A. Research Proposal

Aim: A randomized clinical trial to test the clinical benefit of azithromycin use in non-surgical periodontal therapy in patients with initially poor response to non-surgical periodontal therapy.

The prevalence of moderate to advanced chronic periodontal disease (CP) is estimated to be between 12-27% of the population in people 35 to 44 years of age worldwide.[1] Adults aged 65 years and older, 64% had either moderate or severe periodontitis. Indeed the NHANES (National Health and Nutrition Examination Survey) database in the USA confirms that the prevalence and severity of periodontal disease continues to increase with age,[2] and therefore represents a huge burden for society, associated with very high economic cost.[3] In addition to this, periodontal infections cause other negative effects to patients' health, in particular to cardiac health.[4] Periodontal disease severity increases dramatically in diabetics, affects glycaemic control in diabetes and may contribute to the progressively worsening epidemic in diabetes in developed and developing societies world-wide.[5] Therefore, effective treatment of CP is desperately needed to improve individuals' oral health and also their overall health.

CP is commonly treated using non-surgical periodontal therapy (NSPT).[6] However, access to deeper pockets (≥ 6 mm) to enable adequate root surface debridement is more difficult with less predictable outcomes.[7] As such, it has been suggested that in pockets ≥ 6 mm that periodontal surgery should be used to achieve longer term stability,[8] albeit it at far greater economic [3] and emotional costs[9].

A recent review paper by Bartold et al.[10] highlights the potential effectiveness of azithromycin (AZM):

- for killing the bacterial microbiome that drive periodontal disease
- immunomodulation of the patients' immune response to the bacterial plaque/s
- observational data of dramatic clinical improvements with adjunctive use of AZM

Our group has preliminary data that demonstrates that adjunctive use of AZM in the NSPT of advanced periodontitis results in improved treatment outcomes including the reduced need for surgical therapy.

Our hypothesis is that azithromycin (AZM) used in NSPT has a clinically significant beneficial effect in treatment of refractory advanced periodontal disease (deeper pockets), reducing the need for surgical therapy and increasing the predictability of therapy.

To test this hypothesis, we plan to undertake a randomized clinical trial (RCT) of the efficacy of azithromycin in nonsurgical periodontal therapy in patients with advanced chronic periodontitis that have had a poor response to initial NSPT. (Figure 1.)

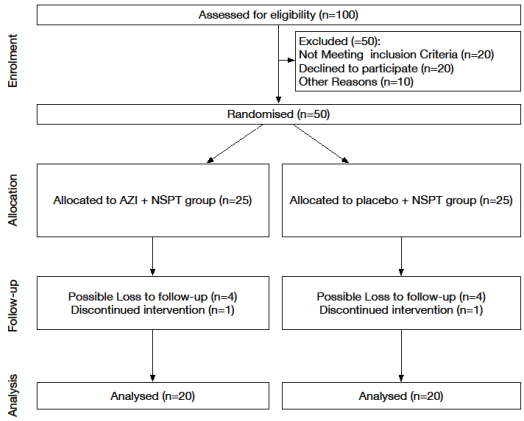

Figure 1 RCT Design

BACKGROUND

AZM is a macrolide antibiotic (9-deoxo-9a-methyl-9a-aza-9a-homoerythromycin) with broad activity including potency against gram negative organisms while retaining the classical erythromycin spectrum. AZM is noted to have low plasma concentrations that are driven by rapid clearance into tissues. Tissue concentrations are much higher than serum concentrations with concentrations in tonsular tissue remaining above the MIC (Mean Inhibitory Concentration) for relevant pathogens. The suggested dosage for soft tissue infections is a single 500 mg dose per day for three days making it a convenient therapy.[11] AZM bioavailability in sites of inflammation also make it an antimicrobial of interest for the treatment of plaque-induced periodontal disease.

The use of systemic antibiotics may enhance the results of NSPT. The adjunctive use of tetracycline, minocycline, metronidazole, and metronidazole and amoxicillin combinations have demonstrated positive results, although there are also a number of papers suggesting no additional benefit. Overall, there appears to be clinical benefit to the use of antibiotics in conjunction with NSPT in advanced disease.[12] The differences in results of these clinical trials can be attributed to differences in study design and in particular inclusion and exclusion criteria, the quality of NSPT undertaken and statistical analysis. Long durations of therapy with multiple doses per day and minor, but significant, side-effects of these antibiotics have been recorded. These factors have led to patient poor compliance with these regimens.

