Other investigators previously noted similarities in the distribution of microbial species in peri-implantitis and peri-implant mucositis sites (Mombelli & Lang, 1992; Hultin *et al.*, 2002; Agerbaek *et al.*, 2006). Increased proportions of Gram-negative anaerobic and facultative anaerobic species have been isolated from implants demonstrating signs of peri-implantitis compared to those seen in health (Leonhardt *et al.*, 1999; Sanz *et al.* 1990; Rams *et al.* 1983, 1984; Mombelli *et al.* 2002; Leonhardt *et al.* 1999; Botero *et al.* 2005).

In the present study, there were an inadequate number of peri-implant health sites for statistical analysis. This is surprising, but may relate to the population examined and/or the criteria defining peri-implant mucositis being employed (i.e. being bleeding on probing as recommended by Berglund *et al.* (2002); Lang & Berglundh, (2011)). However, it may be that the incidence of traumatic false positive readings were encountered more frequently with peri-implant sites than periodontal sites due to the fragility of the peri-implant tissue's vasculature (Lekholm *et al.*, 1986). However,

Luterbacher *et al.* (2000) demonstrated that bleeding on probing is a reliable measure for peri-implant inflammation. Furthermore, in the present study, no significant difference was observed between the baseline relative abundance of the peri-implant microflora in peri-implantitis and peri-implant mucositis cases (Chapter 6, Table 6.4). Also, in the clinical intervention study, bleeding on probing was observed to decrease with the treatment undertaken (Chapter 4, Table 4.1) and the microbial profile appeared to shift towards health following the intervention (Chapter 7, Table 7.5). Therefore, within the limits of the present study, it must be concluded that the prevalence of peri-implant mucositis was high, thereby limiting the opportunity for investigation of sites with peri-implant health.

9.3.4 History of periodontal disease

In the present study, *Treponema* spp. were observed to have a 60% higher percentage relative abundance within the peri-implant and periodontal microflora of patients with a history of severe periodontal disease, compared to patients who did not have a history of severe periodontal disease ($p<0.05$) (Chapter 6, Table 6.6). Also, the percentage relative abundance of *T. forsythia, P. gingivalis* and *A. actinomycetemcomitans* were elevated by 48.6%, 41.5% and 31.9% respectively, in the periodontal and peri-implant microflora of patients with a history of severe periodontal disease compared to those without a history of severe periodontal disease, but differences were not significant ($p>0.05$).

Leonhardt *et al.*, (1999) similarly observed that *P. gingivalis*, *P. intermedia*, *P. nigressens* and *A. actinomycetemcomitans* were recovered from peri-implant sites in patients who had had their teeth extracted for periodontal reasons and had implants placed for five or more years. Also *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* have been more frequently isolated in patients with a history of chronic periodontitis or aggressive periodontitis than patients with no history of periodontal disease (Mengel & Flores-de-Jacoby, 2005). Rams *et al.* (1983) noted high levels of spirochetes, fusiform bacteria and coccoid rods in deep peri-implant sites demonstrating signs of bone loss, whereas implants with pockets not greater than 5 mm yielded predominantly coccoid microbiota and very few rods (Bolero *et al.*, 2005). Bolero *et al.* (2005) observed that *P. gingivalis* was detected in peri-implant sites demonstrating deep probing pocket depths, but not in stable implants.

9.3.5 Smoking status

No significant differences were noted in the percentage relative abundance of *A. actinomycetemcomitans*, *P. gingivalis, T. forsythia* spp. staphylococcal species of current smokers compared to non-smokers ($p>0.05$) included in the study (Chapter 6, Table 6.8). In order to adequately compare the peri-implant and periodontal microflora between smokers and non-smokers, a much larger study would be required (see Section 9.4).

