# The Effect of Azithromycin on Acute and Chronic Inflammation in an *in vivo* Experimental Model

A report submitted to the University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

AH du Bois

# Table of Figures

# Table of Tables

# Declaration

I, Alexandre Du Bois, declare that this work to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text. It contains no material which has been accepted for the award of any other degree of diploma in any university or tertiary institution.

I give consent to this copy of my thesis to be made available to the University Library, for loan or photocopying, subject to the provisions of the Copyright Act 1968, as well for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Declared by:_____ Witnessed by: _____

A Du Bois

Date: _____ Date: _____

# Acknowledgements

I would like to use this opportunity to express my deepest gratitude towards all the people that were involved with this research project, as it is only due to their help and support that the completion of this task was possible.

First and foremost, I would like to thank Professor PM Bartold for giving me the opportunity to embark on this project. His insight, interest and knowledge have been inspirational, to say the least, only surpassed by his humbleness. I am privileged to have had him as my supervisor and mentor.

Furthermore, I would like to thank Mr Victor Marino. He has been inspiring in his tireless effort in helping me with all aspects surrounding this project. His selfless generosity and sacrifices, both in time and effort have deeply moved me. I will surely miss his advice and help, but especially his friendship and camaraderie that I have enjoyed throughout this project. Thank you Victor.

A special thanks also to Ms Ceilidh Marchant for not only helping me with the ELISA assays but also histological analysis. Thank you for always having your door open, sharing your advice and always being prepared to help.

I would also like to thank Marjory Quinn for her indefatigable effort in the preparation of the many histological samples used in this study. Her experience, knowledge, expertise and skill have been invaluable to this project.

Thank you to Dr Robert Hirsch for his input and honesty and sharing his invaluable experience in evaluation of the histological sections. Your help is much appreciated.

Further, I would like to thank Dr Kencana Dharmapatni for all her help and time. Her insight and enthusiasm for this project have been inspirational and it has been a great having her input. However, I would like to thank her most for the patience and kindness towards me.

Thank you also to Michelle Lorimer, who helped in the statistical analysis of the data, all the staff at the IMVS who helped with housing and caring for the animals used for this study, as well as Associate Professor David Haynes and Syatirah Abdhulla in regards to donating and teaching me culturing techniques for the preparation of the bacteria used in this

experiment. Furthermore, I would like to thank Lynn Waterhouse, Dr Gafahr Sarvestani and Dale Caville for the help I received in the capturing of images for this thesis.

Many thanks also to my class mate and friend, Dr Siobhan Gannon for her help and encouragement and my other colleagues Dr Emma Megson, Dr Tina Choo, Dr Geoff Harvey, Dr Kere Kobayashi, Dr Arlene Khaw, Dr Catherine Doyle, Dr Brian Chee and Dr Boram Park for their friendship.

Finally, although by no means least, I would like to thank my family for their continuing support and faith in me. I especially want to thank my beautiful wife, Dr Christine du Bois, for her unconditional love, friendship and endless patience and support as she has always been there for me. I could not ask for a better companion and friend in life.

# Chapter 1. Literature review

## 1.1 Introduction

Chronic periodontitis is a common disease of the oral cavity that affects around 5-20% of the adult population globally (Burt, 2005, Borrell et al., 2005), although in populations with poor oral care prevalence occurrence may be as high as 60% for chronic periodontitis and up to 90% for gingivitis (Page et al., 1997). Nevertheless, differences in susceptibility between individuals have been identified (Löe et al., 1986, Cutress et al., 1982, Reddy et al., 1986). Periodontitis is marked by chronic inflammation of the periodontal tissues initiated by accumulation of dental plaque. As the plaque matures it changes in composition from Gram positive cocci to Gram negative cocci, rods and spirochetes and in particular, the bacteria *Porphyromonas gingivalis, Treponema denticola* and *Tanerella forsythia* are associated with the initiation and propagation of periodontal destruction (Socransky and Haffajee, 2002). However it is thought that the bulk of tissue destruction occurs as a result of the host response (Birkedal-Hansen, 1993) that is triggered by the bacteria as well as their products, and is driven by inflammatory processes in which neutrophils, macrophages, lymphocytes and fibroblasts are the cells that play the biggest role, (Van Dyke et al., 1993). As such it has become evident that periodontal destruction occurs as a result of the interplay between the pathogen and host response, which is influenced by several factors, including genetics and environmental risk and predisposing factors (Kornman, 2008).

Due to the complex nature of the disease, little has changed in terms of treatment strategies for the management of periodontitis over the last few decades. At present, therapy is still mainly aimed at reducing the number of pathogens triggering the host response. To date, this is still primarily achieved by mechanical debridement and was found to improve both clinical and microbiological parameters (Greenstein, 1992).