Recently, it has been suggested that Azithromycin (AZM) may be a useful addition to NSPT when used prior to or after NSPT.(10) Its lower plasma concentration, lower incidence of GI (gastrointestinal) tract complications, effectiveness against putative periodontal pathogens and proposed interaction with host response mechanisms involved in the control of the host response to periodontal pathogens have been suggested as reasons to further test this drug.[10].

Recent research has shown a marked difference in antibiotic resistance and susceptibility of putative periodontal pathogens in different countries to AZM and other antibiotics that have been used to enhance the clinical outcomes of NSPT.[13] Although there have been a number of clinical trials reporting the use of AZM in NSPT, the results have been variable.[10] Some of this variability could result from the short time periods of follow-up following therapy, a variation in the quality of study design and differences in exposure of different populations to AZM. AZM is one of the most frequently prescribed antibiotics in America.[14] Therefore, it could be assumed that there may be a far higher bacterial resistance to AZM in North America and Brazil, the origin of the two studies showing no additional benefit to the use of AZM in NSPT. The prescription of AZM in Australia is relatively restricted to a few specific infections (chlamydia and lung infections in cystic fibrosis).[15] Therefore, significant improvements in the clinical measures of pocket depth reduction and bleeding on probing reduction with the use of AZM in NSPT reported in clinical case reports in Australia[16] might be explained by lower antibiotic resistance of putative periodontal pathogens in an Australian population.

Bartold et al.[10] discuss the immunoregulatory effects of azithromycin with implications in periodontal disease. To date, none of these immunoregulatory effects has been confirmed in clinical practice although some have been confirmed *in vitro*.[10,17] There is general agreement that there are a small number of patients that are highly susceptible to periodontal disease and respond poorly to both non-surgical and surgical therapy and that these patients are at risk of tooth loss.[18]These patients can be identified in retrospect by their lack of resolution of deeper probing depth (> 6 mm) and continued bleeding on probing (BOP) following treatment.

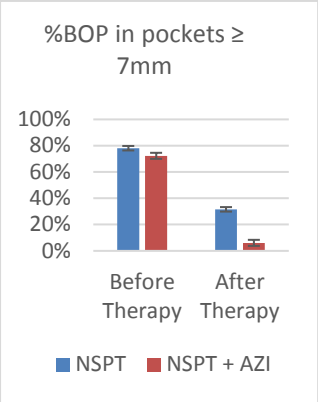

Figure 2: Probing Depth Reduction

Proof of Concept – Pilot Data

Clinical experience in Australia[19] support vastly improved clinical outcomes when utilizing AZM in NSPT of CP in Australia. Dr. O'Rourke has utilized AZM in NSPT for 5 years in a private specialist periodontist practice. These patients ranged in age from 26-56, with slightly more females treated (54.2%). These patients were referred for treatment of advanced CP. These patients had electronic periodontal charting records (Florida Probe) taken prior to treatment, four weeks after the completion of NSPT and 3 weeks following the administration of AZM. Patients were selected for the additional use of AZM based on their initial less than favourable response to NSPT. We have been able to identify 247 patients receiving AZM, and have been able to match these to a further 247 patients seen for NSPT not receiving AZM over the same time period. Probing depth changes are one of the primary clinical measurements of treatment outcome with shallower pockets reflecting a positive treatment outcome.

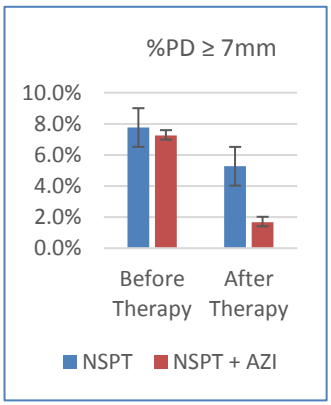

Figure 3: % Bleeding on Probing Reduction

The elimination of pockets of greater than 7mm (to avoid the need for surgical therapy) is one of the major aims of NSPT. Figure 2. demonstrates that the percentage of residual pockets of greater than 7mm (advanced CP) were reduced by the additional use of AZM following NSPT by 32% over NSPT alone.

Bleeding on Probing (BOP) is a sign of ongoing inflammation in the periodontal pocket and lack of BOP has been shown to correlate well with lack of disease activity. Figure 3. shows an additional reduction in BOP of 26%, but also importantly that only 6% of pockets still had BOP when AZM was used.

