TITLE: Systemic administration of azithromycin as an adjunct to non-surgical periodontal therapy in stage III and IV periodontitis- a randomized controlled trial.

Title for lay persons: Benefits of systemically administered azithromycin in treating gum disease.

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INTRODUCTION:

Periodontitis is an oral inflammatory disease initiated by plaque, resulting in the destruction of the tissues supporting teeth.[1] It is mainly a result of host-bacterial interactions.[1] Periodontitis is characterized by an abundance of local factors, bleeding on probing, periodontal pockets and clinical attachment loss(CAL).[2] The prevalence of moderate to severe periodontitis is estimated to be between 12-27% of the population in people 35 to 44 years of age worldwide.[3] Recent use of the National Health and Nutrition Examination Survey (NHANES) database in the USA shows that the prevalence and severity of disease continues to increase with age (of adults aged 65 years and older, 64% had either moderate or severe periodontitis).[4] It seems likely that treatment of periodontitis has a very high economic cost.[5] In addition to this, there is growing evidence that there is a cost to patients' systemic health, in particular cardiac health, as a result of periodontal infections.[6] Periodontal disease severity is increased in diabetics and the glycaemic control in diabetes is affected by periodontal disease, a disease that is becoming an epidemic in developed and developing societies world-wide as discussed in a systematic review by Borgnakke et al.[7] Therefore, effective treatment of CP can be expected to not only improve individuals' oral health but also their overall health.

Based on the amount of CAL, periodontitis is classified as stage I- IV periodontitis. It has also been suggested that in pockets of ≥7mm there is a greater need to undertake periodontal surgery to achieve longer term stability.[8] The need for periodontal surgical procedures to control disease has a high economic cost[5] and emotional cost.[9]

The literature supports the effectiveness of azithromycin (AZI)

- against the bacterial flora involved in periodontal disease.[10]
- in delivery to the area of inflammation in effective dosages with a simple oral dosage regimen.[11]
- control of the patients' immune response to their bacterial plaque.[12,13]

- case reports evidence massive improvements in clinical outcomes when AZI is used.[14]

Associate Supervisor Dr VOR has preliminary data that demonstrates that adjunctive use of AZI in the non-surgical periodontal therapy (NSPT) of severe periodontitis results in improved treatment outcomes including the reduced need for surgical therapy.

Our hypothesis is that AZI used in NSPT has a clinically significant beneficial effect in the treatment of stage III and IV periodontitis, reducing or eliminating the need for surgical therapy and increasing the predictability of results thus reducing financial and emotional costs to patients.

To test this hypothesis, we plan to undertake a double blind randomized clinical trial (RCT) on the efficacy of AZI along with NSPT in patients with stage III and IV periodontitis. (Figure 1.)

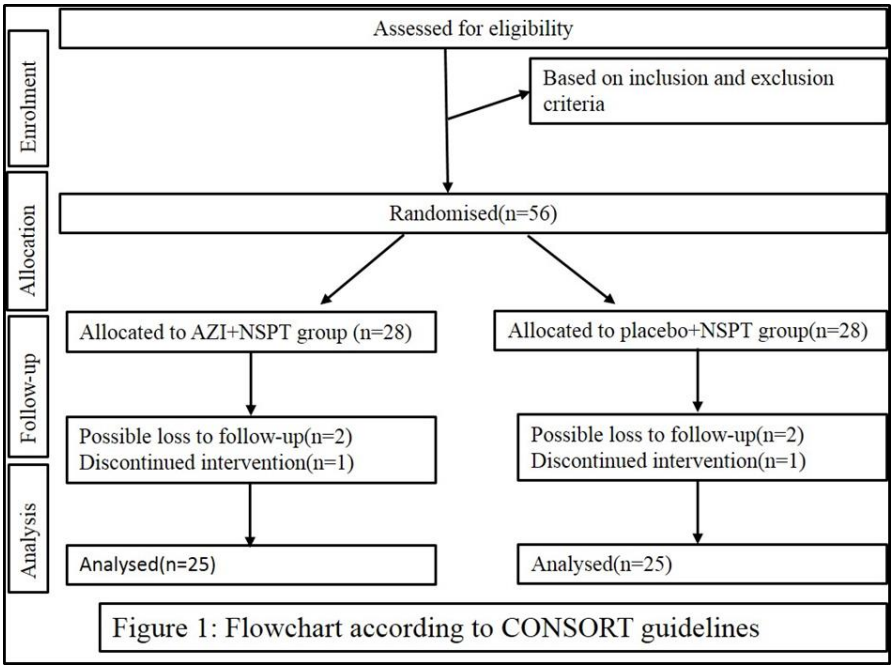


Figure 1: Flowchart according to CONSORT guidelines

BACKGROUND

AZI is a macrolide antibiotic (9-deoxo-9a-methyl-9a-aza-9a-homoerythromycin). Retsema et al.[15] reported on the spectrum and mode of action of AZI. They found that AZI had a significant improvement in potency against gram-negative organisms while retaining the classical erythromycin spectrum. They also reported a high tissue mark-up of AZI with a significantly lower plasma level and quick plasma clearance. It has been reported that in man a single oral dose of 500 mg was bioavailable and produced a peak serum concentration of 0.4 mg/L. Multiple doses only produced a slight increase in plasma concentrations. Serum concentrations rapidly fell, but tissue concentrations were much higher than serum concentrations with peak concentrations of 3 mg/kg reached and a half-life of 3.2 days in tonsillar tissue. This tissue concentration was above the minimum inhibitory concentration (MIC) for bacteria associated with upper respiratory tract infections. Therefore the suggested dosage for soft tissue infections is 500 mg single dose per day for three days.[16] AZI bioavailability in sites of inflammation also make it an antimicrobial of interest in the treatment of plaque induced periodontal disease.[11]