9.3.6 Sites with peri-implant probing depths of >5 mm with bleeding on probing

In the present study, both *T. forsythia* and *P. gingivalis* levels were significantly elevated ($p<0.01$) and *N. mucosa* levels were elevated ($p<0.05$) to a lesser extent in both periodontal and peri-implant sites with probing depths of >5 mm and bleeding on probing, compared to sites without these criteria (Chapter 6, Table 6.5). Cho-Yan Lee *et al.* (2012) noted that probing depths of >5 mm and bleeding on probing of periodontal sites were risk indicators for increased peri-implant bone loss. Agerbaek *et al.* (2006) noted a higher bacterial load from peri-implant sites with probing pocket depths of ≥4 mm compared to sites with probing pocket depths of ≤4 mm. Loesch *et al.* (1983) noted that at periodontal sites with probing pocket depths of >5 mm, favoured the proliferation of anaerobic and facultative anaerobic species. Rams *et al.* (1983) and Rams *et al.* (1984) employed scanning electron microscopy and anaerobic culture to examine the morphology of the plaque samples from peri-implant sites and noted high levels of spirochaetes, fusiform bacteria and coccoid rods in deep peri-implant sites demonstrating signs of bone loss, whereas implants with pockets not greater than 5 mm yielded predominantly coccoid microbiota and very few rods.

9.4 Risk indicators: periodontal disease history

The present investigation studied the relationship between periodontal disease experience and smoking to peri-implantitis or implant loss. Karoussis *et al.* (2003) observed a decreased survival rate of dental implants and an increased peri-implantitis or implant loss in patients with a history of significant periodontal disease compared to patients without a history of periodontal disease. However, the increased risk was not apparent until after five years post-implant placement. Therefore, in the present investigation, only patients who had implants placed for five or more years previously were included in order to detect differences between patient groups.

Many investigators have observed a significant relationship between smoking status and peri-implantitis (Hass *et al.*, 1996; Karoussis *et al.*, 2003; Nitxan *et al.*, 2005; Roos-Jansåker *et al.* 2006; Strietzel *et al.*, 2007; Fransson *et al.*, 2008; Levin *et al.*, 2008; Aglietta *et al.*, 2010; Rinke *et al.*, 2011; Swierkot *et al.*, 2012). Furthermore, it appears that smoking may heighten the risk of peri-implantitis in those patients with a demonstrated susceptibility to periodontal disease (Karoussis *et al.*, 2003; Aglietta *et al.*, 2011). However, it is important to emphasise that not all studies reported a significant relationship between smoking and peri-implantitis (Aalam & Nowzari, 2005; Roccuzzo *et al.*, 2010; 2012; Marrone *et al.*, 2012).

Many previous studies were vague in the criteria employed for defining peri-implant bone loss and did not indicate the extent of bone loss required for a diagnosis of peri-implantitis (Behneke *et al.*, 2002; Karoussis *et al.*, 2002; Ekelund *et al.*, 2003; Ferreira *et al.*, 2006). However, Roos-Jansaker *et al.* (2006) utilised the criteria of radiographic bone loss of ≥1.8 mm to define peri-implantitis. The present study employed the recommended criteria of the European Workshop on Periodontology (2002) for diagnosing peri-implantits: implants demonstrating bleeding or suppuration on probing; probing pocket depth of ≥ 6 mm and radiographic bone loss ≥2.5 mm, were classified as being affected by peri-implantitis (Berglundh *et al.*, 2002). Following the recommendations of the European Workshop on Periodontology (2005), patients were only classified as having experienced severe periodontal disease if ≥30% sites demonstrated inter-proximal clinical attachment loss of ≥5 mm (Tonetti & Claffey, 2005). Patients who were current smokers or had smoked in the year prior to examination were classified as smokers.

In the present investigation, we attempted to employ the use of clear definitions with relatively high thresholds for inclusion in the group displaying at risk characteristics (i.e. peri-implantitis, history of severe periodontal disease and smoking status). Experience of severe periodontal disease was observed to be significantly associated with the diagnosis of peri-implantitis (p=0.03) In the present study, smoking was not observed to be significantly associated with a diagnosis of peri-implantitis (p=0.1) (Chapter 6, Table 6.8). However, it cannot be excluded that the failure to reach statistical significance may be due to a Type I error as the sample size in the present was 51 patients. In contrast, Bain & Moy (1993) examined the outcome of 2194 implants in 540 patients. Therefore, the finding that smoking status was not observed to be significantly associated with peri-

implantitis or implant loss, should be interpreted in light of the current literature and the limitations of the present study.