Figure 4. shows that the number of patients with no pockets of greater than 7 mm is reduced from 53% to 12% (a reduction of 40%).

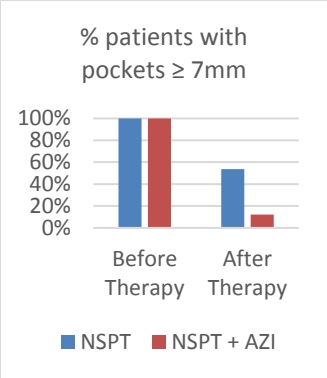

Figure 4: Patients with no pockets greater than 7mm

These data would suggest there is a potential to decrease the amount of surgery required to treat periodontal disease by as much as 26% in the overall population and the requirement for individuals to need surgery by as much as 30%.

During this time only one patient reported a side effect of a slight stomach upset. Compliance (as measured by interview and return of the empty blister pack 1 week after completing the course of AZM) as greater than 95% (far higher than experienced with longer courses of antibiotics). Patients reported that the short course and simple dosage of AZM, the desire to avoid surgical therapy and minimal side-effects resulted in their compliance with the recommended dosage. In addition to this many patients anecdotally reported that "their gums felt better".

The clinical results reported above occur from 6 weeks-3 months after therapy and appear to be relatively stable over some years. About 5% of these patients required a further dosage of AZM 2-3 years after the initial dose because of an episodic decline

in their disease and these patients responded in a similar manner to when they initially received AZM.

While these clinical results are important the subjective nature for the patient selection for use of AZM in NSPT, the use of AZM 3-4 weeks after completion of NSPT and the distinct possibility of operator bias necessitates the use of an RCT to confirm these data. Therefore, it is important to assess the response of an Australian population and individuals with the use of AZM. To date there has been no randomized controlled clinical trial conducted in Australia.

Significance and Innovation

Non-surgical periodontal therapy (NSPT) has been the "gold standard" for treatment of CP for much of the last 50 years. It is recognized that some individuals and some sites respond less favourably to this form of therapy and are far more likely to require surgical periodontal therapy with increased cost and morbidity to patients. The use of systemic antibiotics as an adjunct to non-surgical periodontal therapy has been explored since the causative agent of disease was recognized as bacterial plaque in the 1960's. While some success of these adjunctive antibiotics has been reported, the results reported have been variable and, the courses of antibiotics proposed have had significant side effects and difficulties with compliance. *In vitro* testing has shown Azithromycin to have effect on putative periodontal pathogens and it has also shown modulatory effects on immunological responses that have been implicated in host modulation of the response to the bacterial biofilm that results in periodontal disease. There have been clinical case reports (in Australia and overseas) and controlled clinical trials (overseas) suggesting additional clinical improvements in response to its use in NSPT. Its dosage is simple and we expect to see greater compliance with its use.

We expect that individuals and specific sites will respond with improved clinical measures of response to NSPT with the systemic use of AZM and possibly allow avoidance of surgical periodontal therapy in an Australian population.
We expect this research will provide clinical evidence that use of Azithromycin in NSPT will provide additional benefits in areas that are least responsive to NSPT and this may help to avoid the need for surgical therapy in an Australian population.

This research could lead to further research on:

- The use of this simplified and more predictable therapy in at risk cardiac and diabetic groups to improve outcomes
- Development of new drugs for use in NSPT dependent on the results demonstrating the clinical importance of the immunological or antibiotic properties of AZM

National Benefit

This project would be expected to give evidenced based validation of the additional benefit of Azithromycin as an acceptable addition to non-surgical periodontal therapy.
This would provide the following Australian community benefits:

- More predictable treatment of periodontal disease
- A reduction in the need for retreatment of periodontal disease
- A reduction in the costs and morbidity associated with treatment
- A reduced need for surgical periodontal therapy with further reduced costs and morbidity

Research Plan

a. **Recruitment, screening and obtaining informed consent** - Patients will be enrolled from the Oral Health Centre, School of Dentistry, University of Queensland, Brisbane City Periodontics and Implants, Brisbane and Mount Gravatt that have had initial NSPT but continue to have pockets of ≥ 6 mm with ongoing BOP. These patients will be recruited from the 4th and 5th year undergraduate clinics by informing students of the inclusion and exclusion criteria of the study. Specialist Periodontists in the Brisbane area will also be informed of the clinical trial and protocol so they might invite suitable subjects to join the clinical trial. The principal investigator (Prof Saso Ivanovski) will then discuss with prospective subjects the patient information sheet and the informed consent sheet. These patients would have been given an option of non-surgical retreatment or surgical periodontal therapy by a specialist or postgraduate student regardless of their participation on the clinical trial. Participants will be informed about the objectives and methods of the study and will be included in the study only after signing an informed consent form. The following inclusion criteria will be adopted:
    a. diagnosis of moderate to advanced chronic periodontitis, which is further defined by at least 4 sites with probing depth of ≥ 6mm on at least two non-adjacent teeth with bleeding on probing [20]
    b. age 30 to 75 years ;
    c. At least 16 natural teeth.