The use of systemic antibiotics may enhance the results of NSPT. The adjunctive use of tetracycline, minocycline, metronidazole, and combination of metronidazole-amoxicillin have been reported.[17] There have been positive results in studies utilizing each of these adjuncts; there have also been a number of papers suggesting no additional benefit.[17] Long dosage times and minor but significant side-effects of these antibiotics have been recorded. These factors have led to poor patient compliance with these regimes. The nature of these antibiotics has also led to concerns about the development of antibiotic resistance.[17]

Recently, it has been suggested that AZI may be a useful addition to NSPT. Its lower plasma concentration, lower incidence of 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 contemplate the usage of this drug.[18]

Recent research has shown a marked difference in antibiotic resistance and susceptibility of putative periodontal pathogens in different countries to AZI and other antibiotics that have been used to enhance the clinical outcomes of NSPT.[19] Although there have been a number of clinical trials reporting the use of AZI in NSPT, the results have been variable.[18] Some of this variability could result from the short time periods of follow-up following therapy, a variation in the quality of study design, usage of AZI in mild and moderate periodontal disease and differences in exposure of different populations to AZI. AZI is one of the most frequently prescribed antibiotics in America.[20] The prescription of AZI in Australia is relatively restricted to a few specific infections (chlamydia and lung infections in cystic fibrosis).[21] Therefore, it could be assumed that there may be a far higher bacterial resistance to AZI in North America and Brazil, the origin of the two studies showing no additional benefit to the use of AZI in NSPT.

Proof of Concept – Pilot Data

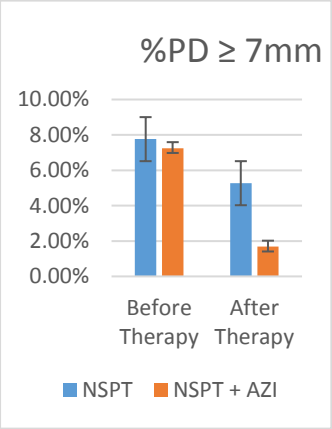


Figure 2: Probing Depth Reduction

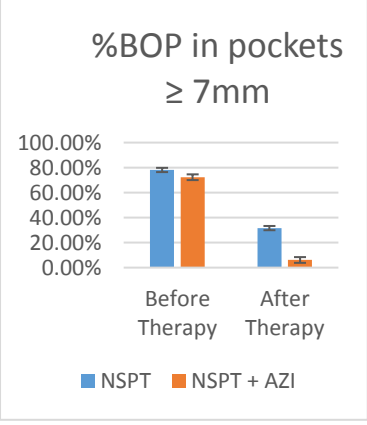


Figure 3: % Bleeding on Probing Reduction

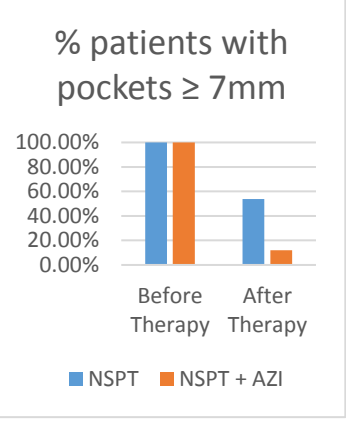


Figure 4: Patients with no pockets greater than 7 mm

Unpublished data from our group (Figures 2, 3 and 4) and clinical experience in Australia[22] are supportive of vastly improved clinical outcomes when utilizing AZI in the NSPT of CP in Australia. Dr VOR has utilized AZI 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 severe 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 AZI. Patients were selected for the additional use of AZI based on their initial less than favourable response to NSPT. We have been able to identify 247 patients receiving AZI, and have been able to match these to a further 247 patients seen for NSPT not receiving AZI 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.
The elimination of pockets of greater than 7 mm (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 7 mm were reduced by the additional use of AZI 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 AZI 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%).

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. Patient compliance (as measured by interview and return of the empty blister pack after completing the course of AZI) was greater than 95% (far higher than experienced with longer courses of antibiotics). Patients reported that the short course and simple dosage of AZI, 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, “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 AZI 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 AZI.

While these clinical results are important, the subjective nature of patient selection for use of AZI in NSPT, the use of AZI 3-4 weeks after completion of NSPT and the distinct possibility of operator bias necessitates the conduction of a randomized controlled trial to confirm these data. Therefore, it is important to assess the response of an Australian population and individuals with the use of AZI.