9.5 The non-surgical treatment of peri-implant infections: clinical response

For patients affected by peri-implant mucositis and those affected by peri-implantitis, significant reductions in the percentage of sites demonstrating bleeding on probing were observed nine months following the completion of the non-surgical treatment on a patient level ($p<0.05$) and on a unit level ($p<0.0001$). Many other investigators have observed improvements in bleeding on probing for both peri-implant mucositis and peri-implantitis patients following non-surgical periodontal treatment of peri-implant infections. (Mombelli *et al.*, 1992; Cianco *et al.*, 1995; Felo *et al.* 1997; Shenk *et al.*, 1997; Leonhardt *et al.*, 2003; Mombelli *et al.*, 2005; Renvert *et al.*, 2009; Heitz-Mayfield *et al.*, 2011; Machtei *et al.*, 2012). However, few studies have investigated the response of the peri-implant tissues to non-surgical treatment without the use of adjunctive antimicrobial agents (Karring *et al.*, 2005; Renvert *et al.*, 2009; Thone-Muhling *et al.*, 2009). In the present study, adjunctive antibiotics or antiseptics were not administered either systemically, or into the peri-implant sulcus professionally. In the present study, the percentage of peri-implant sites with probing depths of ≥6 mm at the nine month examination (T3) were significantly improved compared to the baseline examination (T1) ($p=0.02$), for the peri-implantitis group. However, whilst a reduction in the percentage of sites with probing depths of ≥6 mm was reduced at T3 compared to T1 for the peri-implant mucositis group, the observed improvements were not statistically significant ($p=0.32$). It was postulated that this related to a lack of statistical significance, rather than a lack of clinical significance, as explained in Chapter 4, Section 4.8. Thone-Muhling *et al.* (2010) undertook the only other study which observed reductions in probing pocket depths whilst undertaking mechanical debridement without the use of adjunctive anti-microbial agents. The significant reduction in probing pocket depths ≥6 mm in patients affected by peri-implantitis in the present study was probably related to the repeated debridement and oral hygiene instruction undertaken provided. It is likely that repeated mechanical debridement and improved patient oral hygiene were the principal factors responsible for reductions in the sub-gingival microbial density, in particular those of periodontopathogens. It is important to note that variants in populations (including disease severity at presentation) also likely influence treatment outcomes.

In light of the above clinical improvements, it is interesting to note the changes in the periodontal and peri-implant microflora following non-surgical periodontal treatment. Changes in the percentage relative abundance of DNA of individual bacterial species were used to facilitate understanding of the relative proportions of individual species within the subgingival biofilm complex. In order to further understand the changes in the composition of the peri-implant and periodontal biofilm communities following treatment, the percentage relative abundance of each investigated species within each site sampled was calculated was calculated by computing the percentage of each of the 18 microbial species groupings as a proportion of all of the species detected. This approach of investigating the relative abundance was used to help mitigate the possible differences in the plaque volume at individual sites and the limitations of the microbial sampling techniques employed.

9.6 Microbial sampling technique

To date, no published study has employed both paper point and curette sampling techniques to undertake the microbial sampling in a clinical investigation of the treatment of peri-implant infections. As a part of the present study, the methodological investigation compared paper-point and curette sampling techniques qualitatively and semi-quantitatively utilising the checkerboard DNA-DNA hybridisation technique for microbial analysis. The sensitivity of either paper-point or curette sampling alone in identifying the presence of 18 bacterial species groupings, investigated in the present study, was limited. For example, *P. gingivalis* was identified as being present in 100% of the peri-implant and periodontal sites examined. However, paper-point sampling failed to detect *P. gingivalis* from 18% (3/16) and 17% (4/24) of the peri-implant and periodontal sites, respectively, and curette sampling failed to detect *P. gingivalis* from 25% (4/16) and 21% (5/24) of the periodontal and peri-implant sites, respectively. Therefore, for 18% of the peri-implant sites, a false negative result for the presence of *P. gingivalis* was obtained from paper-point samples, whilst for curette samples, a false negative result was observed for the presence of *P. gingivalis* in 25% of the peri-implant sites. When paper-point and curette samples from individual sites were combined, a greater number of microbial species were identified as being present in the ecological niche sampled, for both the periodontal or peri-implant sites. To the authors' knowledge, only one other study to date has compared the efficacy of paper-point and curette sampling techniques in isolating microbial species from both periodontal and peri-implant sites (Gerber *et al.*,

2005). The Gerber *et al.* (2005) study differed from the present investigation as they employed Paper Strips (Proflow Inc., USA) to gain GCF samples from the periodontal or peri-implant sites. The present study highlights the need for both paper-point and curette sampling to be undertaken, and the samples combined, in order that the microbial samples taken more fully represent the microflora of the site sampled, thereby facilitating the provision of more accurate microbial analysis. It may be hypothesised that certain bacterial species were more firmly attached to the dental implant and root surfaces than each other, thereby resulting in paper point sampling being less efficient in there recovery compared to curette sampling.