   The exclusion criteria for this study will be as follows:
    a. use of antibiotics within 3 months preceding the start of the study;
    b. history of sensitivity to AZM or macrolides
    c. history of cardiac arrhythmia or myocardial infarction
    d. patients with history of cigarette smoking (≥ 10 cigarettes/day)
    e. patients with uncontrolled diabetes mellitus
b. Prior to Treatment collection of
    a. Normal periodontal therapy pre-treatment records
        i. Intra-oral clinical photographs
        ii. OPG radiograph
        iii. Periodontal recording (measuring pockets, plaque and bleeding on probing)

Normal conservative periodontal therapy will be undertaken by periodontists (Prof Saso Ivanovski). This treatment will involve concerted efforts at behavioural modification to improve oral hygiene effectiveness and efficiency and normal non-surgical debridement of the root surfaces of the teeth aimed at removing as much of the microbial biofilm and its products as clinically possible. This therapy is normally undertaken utilizing local anaesthetic but some patients find they do not require local anaesthetic. Subjects will be asked to take either a placebo or Azithromycin (based on a randomized schedule blinded to the operator) commencing immediately with a single 500mg dose and a further 500 mg dosage taken at 1 and 2 days following therapy.
Randomization and operator blinding to drug usage will be undertaken before the commencement of the study. Srinivas Ramachandra will then use https://www.randomizer.org to generate a random allocation of (1)'s and (2)'s and allocate these numbers to the subject number in order and record this on an excel spreadsheet. He will then determine

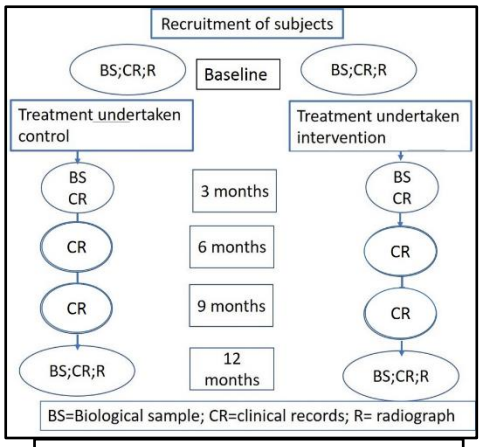

Figure 5: Sample collection design- after enrolment of subjects .

whether (1) or (2) are the placebo or the test drug and record this on the excel spreadsheet and the spreadsheet will be locked by a password known only to him and to the pharmacist preparing the drugs. The azithromycin capsules will then be prepared using pure azithromycin or fillers such as avicel, which create a matching placebo. These will be prepared by My Life My Health Compounding Pharmacy, Brisbane. The prepared placebo/drug will then be placed into individual containers (with exactly the same external appearance) numbered 1 through 50 by the schedule allocated in the spreadsheet. These will be given to the patients at the completion of the initial treatment by the operator.

All clinical recordings and biological samples for each patient will de-identified with the code held by Prof Saso Ivanovski in a secure location in the Oral Health Centre. The codes identifying patients receiving placebo or AZM will be held by Prof Saso Ivanovski and not broken until completion of each phase of the trial. All data will be stored on a secure server at the University of Queensland, School of Dentistry. In addition to this, a register of adverse events will be kept and all adverse events reported by patients and described by patients after interview at each appointment will be recorded. All patients will be given work and after hours contact details of the principle investigator Prof Saso Ivanovski.

If any subjects exhibit any symptoms after taking their drugs they will be asked to contact him and appropriate advice will be given dependent on the symptoms described. Codes would be broken if there is any suspicion of anaphylaxis to the drug taken. A thorough written and oral dental and medical history will be obtained and this will include investigation to ensure no drug interactions with medications currently taken by the subject and a record of the subjects' medical practitioner and his/her contact details. These measures ensure compliance with CONSORT guidelines.[21]

Medical histories will be checked at each visit as is normal clinical protocol. The use of antibiotics during the trial will be noted and subjects' data following the use of any antibiotic will be excluded from the study from that time period onward and no further biological samples (Saliva, GCF or plaque) will be collected.