Significance and Innovation

NSPT has been the “gold standard” for treatment of periodontitis 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 NSPT 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 AZI to have effect on putative periodontal pathogens and it has 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 impressive 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 AZI and possibly allow avoidance of surgical periodontal therapy in an Australian population.

We expect this research will provide clinical proof that use of AZI with NSPT will provide additional benefits in stage III and stage IV periodontitis and this may help to avoid the need for surgical therapy in an Australian population.

This research and the novel use of microbiological and immunological investigations could lead to further research on:

- Simplified tests to predict a patient response to NSPT and allow development of individualized, evidence based treatment plans.
- 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 AZI.

National Benefit

This project would be expected to give evidenced based validation of the additional benefit of AZI as an acceptable addition to NSPT.

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
- Prediction of response to therapy prior to treatment would allow for evidence based individualized treatment plans

The benefits to science would be

- A better understanding of relative roles of modulation of the immune response and changes in bacterial biofilm in clinical response to treatment of periodontal disease
- Greater understanding of the temporal sequence of host response and bacterial response to treatment, which could lead to even more targeted therapy, possibly with an appropriate component of the AZI molecule.
- Development of appropriate tests to allow for prediction of treatment outcome.

Study type and design

Study will be a randomised, parallel design, double blind, and placebo-controlled trial.

AIMS: The aims of this clinical trial are

1) To evaluate the clinical changes in cases of stage III and IV periodontitis following adjunctive systemic administration of AZI with NSPT.
2) To evaluate the changes in the levels of pro-inflammatory cytokines, matrix metalloproteinases (MMPs), Tissue Inhibitors of MetalloProteinases(TIMPs) and bone biomarkers in the gingival crevicular fluid (GCF) and saliva of patients with stage III and IV periodontitis treated with and without systemic AZI as an adjunct to NSPT.
    i. The pro-inflammatory cytokines to be studied are IL-1β, IL-6, IL-8, IL-10, IL-17, Tumor Necrosis Factor-α (TNF-α), lipoxins, resolvins and prostaglandin E2 (PGE2).
    ii. The bone biomarkers to be studied are pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP), receptor activator of nuclear factor-κB-ligand (RANK-L), osteoprotegerin (OPG) and osteopontin.
    iii. The MMPs to be studied are MMP-3, MMP-8, and MMP-13, as well as Tissue Inhibitors of MetalloProteinases-1 (TIMP-1).
3) To evaluate the changes in the microbiology and resistance to antibiotics in the subgingival plaque of patients with stage III and IV periodontitis treated with and without systemic AZI as adjunct to NSPT.

Research Plan

Method

Subjects/Patients:

A. **Recruitment, screening and obtaining informed consent** - Patients enrolled in the screening service of the Oral Health Centre, Herston, Queensland, Brisbane City

Periodontics and Implants, Brisbane, Mount Gravatt and Specialist Dental Centre, 34 Castlereagh Street, Penrith, NSW 2750 will be invited to participate if they have clinical signs that suggest suitability. 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 stage III and IV periodontitis.[2]
   at least 30% of the sites with probing depth or clinical attachment level (CAL) ≥4 mm with bleeding on probing (BOP). Additionally presence of PD≥ 6mm with bleeding on probing of at least 6 sites(total 6 sites) on at least 3 teeth.[2,23]
b. grade C periodontitis as per the new periodontitis classification.[2]
   Grade C periodontitis is further defined by presence of ONE of the following features:
   • ≥2 mm of CAL loss over 5 years,
   • % bone loss/age > 1.0,
   • destruction exceeds expectation given biofilm deposits, specific clinical patterns suggestive of periods of rapid progression and early onset disease (e.g., molar/incisor pattern),
   • smokers ≥10 cigarettes/day,
   • HbA1c ≥ 7.0% in patients with diabetes mellitus
c. age 25 to 70 years;
d. At least 16 natural teeth.

The exclusion criteria for this study will be as follows:

a. use of antibiotics within three months preceding the start of the study;
b. history of sensitivity to AZI or macrolides
c. prolonged QT interval or history of cardiac arrhythmia or history of myocardial infarction.
d. periodontal therapy including root surface debridement in the past 6 months
e. pregnant women