9.7 Microbial changes following non-surgical treatment of peri implant infections

In the present investigation, a decrease in bacterial DNA detected at peri-implant sites was observed, indicating a reduction in overall bacterial load at peri-implant sites, nine months following debridement of the periodontal and peri-implant sites and the instigation of improved oral hygiene practices (Chapter 4, Section 4.4). This is in line with the findings of (Mombelli & Lang, 1992; Strooker *et al.*, 1998; Mombelli *et al.*, 2001; Mombelli *et al.*, 2005; Renvert *et al.*, 2009; Persson *et al.*, 2006) who similarly noted a reduction in the microbial load following non-surgical treatment, with or without adjunctive antimicrobial agents.

Furthermore, in the present investigation, a decrease was noted in the relative abundance of periodontopathogens coupled with an increase in the more beneficial species, for both peri-implant mucositis and peri-implantitis sites, following treatment. For peri-implant sites, highly significant decreases were noted in the relative abundance of *P. gingivalis* and *A. actinomycetemcomitans* following treatment ($p<0.01$) (Chapter 7, Table 7.3). The decrease in periodontopathogens, for example *P. gingivalis*, was more evident at peri-implant mucositis sites than at peri-implantitis sites (Chapter 7, Tables 7.7 and 7.8). Strooker *et al.*, (1998) and Persson *et al.* (2006 & 2010) similarly observed significant reductions in *A. actinomycetemcomitans* and *P. gingivalis* levels following non-surgical treatment. The microbiological findings of a reduction in both the microbial density and the percentage relative abundance of many periodontopathogens following non-surgical periodontal treatment of both peri-implant mucositis and peri-implantitis, helps to support and illuminate the clinical observations. It is hypothesised that the non-surgical treatment undertaken resulted in less microbial deposits being present

subgingivally/ submucosally allowing the periodontal and peri-implant inflammation to resolve in many sites. Furthermore the repeated oral hygiene instruction may have helped to improve patient performed oral hygiene practices, thereby decreasing the accumulation of new microbial deposits. The combination of the above factors are hypothesised to have resulted in the clinical and microbiogical improvements observed in the present study.

9.8 Future directions

Checkerboard DNA-DNA hybridization is a useful tool for the semi-quantitative enumeration of bacterial species in microbiologically complex systems and has been used widely for this purpose. Furthermore, this method permits the enumeration of large numbers of microbial species in large numbers of clinical samples (Socransky *et al.*, 2004). Checkerboard DNA-DNA hybridization employs digoxigenin-labelled whole-genome DNA probes and allows the simultaneous semi-quantitative analysis of up to 28 individual species in mixed microbiota samples on a single membrane (Gellen *et al.*, 2007). However, checkerboard analysis has significant limitations. Firstly, because related bacterial species and even genera can share particular genes, especially those located on mobile genetic elements, concerns have been raised over the possibility of false-positive results caused by cross-reactivity using the checkerboard system. Secondly, the inability of the checkerboard method to distinguish between living and dead bacteria has been highlighted as a significant concern (Chan *et al.*, 2004). Thirdly, concerns have been raised regarding the sensitivity of checkerboard analysis and its inability to detect species present in low abundance. There is no doubt that checkerboard analysis has provided valuable insights into subgingival microbial populations in health and disease, but its usefulness is being superseded by advances in molecular technology, in particular microbiome analysis and metagenomics. Both of these developing technologies rely on the amplification of specific target sequences (e.g. ribosomal RNA gene sequences) and the application of high-throughput DNA sequence analysis methods such as pyrosequencing.