The rationale for our longitudinal design is to ensure a valuable clinical trial will assess early and longer-term (clinically important) data, and to be able to assess the temporal sequence of the response to allow a proper understanding of the clinical, microbiological and immunological response to this therapy. Pre-treatment biological and immunological samples will form an important part of assessing the identification of markers that may predict clinically significant improved treatment outcomes. It is important to note that the 3 month, 6 month, 9 month and 12 month periodontal support therapy appointments, including clinical measurements, ongoing oral hygiene improvement discussions, tooth debridement and prophylaxis, is considered the current standard of care for patients that have clinically demonstrated prior susceptibility to disease.

Aim 1. A randomized clinical trial to test the efficacy of the addition of azithromycin to non-surgical periodontal therapy.

Rationale: Our rationale for this Aim is to perform a randomised Phase II placebo-controlled intervention trial. Previous studies on the use of antibiotics as an adjunct to NSPT have shown that although initial (6-12 week) responses can be favourable, longer term results (up to 12 months) can show a diminution of the differences.

Approach: Clinical recordings will be undertaken at the time points above and validated by experienced clinician not undertaking the therapy.

Methods:

Records of probing depths (PD), recession to allow calculation of clinical attachment levels (CAL) and bleeding on probing will be recorded at 6 sites per tooth. This will allow for calculation of ΔPD, ΔCAL and Δ%BOP. Assessment of compliance with antibiotic dosage will be assessed by participant interview and return of the antibiotic packaging and recorded. Assessment of adverse events will be undertaken by patient self-reporting and by assessment of response to interview questions regarding adverse events at each time point. These will be recorded in a register of adverse events.

Our primary analysis for this Aim will be to test means between groups across the different endpoints. With at least 17 subjects per arm, we will be able to detect an effect size (mean divided by standard deviation) of 1.25 at 90% power and a two-sided alpha level of 0.01. This reduced alpha allows for post hoc adjustments for multiple comparisons. It is proposed to enrol 40 (20 controls and 20 AZM) patients to compensate for drop out.

Additional analyses will take into account longitudinal effects and the cluster of measurements per tooth using a hierarchical random effects model to control for intra-tooth and probe variability.

Aim 2. To quantify the changes in systemic immune response and cardiac markers with the use of AZM in NSPT.

Rationale: Assessment of the systemic immune response to therapy is important because of the proposed linkage of periodontal disease to increased risk of coronary heart disease, poor metabolic control in diabetes and a number of other periodontal/systemic disease relationships.[22]

Approach: The use of multiplex assays for cardiac disease markers and inflammatory cytokine markers gives an accurate and reproducible method to measure the systemic response to therapy and allows for the evaluation of multiple immune pathways implicated in the systemic relationships to CP.

Methods: Approximately 5 ml of saliva will be collected by in untreated collection tubes (Becton-Dickinson, North Ryde, NSW, AUSTRALIA).

Saliva will be centrifuged at 1300 g for 10 min. and aliquoted and stored at -70°C until further analysis. The concentrations of 31 biomarkers will be assessed using a multiplex assay (Merc Millipore, Frenches Forrest, NSW, Australia) for Luminex technology according to the manufacturers' instructions. The assays used will be (i) Human CVD Panel 2, for the assessment of ADAMTS13, growth differentiation process 15 (GDF-15), D-Dimer, soluble intercellular adhesion molecule-1 (sICAM-1), NGAL/Lipocalin-2, myeloperoxidase (MPO), Myglobin, sP-Selectin, Serum Amyloid A and soluble vascular adhesion molecule 1 (sVCAM-1); and (iii) the high-sensitivity T Cell Panel for the assessment of the following cytokines: FractalkineIL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17/CTLA8, IL-21, IL-23, IL-23, I-TAC/CXCL11, MIP-1α/CCL3, MIP-1β/CCL4, MIP-3α/CCL20 and TNF-α. In brief, saliva samples will be incubated with a mix of beads coated with antibodies for different serum mediators. After washing, samples are incubated with a biotin labelled Detection Antibody Cocktail that is then marked with a streptavidin–phycoerythrin dye. Readout is performed with a Luminex 100 (Luminex, Austin, TX, USA). Bio-Plex Manager 3.0 (BIO-RAD, Hercules, CA, USA) will be used to analyse the readout. Samples from different time points from the same patient will be always run on the same plate, and a total of three consecutive runs will be used to run all samples from the entire study.