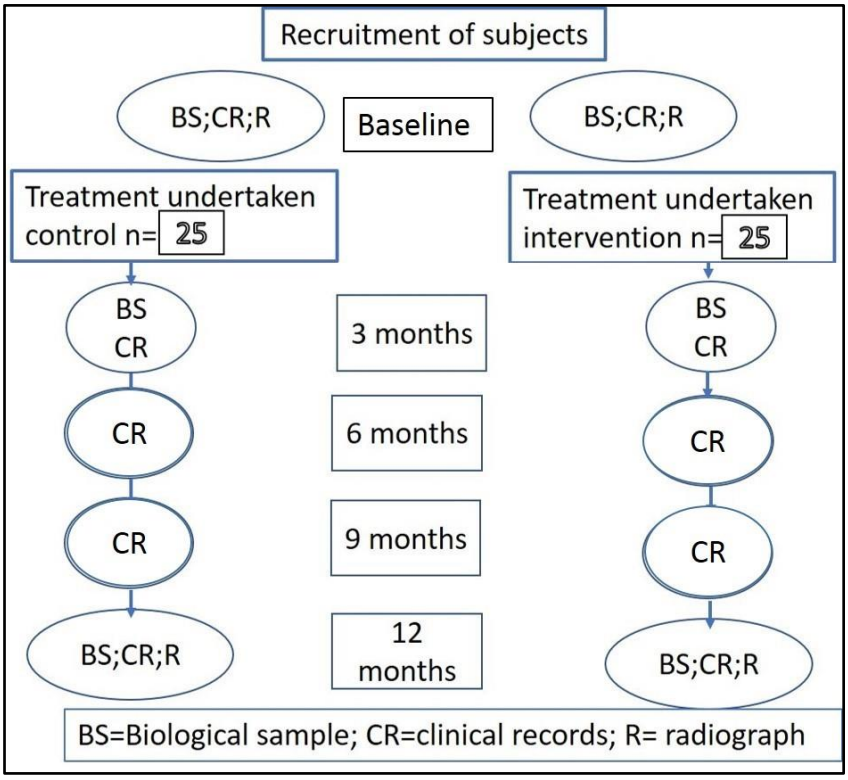
B. Prior to treatment collection of
   a. Normal periodontal therapy pre-treatment records
      i. Intra-oral clinical photographs

ii. Orthopantomograph (OPG) radiograph
iii. Periodontal recording (measuring periodontal pockets, clinical attachment loss, plaque and bleeding on probing, tooth mobility, furcation involvement, infrabony pockets, prognosis)

b. Biological Sampling
   i. Collection of saliva to assess the possible alteration of the oral immune responses to the periodontal therapy/intervention.[24]
   ii. Collection of gingival crevicular fluid (GCF) a normally occurring fluid exuded from the gingiva to assess the possible alteration of the local immune responses to the periodontal therapy/intervention.
   iii. Collection of subgingival plaque samples to assess the bacterial response and changes in antibiotic resistance to periodontal therapy/intervention.[25]

Prognosis of the patients will be classified into secure, doubtful, poor and irrational to treat.[26] Patients will be provided with all the possible treatment options based on the severity of the disease. Patients with stage I and stage II periodontitis will be treated with NSPT by postgraduate students at the Oral Health Centre, Herston. Patients with stage III and IV periodontitis will be invited to participate in this study. In case of patients' opting for surgery, second and/or final year postgraduate students will provide surgical periodontal therapy. Patients with stage III and IV periodontitis agreeing to participate in the study will be randomly assigned into two groups: control group (NSPT with placebo) and test group (NSPT with systemic AZI).

Statistical analysis: Sample size calculations: With at least 25 subjects per arm, we will be able to detect an effect size of reduction of periodontal inflamed surface area score (PISA) of 297 mm^2 at 80% power and a two-sided alpha level of 0.05. Periodontal inflamed surface area score is an integrated score that measures the inflammatory burden on the periodontium calculated from clinical attachment loss, pocket depth, gingival recession and bleeding and probing.[27,28] It is proposed to enrol 56 (28 controls and 28 test group) patients to compensate for drop out.



INTERVENTION: NSPT (root surface debridement) will be undertaken over two treatment sessions by calibrated periodontists for all patients within three days. The patients will be asked to take either a placebo or AZI (based on a randomized schedule blinded to the operator and the patient) commencing immediately with a single dose on the first day of NSPT and further two dosages taken over the next two days. All clinical recordings and biological samples for each patient will de-identified with the code held by the PI (Prof SI) in a secure location in the Oral Health Centre. The codes identifying patients receiving placebo or AZI will be held by PI (Dr. RL) in a sealed opaque envelope securely and not broken until completion of each phase of the trial. These measures ensure compliance with Consolidated Standards of Reporting Trials (CONSORT) guidelines.[29]

The rationale for our longitudinal design is to ensure a valuable clinical trial that 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.

The next paragraphs summarize the protocols to be followed to each of the aims in the study.

Aim 1. A double blind randomized clinical trial to test the efficacy of the addition of AZI to NSPT.

Methods:

Rationale: Ultimately, it is the clinical response to therapy that is the important outcome. Previous studies on the use of antibiotics as an adjunct to NSPT have shown that although initial (6-12 week) responses can be favorable, longer term results (up to 12 months) can show a diminution of the differences. In addition to this, the design of this study and the determination of subject numbers will allow assessment of the true clinical significance of the adjunctive use of AZI; that is the ability to reduce pocket depth by 2 mm thus reducing the need for surgical therapy.

Approach: Clinical recordings will be undertaken at the time points above and validated by experienced periodontists.