A microbiome is the community of commensal, symbiotic and pathogenic microorganisms that are present in a particular anatomical location, or indeed the entire body. Metagenomics is the study of genetic material, mainly DNA, recovered directly from clinical or environmental samples. These approaches are being used to investigate the diversity of microorganisms present at different body sites and to monitor changes in microbial populations in health and disease. There is little doubt that the application of

these technologies will be used to revolutionise our understanding of the role of microorganisms in disease. Several such studies have already been reported in relation to oral microorganisms and it is to be anticipated that such approaches in the future will be used to help comprehensively unravel the complex and dynamic microbiology of periodontal disease and peri-implantitis (Zarco *et al.*, 2011; Maddi and Scannapieco, 2013; Foxman *et al.*, 2014). It is highly likely that using these approaches hitherto unrecognised microbial species will be found to contribute significantly to periodontal and peri-implant infections, raising the possibility for improved treatments and control.

9.9 Concluding remarks

For a significant proportion of patients whose dentitions are rehabilitated with dental implant restorations peri-implant infections are likely to continue to influence the outcomes of dental implant treatment for the forseable future. Through understanding peri-implantitis risk indicators/factors, greater disgression can be exercised in the selection of patients for dental implant treatment, maintenance recall intervals can be estimated at the treatment planning stage and more easily cleansable dental implant designs can be appropriately selected.

It is crucial that the sampling techniques employed in gaining microbial samples yield samples which best represent the subgingival/submucosal niche from which the samples are taken. Therefore, both paper-point and curette sampling techniques combined should be employed in providing microbial samples of the periodontal and peri-implant niche.

The present study was a proof of principle study investigating the benefits of non-surgical debridement and oral hygiene instruction in the management of peri-impalnt infections. In many cases it will be worthwhile to utilise chemotherapeutic agents to gain their previously demonstrated adjunctive benefits in the treatment of peri-implant infections. Furthermore, it is recognised that whilst non-surgical treatment may help to improve peri-implant health, additional surgical treatment will be required in many cases to achieve as much repair as possible of the peri-implantitis damaged tissues. Optimising the periodontal health throughout the mouth, through non-surgical debridement and oral hygiene instruction, has benefits for the peri-implant site even if further treatment is required (as these interventions often lead to a decrease in the inflammation of the peri-implant tissues thereby improving peri-implant tissue handling for surgical procedures). Furthermore, successful treatment of peri-implant infections will only come about,

irrespective of whether a surgical or non-surgical treatment approach is adapted, in a mouth in which plaque deposits are regularly efficiently removed through patient performed oral hygiene practices and professionally performed maintenance care.

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Appendix 1.

Ethical Approval

Patient Information Leaflet

An investigation of the PERI -IMPLANT sub-gingival flora

Dear Patient,

We would like to inform you of the study we would like you to participate in. In an attempt to further our understanding of periodontal diseases we are studying which microorganisms are present beneath the gum of patients with dental implants, and to investigate these are different to microbes found beneath the gum adjacent to natural teeth.

If you decide to participate, small paper points will be placed along side your teeth and implants beneath your gum and samples of the deposits will be gathered.

Another sample, taken from the deposits removed while cleaning your teeth and implants will be collected. Samples will be taken from a third area of your mouth. These samples will be gained through collecting a rinse of water into a cup. Also a sample will be taken by gently rotating cotton wool in your nose.

All of these samples will provide information about the microorganisms present in your mouth and how your body prevents infection and helps in improving our understanding of gum disease.

We expect the gathering of these samples to take approximately ten minutes and do not expect that you will experience any pain during these procedures.

If you are a woman of childbearing age and you should become pregnant please inform us immediately. *Your doctors are covered by standard medical malpractice insurance. Your participation in this study is voluntary. Nothing in this document restricts or curtails your rights. If you volunteer to participate in this study you may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.*

At this stage may I kindly ask you to reflect on the previous information given and encourage you to ask any question you feel to be important.

Thank you for your kind assistance.

With regards,

Dr. Rory Maguire

Consent Form

An investigation of the PERI -IMPLANT sub-gingival flora

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement.

PARTICIPANT'S NAME:

PARTICIPANT'S SIGNATURE:

Date:

Date on which the participant was first furnished with this form:

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician's signature:

Date:

(Keep the original of this form in the participant's medical record, give one copy to the participant, keep one copy in the investigator's records, and send one copy to the sponsor (if there is a sponsor).

Ethical Approval

Dear Rory,

Please be advised that your proposed amendment has been approved for both studies as outlined below.