The statistical analyses for this Aim will compare treatment versus control across time points while adjusting for baseline labs. This will primarily be an exploratory and hypothesis-

generating analysis to identify possible mechanisms for CP. We will apply false discover rate (FDR) adjustments to reduce the influence on multiple comparisons on these analyses.

Aim 3. To quantify the changes in local immune response with the use of AZM in NSPT.
Rationale: The local immune response is the first line of defence in the response to microbial plaque to periodontal disease. IL-8 is known to be a potent potentiator of *Net* formation and IL-17 is a marker of *Net* formation. The use of multiplex assays to assess these cytokines gives a very accurate method particularly in the small volumes of sample available.
Approach: Sampling of the gingival crevicular fluid (GCF) will reflect to the local immune response to microbial plaque and to therapy.
Methods:
Gingival Crevicular Fluid Sampling:
GCF will be collected from all sites of ≥ 6 mm in each subject. GCF will be collected by using filter paper (Drummond Scientific, Broomall, PA) following the method described by Chapple et al.[23]
Gingival crevicular fluid (GCF) samples will obtained from the site of the deepest pockets on teeth with PD greater than 6 mm. Following isolation of the site with cotton rolls, supragingival plaque is removed, the area air dried and a 30-sec GCF sample collected with filter strips (Periopapers, Interstate Drug Exchange, Amityville, NY, USA). Periopaper strips are gently inserted into the orifice of the periodontal pocket, 1–2 mm subgingivally. Gingival crevicular fluid volume is determined using a Periotron 8000 (Oraflow Inc., Plainview, NY, USA), calibrated following the protocol described by Chapple et al..[23] Samples will be immediately placed in Eppendorf tubes on ice, transported to the laboratory and stored at -80° C. Before the assay, GCF samples are eluted using 60 μl of the assay buffer provided in the Millipore kit by vortexing for 30 min. and then centrifuging for 10 min. at 11,200 g.
Samples will then be tested for the presence of IL-17 and IL-8 utilizing monoclonal antibodies. GCF concentration of IL-17 and IL-8 will be analyzed using a modified enzyme-linked immunosorbent assay from a commercially available kit (MabTech Inc., Cincinnati, OH). Free IL-17 and IL-8 concentrations will be determined using the capturing antibody and biotinylated detection antibody provided in the kit. In brief, assay plates are incubated overnight with capture antibody in phosphate-buffered saline. The plates are then washed and blocked for 1 hour using 0.1% bovine serum albumin in assay buffer. Diluted test serum samples or standards are added, and the plates are incubated for 2 hours. Detection antibody is then added and followed by a 1-hour incubation. After a wash step, diluted streptavidin–horseradish peroxidase is added to each well and the plates are incubated for 1 hour, followed by addition of tetramethylbenzidine substrate. All incubations are done at room temperature. The reaction is stopped by the addition of 2N H_2SO_4. Spectrometric absorbance is read at 450 nm with 570-nm subtraction. In the presence of IL-17–mAb A complex and IL-8–mAb A complex, the assay is able to accurately quantify free IL-17 and IL-8 concentrations.
The statistical approach for this Aim will consider the possible associations between the immunoregulatory responses in general, as well as functions of PMN's in CP and changes in the PMN response to therapy over time. The statistical analysis for this Aim will be similar to the analysis as described in Aim 2.

Aim 4. To investigate the alterations in an individuals' microbiome with the use of AZM in NSPT.
Rationale: Although it is tempting to continue to undertake ongoing measurement of changes in cultivatable or *ELISA* (Enzyme-linked immunosorbent assay) evidence of putative periodontal pathogens it now appears clear that although there are increased numbers of the putative pathogens in CP, and these putative pathogens can be identified in healthy individuals

as well. It has recently been proposed that each individual has a unique plaque biofilm and that now and in the future research should focus on the individuals' biofilm changes as a result of therapy.[24]

Approach: The use of 16S rRNA gene reference sequences methodology will give the best possible method to measure the complete microbial population for individuals and to monitor changes as a result of therapy.