Methods:

Records of probing depths (PD) and recession to allow calculation of 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. Expectations from clinical practice and previous research are that 50% of those receiving standard care will respond clinically with a significant improvement in ΔPD, ΔCAL and Δ%BOP and we would expect the AZI group will achieve a significant improvement. The experimental unit for statistical evaluation will be a patient. The mean reduction in periodontal inflamed surface area measured at baseline and followed at 12 months follow up is the primary outcome. To evaluate statistical significance of the mean difference between the two groups (control and intervention groups) at various time points ANOVA (analysis of variance) will be used. To assess the difference in means between the control and intervention groups an ANCOVA model will be used to adjust the baseline parameters, wherein the treatment groups will be the factors and baseline PISA scores will be the co-variates. A repeated measures ANOVA will be used to perform a group comparison across the different time points (baseline, three, six, nine and twelve months) by treatment groups. The level of statistical significance is kept at 0.05. Secondary analysis will use generalised linear mixed models allowing for attrition to compare these groups across time

profiles of follow up for PD, CAL and BOP.

Any information on adverse effects/harms occurring due to the intervention/administration of AZI will be collected. The form for reporting of suspected adverse reaction to medicines has been attached along with the application.

Aim 2. To quantify the changes in local immune response with the use of AZI in NSPT.

Rationale: Bartold et al.[18] discussed the immunoregulatory effects of AZI 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*.[18,30]

There has been much interest in assessing saliva and GCF for markers of inflammation to further define periodontal disease entities and for identification of "at risk" individuals.[23,31] These have focused on the measurement of pro-inflammatory markers such as IL-1β, IL-6, IL-8, IL-10, IL-17, Tumor Necrosis Factor-α (TNF-α) and prostaglandin E2(PGE2), pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP), receptor activator of nuclear factor-κB-ligand (RANK-L), osteoprotegerin (OPG), osteopontin, MMP-3, MMP-8, and MMP-13, TIMP-1, as well as lipoxins and resolvins.[32,33] Serhan and his group have identified a number of pro-resolving cytokines called lipoxins.[33] It is proposed that imbalance in an individual's pro-inflammatory and pro-resolving immune responses to bacterial plaque may explain the variable population response.[34] To date no clinical studies have investigated lipoxins in periodontal disease.

Approach: Sampling of saliva and GCF will reflect to the local immune response to microbial plaque and to therapy. Pro-inflammatory and pro-resolving cytokines can be detected in saliva and GCF. The use of multiplex assays to assess these cytokines gives a very accurate method particularly in the small volumes of sample available.

Methods:

Salivary Fluid Sampling:

Subjects will be asked to rinse intensively with 15–20 mL tap water for 20 s to remove food debris. Afterwards the water is discarded. Then they will wait for 1 min, while saliva is swallowed. Thereafter, they will take 5.5 mL of rinsing fluid (sterile purified water) to rinse the oral cavity for 30 s. This rinsing specimen will be spitted into a cup and 3 mL of this solution will be filled into a syringe. To filter the sample, a 0.45 ml micro-filter will be placed on the

tip of the syringe. The rinsing specimen is subsequently filtered a second time using a new filter to receive an aliquot of 0.3 mL filtrate. This filtrate will be stored at -70°C and stored for quantitative analysis at a later date. These tests will be undertaken using multiplex assay.

Protocol for collection of GCF: Periotron 8000(Oralflow Inc., New York, NY, USA) will be used to quantify the amount of GCF.[35] The deepest periodontal pockets in the mouth will be chosen for collection of GCF. The tooth with deepest periodontal pocket will be isolated with cotton rolls and drying the external surface of the gingiva with a stream of air. Visible supragingival plaque will be removed with the help of a scaler. Periopaper strips are paper strips used to soak GCF up to 1.2 µL.[35] The absorbent portion is placed within the gingival sulcus, whereas the other end is coated with plastic for handling with forceps or tweezers. The Periopaper strip will be placed into the buccal sulcus of the periodontal pocket up to a maximal depth of 1 mm for 30 secs. If the Periopaper strip is contaminated with blood or saliva, such strips will be discarded. New strips will be used to collect a fresh sample of GCF after 30 minutes. The amount of fluid will be measured by Periotron device.[35]

In brief, GCF and 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 multiplex assay(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.

Aim 3. To investigate the alterations in the microbiology and resistance to antibiotics among the subgingival plaque bacteria with the use of AZI in NSPT.

Rationale for changes in microbiology*:* It is tempting to continue to undertake ongoing measurement of microbiological changes by cultures or ELISA (Enzyme-linked immunosorbent assay) evidence of putative periodontal pathogens. However, it now appears clear that although there are increased numbers of the putative pathogens in periodontitis they are present 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 individual's biofilm changes as a result of therapy.[36]

Approach to study the microbiological changes: 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 to study the microbiological changes: For each subject, subgingival biofilm samples will be collected from the four deepest sites 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. The adhering material is wiped off onto a sterile paper point. All samples collected will be pooled together for each patient and shipped in separate transport test tubes, each containing 100 μL of guanidine buffer. Sample collection will be done in a standardized manner for all patients. The samples will be stored at -80^0C 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.

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

Approach to study the changes in antibiotic resistance among plaque bacteria: The use of a standard microbial antibiotic resistance method that is reproducible and internationally recognized is proposed.

Methods to study the changes in antibiotic resistance among plaque bacteria.