Best Regards, Louise Whelan Senior Executive Officer School of Medicine Chemistry Building Trinity College Dublin Dublin 2 Tel. +353 1 8961476 Fax. +353 1 6713956 www.medicine.tcd.ie

Ms Louise Whelan,
School of Medicine
Chemistry Building
Trinity College
Dublin 2

21st January 2008

Dear Ms. Whelan,

We have previously submitted two protocols to the Faculty of Health Science Research Ethics committee:

AN INVESTIGATION OF THE CHANGES IN THE PERI-IMPLANT SUBGINGIVAL FLORA OVER TIME (Submitted to the December 2008 meeting)

And **AN INVESTIGATION OF THE PERI-IMPLANT SUB-GINGIVAL FLORA** (Submitted to the November 2008 meeting).

Ethical approval was given to the **AN INVESTIGATION OF THE PERI-IMPLANT SUB-GINGIVAL FLORA** study. The addition of three points to the **AN INVESTIGATION OF THE CHANGES IN THE PERI-IMPLANT SUBGINGIVAL FLORA OVER TIME** study was required and this document has since been amended accordingly and submitted.

We wish that in conjunction with the oral microbial samples which are outlined in our previously submitted protocols, to take a nasal microbial swab sample from the same subjects. This is a simple non-invasive procedure with no potential adverse consequences for the patient, which will contribute to furthering our understanding of peri-implant infections.

Please find attached amended patient information leaflet and ethical approval protocol forms.

As this is a simple addition to previously considered protocols we would be grateful if this amendment could be considered at the January the 29th meeting as patients for the first study (**AN INVESTIGATION OF THE PERI-IMPLANT SUB-GINGIVAL FLORA** study) are to attend on the 6th of February 2008.

Many thanks for your help in this matter,

Yours sincerely,

_____

Dr Rory Maguire

Appendix 2.

Patient Characteristics

Table A2.1 Summary of the baseline characteristics of the patients participating in the present investigation.

Patient no.	Age	Relevant medical history	Smoking status	Reason for tooth loss	Grafting undertaken prior to implant placement	Caries present at baseline examination
1	36 years 1 month	Addison's disease	Never	Trauma	No	Yes
2	27 years 1 month	Gastric ulcer	20 /day	Hypodontia	No	Yes
3	51years 1 month	No relevant	Never	Caries	No	No
4	50 years 3 month	Antibiotics for kidney	Ex-smoker 3 yrs	Periodontitis	No	No
5	45 years 9 month	No relevant	10 /day	Trauma	No	Yes
6	55 years 3 month	No relevant	Ex-smoker 1yr	Periodontitis	No	No
7	61 years 3 month	No relevant	Never	Caries	No	No
8	63 years 8 month	Gastric	Never	Failed bridge work	No	No
9	29 years 4 month	No relevant	Never	Caries	No	No
10	54 years 8 month	Diabetes	Never	Caries	No	No
11	31 years 4 month	Anti-inflammatories for arthritis	Never	Caries	No	No
12	49 years 11 month	No relevant	10 /day	Failure of eruption	No	No
13	29 years 4 month	No relevant	Never	Fracture	Yes	Yes
14	24 years 11 month	No relevant	5 /day	Trauma	Yes	No
15	58 years	No relevant	Never	Trauma	No	No
16	64 years 1 month	No relevant	Never	Hypodontia	No	No
17	60 years 3 month	Epilepsy	Never	Failed bridgework	No	No
18	58 years 2 month	Gastric	Never	Failed bridgework	No	Yes
19	50 years 3 month	Discoid lupus	Never	Failed bridgework	No	No
20	40 years 6 month	No relevant	Never	Periodontitis	No	No
21	50 years 4 month	No relevant	Ex-smoker 20 years	Failed bridgework	Yes	No
22	42 years 5 month	No relevant	Never	Trauma	No	No
23	53 years 5 month	Lipitor for cholesterol	Ex-smoker 20 years	Periodontitis	No	No