However not all patients respond well to periodontal treatment and some may still lose teeth despite both non surgical and surgical periodontal therapy (Hirschfeld and Wasserman, 1978, McFall, 1982, Pearlman, 1993) which seems to be especially true for those with aggressive forms of periodontal disease (Drisko, 2001). Furthermore, areas with deep pocketing are hard to debride and may need surgery to facilitate calculus removal (Caffesse et al., 1986). Recognizing these limitations, adjuncts to conventional therapy have been used in order to obtain better outcomes.

It has thus been suggested that for patients suffering from aggressive periodontitis and multiple failing sites, the use of systemic antibiotics in conjunction with mechanical debridement may be of value (Heitz-Mayfield, 2009). In the past, several different antibiotics

have been used for the treatment of aggressive periodontal disease. The most popular to date are tetracycline (Al-Joburi et al., 1989), metronidazole combined with amoxicillin (Yek et al., 2010) and clindamycin (Brook et al., 2005), which have all been found to produce varying degrees of success (Haffajee et al., 2003).

Several other pharmaceutical agents have also been used in an attempt to modulate the host inflammatory response as well as bone destruction with varying success (Bartold et al., 2010).

Recently, it has become evident that macrolide antibiotics seem to possess not only antimicrobial properties, but also modulate inflammation. Due to this unique combination of both anti-inflammatory and antimicrobial properties, macrolides and especially azithromycin are currently used for several severely debilitating pulmonary conditions such as cystic fibrosis and panbronchiolitis which have both an inflammatory and microbial component (Giamarellos-Bourboulis, 2008). For the same reason, azithromycin may be of value as an adjunct in the management of periodontitis.

## 1.2 Pharmacology and background

### 1.2.1 Pharmacology of azithromycin

Azithromycin falls under the antimicrobial drug group of macrolides and is derived by chemically altering erythromycin. The macrolides are a commonly used family of antimicrobials isolated from the gram-positive bacteria *Streptomycetes*. The molecular structure of the macrolides is typically that of a macrocyclic 14-membered lactone ring with ten asymmetric centres and two sugars. Erythromycin A was the first member of the group and was obtained from cultures of *Streptomyces erythraea* in 1952. However there are several limitations associated with erythromycin such as its instability under the acidic conditions in the stomach (Addy and Martin, 2004), resulting in poor absorption and blood concentrations. This poor absorption, together with the short half life of erythromycin (T½= 1.5 hrs), in turn leads to the necessity to administer comparatively high doses over short intervals (6 hourly) which are associated with poor patient tolerance and increased side effects such as epigastric distress, nausea and vomiting. As a result the maximum dosage as well as patient compliance with erythromycin are limited (Addy and Martin, 2004).

Azithromycin differs chemically from erythromycin in that it is comprised of a 15 membered ring and contains nitrogen and is thus more correctly referred to as an azalide (Culic et al., 2001) (Figure 1). It is formed by inserting methyl-substituted nitrogen in place of the carbonyl group at the 9a position of the glycone ring, which results in a highly stable compound. This makes azithromycin more resistant to acid breakdown, giving it a longer half

life and tissue penetration as well as greater activity against gram negative bacteria compared to erythromycin (Zuckerman et al., 2009).

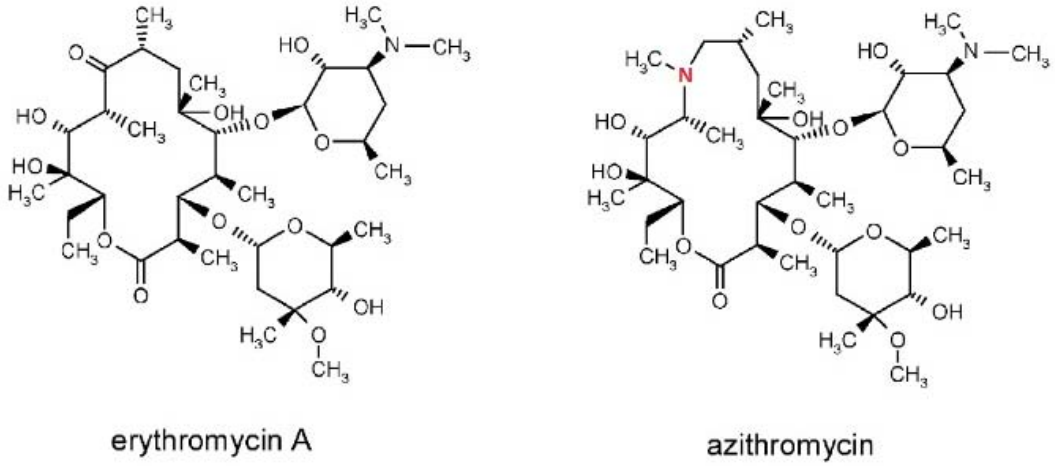



**Figure 1.** Demonstrating the molecular structure of macrolides and azides (Adapted from Culic et al., 2001). Note the presence of nitrogen in the macrolide ring.