This will be carried out at baseline and at the end of the study(12 months).

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, subgingival plaque samples will be collected using a curette as previously suggested. The collected sample from all four selected periodontal sites will be pooled in 2.0ml of reduced transport fluid (RTF).[35] Samples will be processed within 2 hrs after sampling.

To assess the effectiveness of AZI against periodontitis associated pathogens, next generation sequencing (Illumina HiSeq series, Australian Centre for Ecogenomics, University of Queensland) will be used to identify and enumerate the microbiota present in plaque samples collected with a curette from the periodontal pockets throughout the 12-month healing period of the study. Primer sets used for sequencing cover the V1 - V3, V3 - V4, V5 - V8 and V6 - V8 hyper-variable regions of the 16S rRNA gene facilitating the identification of different microorganisms present.

Antibiotic Resistance: The emergence of resistance to macrolides, including AZI, in microbes associated with peri-implantitis, such as the staphylococci, has been reported. In most cases, macrolide resistance has been linked to target site alteration due to methylation of 23S rRNA within the large ribosomal subunit by a family of rRNA methyltransferases designated Erm. Erm enzymes catalyze mono or dimethylation of a specific adenine residue in the 23S rRNA. The methyl group sterically hinders the macrolide binding site and disrupts the hydrogen bonding between the macrolides and the rRNA, thus rendering bacteria resistant. Methylation of adenosine 2058 or 2059 is the most common mechanism of resistance. Methylation-sensitive high-resolution melt polymerase chain reaction (MS-HRM PCR), a sensitive method for the assessment of methylation, will be used to determine the presence of erm mutations within the

microbiota of the subgingival plaque samples to be collected. Primers will be designed against the completely methylated sense strand sequence of 23S rRNA erm genes and PCR amplification of the DNA will be carried out using a PCR thermocycler and high resolution melt analysis (Roche Applied Science).

Follow up: Patients will be followed at two weeks, one month, 3 months, 6 months, 9 months and 12 months after treatment.

At each of the following time points, periodontal maintenance therapy will be performed. Periodontal maintenance therapy performed will be similar in both the test and control group of patients. Periodontal maintenance therapy will be performed by experienced periodontists and will adhere to the standardized protocol for maintenance as described below.[38] Dental examination will include assessment of caries status, changes in the status of any restorations or prosthesis (including dental implants). All periodontal parameters including general levels of plaque and calculus, PPD, CAL, BOP, furcation invasion, tooth mobility, and exudation of pus will be examined at each of the time-points. Maintenance therapy for each of the patients will involve removal of supragingival and subgingival plaque and calculus (if any), and reinforcement of oral hygiene instructions. Radiographs (OPG) will be taken at the one-year follow-up appointment. Compliance with the suggested recall intervals will be monitored. A time gap of two weeks will be accepted for reporting of patients for the purpose of follow-up to a given follow-up appointment.

Patient-reported outcome measures will be collected from all patients using a questionnaire.

End Points: The following will be the endpoints expected at the end of the study:

Primary end-point: The mean reduction in periodontal inflamed surface area measured at baseline and followed at 12 months follow up.

Secondary end-point: A significant decrease in the levels of the studied cytokines in the GCF and saliva of patients treated with azithromycin plus NSPT compared to NSPT plus placebo. The studied cytokines are IL-1β, IL-6, IL-8, IL-10, IL-17 and TNF-α, prostaglandin E2(PGE2), pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP), receptor activator of nuclear factor-κB-ligand (RANK-L), osteoprotegerin (OPG) and osteopontin, MMP-8, MMP-3, MMP-13, TIMP-1, lipoxins and resolvins.

Secondary end-points

Microbiology and antibiotic resistance: A significant change in the microbiology (as evidenced by reduction in the number of bacterial counts) of the deepest periodontal pocket from baseline to all the time points throughout the study(baseline, three months, six months, nine months and 12 months). Any significant change in the resistance to antibiotics among subgingival bacteria from baseline versus 12 months in the patients treated with or without AZI.

Study Plan:

1. Approval from ethics committee and clinical trial registration (6 months)
2. Recruitment of patients + data collection and oral prophylaxis+ treatment with intervention including follow-up (18 months). Follow-up will be done at the following time intervals: baseline, 3 months, 6 months, 9 months and 12 months.
3. Statistics & preparation for publication (6 months)

Component/Year	Year 1	Year 2	Year 3
Ethics approval, patient recruitment			
Patient Treatment			
Biological sample testing			
Statistics & Preparation for publication			

Analysis

Mean pocket depths and mean gain in CAL with standard deviations (SD) will be calculated at baseline, 3 months, 6 months, 9 months and 12 months post-intervention in both the control and test groups. Similarly changes in the levels of cytokines in the GCF and saliva of test and control group of patients will be compared. An independent *t*-test will be used to analyse and compare the changes in pocket depths and CAL between control and test groups. Significance level will be set at $p < 0.05$.