Continued overleaf

Table A2.1 continued

24	33 years	No relevant	Never	Failure of eruption	Yes	No
25	52 years	Gastric ulcer	Never	Periodontitis	No	No
26	51 years 4 month	Irritable bowel syndrome	Never	Caries	No	No
27	61 years 2 month	HRT, Lichen planus	Never	Periodontitis	Yes	No
28	44 years 5 month	Irritable bowel syndrome	Ex-smoker 17 years	Periodontitis	No	No
29	30 years 10month	No relevant	Never	Hypodontia	No	No
30	27 years 8 month	No relevant	Never	Trauma	Yes	No
31	33years 6 month	No relevant	20 /day	Periodontitis	No	No
32	28 years 8 month	No relevant	Never	Hypodontia	No	Yes
33	31 years 2 month	No relevant	Never	Hypodontia	No	No
34	28 years 1 month	No relevant	Never	Hypodontia	No	No
35	29 years 5 month	No relevant	Never	Hypodontia	No	No
36	29 years 5 month	No relevant	Never	Hypodontia	No	No
37	63 years 6 month	No relevant	20 /day	Periodontitis	No	No
38	56 years 1 month	No relevant	Never	Caries	No	No
39	62 years 4 month	Aspirin	Never	Periodontitis	No	No
40	51 years 4 month	Arthritis, lipitor	Never	Periodontitis	No	No
41	29 years 7 month	No relevant	Never	Trauma	Yes	No
42	31 years 9 month	No relevant	10 /day	Hypodontia	No	No
43	28 years 3 month	No relevant	10 /day	Hypodontia	No	No
44	43 years 6 month	No relevant	Never	Trauma	No	No
45	48 years 2 month	Osteoporosis	10 /day	Periodontitis	No	No
46	58 years 2 month	DVT	20 /day	Periodontitis	No	No
47	31 years 7 month	No relevant	Never	Hypodontia	No	No
48	62 years 5 months	Osteoporosis	10 /day	Periodontitis	No	No
49	59 years	Osteoporosis	Never	Periodontitis	No	No
50	52 years 3 months	Gastric ulcer	10 /day	Periodontitis	No	No
51	58 years 1 months	Osteoporosis	Never	Periodontitis	No	No
52	31 years 5 months	OCP	Never	Hypodontia	No	No

Table A2.2 Summary of patient participation in individual studies in the present investigation.

Patient no.	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7
1	Yes	Yes	No	Yes	No
2	Yes	Yes	No	Yes	No
3	Yes	Yes	No	No	No
4	Yes	Yes	Yes	Yes	Yes
5	Yes	Yes	Yes	Yes	Yes
6	Yes	Yes	Yes	Yes	Yes
7	Yes	Yes	Yes	Yes	Yes
8	Yes	Yes	Yes	Yes	Yes
9	Yes	Yes	No	Yes	No
10	Yes	Yes	Yes	Yes	Yes
11	Yes	Yes	Yes	Yes	Yes
12	Yes	Yes	Yes	Yes	Yes
13	Yes	Yes	No	Yes	Yes
14	Yes	Yes	Yes	Yes	Yes
15	Yes	Yes	Yes	Yes	Yes
16	Yes	Yes	Yes	Yes	No
17	Yes	Yes	Yes	Yes	Yes
18	Yes	Yes	Yes	Yes	Yes
19	Yes	Yes	No	Yes	No
20	Yes	Yes	Yes	Yes	No
21	Yes	Yes	Yes	Yes	Yes
22	Yes	Yes	Yes	Yes	Yes
23	Yes	Yes	No	Yes	Yes
24	Yes	Yes	Yes	Yes	No
25	Yes	Yes	Yes	Yes	Yes
26	Yes	Yes	Yes	Yes	Yes
27	Yes	Yes	No	Yes	Yes
28	Yes	Yes	No	Yes	No
29	Yes	Yes	Yes	Yes	Yes
30	Yes	Yes	No	No	No
31	Yes	Yes	Yes	Yes	Yes
32	Yes	Yes	Yes	Yes	Yes
33	No	Yes	No	No	No
34	Yes	Yes	No	Yes	Yes
35	Yes	Yes	No	Yes	No
36	Yes	Yes	No	No	No
37	Yes	No	No	No	Yes
38	Yes	Yes	No	No	No
39	Yes	Yes	Yes	No	Yes
40	Yes	Yes	Yes	Yes	No
41	Yes	Yes	Yes	Yes	Yes
42	Yes	Yes	No	No	Yes
43	Yes	Yes	No	Yes	No
44	Yes	No	No	No	No
45	Yes	Yes	No	Yes	No
46	Yes	Yes	Yes	Yes	No
47	Yes	Yes	No	Yes	Yes
48	Yes	Yes	No	Yes	No
49	Yes	No	Yes	Yes	No
50	Yes	No	Yes	No	No
51	Yes	No	No	No	No
52	Yes	No	No	No	No