Methods: For each subject, subgingival biofilm samples will be collected from the four deepest sites (pocket depth ≥ 6 mm) that do not exhibit pus secretion as observed by prior clinical examination. The subgingival biofilm samples for microbiological analyses will be collected by the curette collection method. Before sampling, the teeth are isolated from the cheek and tongue with cotton rolls and the supragingival surfaces are cleaned with rubber cups and polishing paste. Care will be taken not to provoke any bleeding in the adjacent tissues. A Gracey curette will be gently inserted into the pocket and the subgingival biofilm is collected with a single stroke. All samples collected will be pooled together for each patient and shipped in separate transport test tubes, each containing 100 μL of guanidine buffer. All participating clinicians will be trained in advance to perform the sample collection in a standardized manner. The samples will be stored at -70°C until DNA extraction is performed. DNA will be extracted from isolated colonies or from the remaining volume of the sample using a NucliSENS Easymag automated DNA extractor (BioMerieux). Extracted DNAs are then quantified by Qubit dsDNA HS kit (Life Technologies). For mixing studies, the relative contribution of 16S rRNA alleles from each organism will be estimated from quantified input DNA, average genome size of sequenced reference strains, and average 16S rRNA locus copy number for the species. Bacterial genomic DNA isolated from isolates of clinical specimens will be sequenced by the using the Sanger method to establish 16S rRNA gene reference sequences or to attempt molecular diagnosis, where applicable.

Aim 5. To investigate the levels of subgingival plaque azithromycin resistance before and after therapy.

Rationale: The recently demonstrated regional population differences in AZM microbial resistance make it important to determine the microbial AZM resistance in an Australian population. Further, it is important to assure that the use of AZM in NSPT does not result in increased microbial resistance.

Approach: The use of a standard microbial antibiotic resistance method that is reproducible and internationally recognized is proposed.

Methods:

Microbiological sampling

In each quadrant of the dentition, the deepest pocket, showing bleeding on probing and with the maximum of attachment loss will be selected for microbiological sampling. After careful removal of supragingival plaque deposits, isolation of the sampling sites with cotton rolls and after gentle air-drying, two consecutive sterile paper points are inserted to the depth of the pockets and left in place for at least 10 s. Paper points from all four selected periodontal sites will be pooled in 2.0ml of reduced transport fluid (RTF).[25] Samples will be processed within 2 h after sampling.

Microbiological procedures

After vortexing for 30 s, samples are 10-fold serially diluted in RTF and 100 ml of appropriate dilutions are plated on non-selective 5% horse blood agar plates (Oxoid no. 2, Oxoid Ltd., Basingstoke, UK) supplemented with haemin (5 mg/l) and menadione (1 mg/l). Samples are also plated onto trypticase soy serum–bacitracin–vancomycin plates (TSBV)[26] or on Dentaid-1 plates[27] for selective isolation of *Aggregatibacter actinomycetemcomitans*. Blood agar plates

are incubated for up to 14 days at 37 ^{0}C in 80% N_2, 10% CO_2 and 10% H_2. TSBV and Dentaid-1 plates are incubated in air plus 5% CO_2 at 37 ^{0}C for 5 days.
The periodontal pathogens *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum* and are identified using standard anaerobic techniques.[28] *A. actinomycetemcomitans* is identified on the basis of its characteristic colony morphology (star-like inner structure), on the basis of a positive catalase reaction with 3% hydrogen peroxide and on a set of specific enzymes (APIZYM, BioMerieux, Boxtel, the Netherlands). Pure isolates are kept on plates, or are preserved at -70^{0}C, until used for MIC determinations.

B. References

1. Dye BA. Global periodontal disease epidemiology. Periodontol 2000. 2012 Feb;58(1):10–25.

2. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ, CDC Periodontal Disease Surveillance workgroup: James Beck (University of North Carolina, Chapel Hill, USA), Gordon Douglass (Past President, American Academy of Periodontology), Roy Page (University of Washin. Prevalence of periodontitis in adults in the United States: 2009 and 2010. J Dent Res. SAGE Publications; 2012 Oct;91(10):914–20.

3. Brown LJ, Johns BA, Wall TP. The economics of periodontal diseases. Periodontol 2000. 2002;29:223–34.

4. Cullinan MP, Seymour GJ. Periodontal disease and systemic illness: will the evidence ever be enough? Periodontol 2000. 2013 Jun;62(1):271–86.

5. Borgnakke WS, ostalo PVY, Taylor GW, Genco RJ. Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. Journal of Periodontology. American Academy of Periodontology; 2013 Apr 30;84(4-s):S135–52.