Ethical issues: Patients attending the Oral Health Center, Herston, Brisbane City Periodontics and Implants, Brisbane, QLD, 4000, Brisbane City Periodontics and Implants, Mount Gravatt, 4122 and Specialist Dental Centre, 34 Castlereagh Street, Penrith, NSW 2750 will be screened and eligible participants as per the inclusion criteria will be invited to participate in the study. Patients will be provided with patient information sheet and consent forms. Patient information sheet provides all the required information about the study. Consent forms include both the

informed consent form and the form for withdrawal of consent. Patients will be included in the study only after signing the informed consent form. Informed consent will be the only method of taking consent in this clinical trial. Both the patient information sheet and consent forms are attached along with this application. Patients are free to withdraw from the trial unconditionally anytime throughout the study period. Patients will be provided with phone numbers of treating specialists and postgraduate student (SSR) in case of any adverse events related to the treatment or drug.

Since NSPT is the standard of care for chronic periodontitis no major adverse events are expected due to this treatment. AZI has been considered a safe antibiotic with minimal adverse effects including gastric upset, vomiting, diarrhoea and headache. In case patients report of any adverse effects, patients will be asked to discontinue with the drug. Patients with cardiac arrhythmia, history of myocardial infarction have been listed as exclusion criteria in the study to ensure safety of the patients.

Non-compliance to the prescribed antibiotic regimen is one of the reasons for emergence of resistant micro-organisms.[39,40] Patients taking once daily antibiotic regimens were found to be more compliant than those taking twice daily or thrice daily antibiotic regimens for upper respiratory tract infections.[40] Suggestions have been made to prescribe once daily antibiotic regimens/formulations, wherever feasible than twice daily or thrice daily antibiotic regimens.[39]Along similar lines, patients on AZI once daily would be more compliant than patients on a combination of amoxicillin-metronidazole, a popular choice as adjunct in the treatment of periodontal diseases. This is especially true for drugs like AZI having long-half life, as they continue to be effective even if there is a delay in taking a dose or even skipping a dose.[40] So, these drugs are called "forgiving drugs".[39] So prescription of AZI may not add to the emergence of resistant microorganisms.

Resource requirements:

1) AZI and placebo tablets will be prepared at Pharmacy Australia Centre of Excellence, Brisbane.
2) Examination, recruitment and treatment of patients for the trial will be carried out at Oral Health Centre, Herston, Brisbane City Periodontics and Implants, Brisbane and Mount Gravatt and specialist Dental Centre, 34 Castlereagh Street, Penrith, NSW 2750.

Samples of saliva, GCF and subgingival plaque will also be collected at the Oral Health Centre, Herston.

3) All the patient samples will be stored in Eppendorf tubes, in -70°c fridge, at Level 6, Research lab, School of Dentistry, The University of Queensland.
4) Microbiological assessments of subgingival plaque, measurement of cytokine markers, genome and proteome analysis from saliva and gingival crevicular fluid will be done at Centre for Clinical Research, University of Queensland (UQCCR).

Supervision: Supervision of the PhD work will be done by Prof Saso Ivanovski (Principal supervisor), Dr Valerie Woodford, Dr Roderick Marshall and Dr Tino Mercado, Dr Ryan Lee and Dr Pingping Han. All are full time faculty members at the School of Dentistry, the University of Queensland. Prof Saso Ivanovski, and Dr Ryan Lee are registered specialists in Periodontics working at Oral Health Centre, Herston.

Dissemination of findings

Report of the clinical trial will be submitted as a report to the School of Dentistry, The University of Queensland. Manuscripts will be submitted to international peer-reviewed journals for publication to disseminate the findings of this clinical trial. In addition, since the clinical trial will be registered at the Australian New Zealand Clinical Trials Registry, the results of the study will be updated in the registry.

References:

1. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. Periodontol 2000. 1997;14:9-11.
2. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, Flemmig TF,Garcia R, Giannobile WV, Graziani F, Greenwell H, Herrera D, Kao RT, Kebschull M,Kinane DF, Kirkwood KL, Kocher T, Kornman KS, Kumar PS, Loos BG, Machtei E, Meng H, Mombelli A, Needleman I, Offenbacher S, Seymour GJ, Teles R, Tonetti MS. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Periodontol. 2018 Jun;89 Suppl 1:S173-S182.

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

4. 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. 2012;91(10):914–20.
5. Brown LJ, Johns BA, Wall TP. The economics of periodontal diseases. Periodontol 2000. 2002;29:223–34.
6. Cullinan MP, Seymour GJ. Periodontal disease and systemic illness: will the evidence ever be enough? Periodontol 2000. 2013;62(1):271–86.
7. 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;84(4-s):S135–52.
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;38(12):1115–21.
10. Belibasakis GN, Thurnheer T. Validation of antibiotic efficacy on in vitro subgingival biofilms. Journal of Periodontology. 2014;85(2):343–8.
11. Gomi K, Yashima A, Iino F, Kanazashi M, Nagano T, Shibukawa N, et al. Drug concentration in inflamed periodontal tissues after systemically administered azithromycin. Journal of Periodontology. 2007;78(5):918–23.
12. Čulić O, Eraković V, Parnham MJ. Anti-inflammatory effects of macrolide antibiotics. Eur J Pharmacol. 2001;429(1-3):209–29.
13. Doyle CJ, Fitzsimmons TR, Marchant C, Dharmapatni AASSK, Hirsch R, Bartold PM. Azithromycin suppresses P. gingivalis LPS-induced pro-inflammatory cytokine and chemokine production by human gingival fibroblasts in vitro. Clin Oral Investig. 2015;19(2):221–7.
14. Hirsch R. Periodontal healing and bone regeneration in response to azithromycin. Aust Dental J. 2010;55(2):193–9.
15. Retsema J, Girard A, Schelkly W, Manousos M, Anderson M, Bright G, Borovoy R, Brennan L, Mason R. Spectrum and mode of action of azithromycin (CP-62,993), a new 15-membered-ring macrolide with improved potency against gram-negative