6. Badersten A, Nilvéus RR, Egelberg J. Effect of non-surgical periodontal therapy. VI. Localization of sites with probing attachment loss. Journal of Clinical Periodontology. 1985 Apr 30;12(5):351–9.

7. Wylam JM, Mealey BL, Mills MP, Waldrop TC, Moskowicz DC. The clinical effectiveness of open versus closed scaling and root planing on multi-rooted teeth. Journal of Periodontology. 1993 Nov;64(11):1023–8.

8. Lindhe J, Socransky SS, Nyman S, Haffajee A, Westfelt E. "Critical probing depths" in periodontal therapy. Journal of Clinical Periodontology. 1982;9(4):323–36.

9. Saito A, Ota K, Hosaka Y, Akamatsu M, Hayakawa H, Fukaya C, et al. Potential impact of surgical periodontal therapy on oral health-related quality of life in patients with periodontitis: a pilot study. Journal of Clinical Periodontology. 2011 Nov 30;38(12):1115–21.

10. Bartold PM, Bois du AH, Gannon S, Haynes DR, Hirsch RS. Antibacterial and immunomodulatory properties of azithromycin treatment implications for periodontitis. Inflammopharmacology. 2013 Jul 31;21(4):321–38.

11. Foulds G, Shepard RM, Johnson RB. The pharmacokinetics of azithromycin in human serum and tissues. Journal of Antimicrobial Chemotherapy. 1990;25(suppl A):73–82.

12. Feres M, Figueiredo LC, Soares GMS, Faveri M. Systemic antibiotics in the treatment of periodontitis. Periodontol 2000. 2015 Feb;67(1):131–86.

13. Veloo ACM, Seme K, Raangs E, Rurenga P, Singadji Z, Wekema-Mulder G, et al. Antibiotic susceptibility profiles of oral pathogens. 2012 Nov;40(5):450–4.

14. Hicks LA, Taylor TH Jr., Hunkler RJ. U.S. Outpatient Antibiotic Prescribing, 2010. N Engl J Med. 2013 Apr 11;368(15):1461–2.

15. McMullan BJ, Mostaghim M. Prescribing azithromycin. Australian Prescriber. 2015 May 26;38(3):87–9.

16. Hirsch R. Periodontal healing and bone regeneration in response to azithromycin. Aust Dental J. 2010 May 31;55(2):193–9.

17. Parnham MJ, Eraković Haber V, Giamarellos-Bourboulis EJ, Perletti G, Verleden GM, Vos R. Azithromycin: mechanisms of action and their relevance for clinical applications. Pharmacol Ther. 2014 Aug;143(2):225–45.

18. Hirschfeld L, Wasserman B. A long-term survey of tooth loss in 600 treated periodontal patients. Journal of Periodontology. Am Acad Periodontology; 1978;49(5):225–37.

19. Hirsch R, Deng H, Laohachai MN. Azithromycin in periodontal treatment: more than an antibiotic. J Periodont Res. 2012 Mar 31;47(2):137–48.

20. Armitage GC. Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000. 2004.

21. Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC, Devereaux PJ, et al. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. BMJ. 2010;340(mar23 1):c869–9.

22. Seymour GJ, Ford PJ, Cullinan MP, Leishman S, Yamazaki K. Relationship between periodontal infections and systemic disease. Clin Microbiol Infect. 2007 Sep 30;13:3–10.

23. Chapple IL, Garner I, Saxby MS, Moscrop H, Matthews JB. Prediction and diagnosis of attachment loss by enhanced chemiluminescent assay of crevicular fluid alkaline phosphatase levels. Journal of Clinical Periodontology. Munksgaard International Publishers; 1999 Mar;26(3):190–8.

24. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol. Nature Publishing Group; 2015 Jan 1;15(1):30–44.

25. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. Appl Microbiol. American Society for Microbiology (ASM); 1972 Oct;24(4):638–44.

26. Slots J. Selective medium for isolation of Actinobacillus actinomycetemcomitans. J Clin Microbiol. American Society for Microbiology (ASM); 1982 Apr;15(4):606–9.

27. Alsina M, Olle E, Frias J. Improved, low-cost selective culture medium for Actinobacillus actinomycetemcomitans. J Clin Microbiol. American Society for Microbiology; 2001 Feb;39(2):509–13.

28. van Winkelhoff AJ, Carlee AW, de Graaff J. Bacteroides endodontalis and other black-pigmented Bacteroides species in odontogenic abscesses. Infect Immun. 1985 Sep;49(3):494–7.