organisms. Antimicrob Agents Chemother. 1987;31(12):1939-47.

16. 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.
17. Feres M, Figueiredo LC, Soares GMS, Faveri M. Systemic antibiotics in the treatment of periodontitis. Periodontol 2000. 2015;67(1):131–86.
18. Bartold PM, Bois du AH, Gannon S, Haynes DR, Hirsch RS. Antibacterial and immunomodulatory properties of azithromycin treatment implications for periodontitis. Inflammopharmacology. 2013;21(4):321–38.
19. Veloo ACM, Seme K, Raangs E, Rurenga P, Singadji Z, Wekema-Mulder G, et al. Antibiotic susceptibility profiles of oral pathogens. Int J Antimicrob Agents. 2012;40(5):450–4.
20. Hicks LA, Taylor TH Jr., Hunkler RJ. U.S. Outpatient Antibiotic Prescribing, 2010. N Engl J Med. 2013;368(15):1461–2.
21. McMullan BJ, Mostaghim M. Prescribing azithromycin. Australian Prescriber. 2015;38(3):87–9.
22. Hirsch R, Deng H, Laohachai MN. Azithromycin in periodontal treatment: more than an antibiotic. J Periodont Res. 2012;47(2):137–48.
23. Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. J Periodontol. 2007 Jul;78(7 Suppl):1387-99.
24. Ebersole JL, Nagarajan R, Akers D, Miller CS. Targeted salivary biomarkers for discrimination of periodontal health and disease(s). Front Cell Infect Microbiol. Frontiers; 2015;5:62.
25. Armitage GC. Learned and unlearned concepts in periodontal diagnostics: a 50-year perspective. Periodontol 2000. 2013;62(1):20–36.
26. McGowan T, McGowan K, Ivanovski S. A Novel Evidence-Based Periodontal Prognosis Model. J Evid Based Dent Pract. 2017 Dec;17(4):350-360.
27. Nesse W, Abbas F, van der Ploeg I, Spijkervet FK, Dijkstra PU, Vissink A. Periodontal inflamed surface area: quantifying inflammatory burden. J Clin Periodontol. 2008 Aug;35(8):668-73.
28. Rosenberg DR, Andrade CX, Chaparro AP, Inostroza CM, Ramirez V, Violant D, Nart J. Short-term effects of 2% atorvastatin dentifrice as an adjunct to periodontal therapy:a randomized double-masked clinical trial. J Periodontol. 2015 May;86(5):623-30.

29. 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(1):c869–9.
30. 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;143(2):225–45.
31. Nomura Y, Shimada Y, Hanada N, Numabe Y, Kamoi K, Sato T, et al. Salivary biomarkers for predicting the progression of chronic periodontitis. Arch Oral Biol. 2012;57(4):413–20.
32. Lorenz K, Keller T, Noack B, Freitag A, Netuschil L, Hoffmann T. Evaluation of a novel point-of-care test for active matrix metalloproteinase-8: agreement between qualitative and quantitative measurements and relation to periodontal inflammation. J Periodontal Res. 2017;52(2):277-284.
33. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol. 2008;8(5):349–61.
34. Van Dyke TE, Serhan CN. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. J Dent Res. 2003;82(2):82–90.
35. Bevilacqua L, De Biasi M, Lorenzon MG, Frattini C, Angerame D. Volumetric Analysis of Gingival Crevicular Fluid and Peri-Implant Sulcus Fluid in Healthy and Diseased Sites: A Cross-Sectional Split-Mouth Pilot Study. The Open Dentistry Journal. 2016;10:131-138.
36. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol. Nature Publishing Group; 2015;15(1):30–44.
37. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. Appl Microbiol. American Society for Microbiology (ASM); 1972;24(4):638–44.
38. Parameter on periodontal maintenance. American Academy of Periodontology. J Periodontol. 2000;71(5 Suppl):849-50.
39. Kardas P. Patient compliance with antibiotic treatment for respiratory tract infections. J Antimicrob Chemother. 2002;49(6):897-903.
40. Falagas ME, Karagiannis AK, Nakouti T, Tansarli GS. Compliance with once-daily versus twice or thrice-daily administration of antibiotic regimens: a meta-analysis of randomized controlled trials. PLoS One. 2015;10(1):e0116207.