As such azithromycin is approximately 300 times more acid stable then erythromycin, improving its uptake (Fiese and Steffen, 1990) and is associated with significantly fewer gastro-intestinal problems than erythromycin (Langley et al., 2004). Furthermore, azithromycin uptake is not dependent on concurrent food intake (Foulds et al., 1996). A further advantage is that owing to its lipophilic nature (Zuckerman et al., 2009), azithromycin has the ability to penetrate cells, including tissue fibroblasts and epithelial cells (Gladue and Snider, 1990) and phagocytic cells such as macrophages and polymorphonuclear leukocytes (Gladue et al., 1989, Bosnar et al., 2005).

In *in vitro* models, aimed at analysing cell penetration by the drug, it was found to have 226 times the intracellular concentration compared to the extracellular surrounding, and *in vivo* was found to have a cellular uptake 26 times higher than erythromycin (Gladue et al., 1989), reaching concentrations of up to 800 times the serum concentration in lung macrophages (Rodvold et al., 1997). Additionally, once inside phagocytic cells, the drug is transported within these cells and actively released at the site of infection (Gladue et al., 1989, Mandell and Coleman, 2001) including the gingiva (Blandizzi et al., 1999). Consequently, it appears there are two methods of azithromycin accrual in tissue, one being a more passive process in which it is directly taken up into tissue, as is the case for fibroblasts (Gladue and Snider, 1990) and the other being active transport to the area of infection via phagocytes (Gladue et al., 1989). The intracellular accumulation of azithromycin is further augmented when it is administered over longer periods of time, especially as no saturation point for intracellular uptake could be identified (Bosnar et al., 2005). As a result it has been found in intracellular drug concentrations exceeding 3000 times serum levels in patients on long-term

therapy (Wilms et al., 2006). Furthermore, the high intracellular levels achieved in macrophages were found to be further increased by up to 200% if azithromycin was administered to macrophages which had been stimulated with TNF-α or IFN-γ (Bermudez et al., 1991).

Once inside the cells, macrolides preferentially accumulate inside lysosome and azurophilic granules from which they can readily be released (Carlier et al., 1987). Of the members of the macrolide group, azithromycin has the ability to remain inside the cell even after serum concentrations have dropped. Nevertheless, the exact method of cellular uptake of azithromycin is still not totally clear at present. It is thought that the majority of azithromycin is absorbed by means of passive absorption, driven by pH gradients between the basic amine groups in the molecular structure of the drug and the acidic lysosome in phagocytes (Gladue and Snider, 1990). Although it was originally suggested that some active transport may contribute to cellular uptake (Gladue et al., 1989), more recent studies have found that blocking and inhibition of metabolic pathways did not reduce the cellular uptake by azithromycin although alterations in pH had a significant impact (Hand and Hand, 2001). These findings were confirmed in a study on cultured human macrophages (Hall et al., 2002). Cellular uptake of azithromycin was determined at different pH levels showing that there is a pH dependent shift in the mechanism of azithromycin uptake. At pH 7, 4 uptake of the drug was found to be a saturable process suggesting it may be a carrier mediated process. However as the pH was reduced, the cellular uptake of azithromycin started following a linear pattern, indicative of passive diffusion (Hall et al., 2002). Recently at least one cell surface transporter has been identified in alveolar epithelial cells as the MDR-1 transporter (Togami et al., 2011). From the available evidence it can be suggested that both active and passive cellular drug accumulation seem to play a role, although how big this role is depends on the surrounding tissue pH.

Further advantages of azithromycin over erythromycin are the faster uptake and peak plasma concentrations reached within 2 hours and the extended half life (T½) (Zuckerman et al., 2009). It was thus found that T½ for azithromycin is generally between 40-68 hours (Zuckerman et al., 2009). However different T½ times have been reported for different compartments, ranging from 0.1 hours in plasma to 289 ±166 hours in neutrophils (Wilms et al., 2006)with some reports detecting it in human neutrophils for up to 28 days (Culic et al., 2002)and between 2.3 days in human prostate and 3.2 days in tonsilar tissue (Foulds et al., 1990). More applicable to periodontics, the drug could be detected in concentrations exceeding Minimal Inhibitory Concentration (MIC) in the periodontal tissues for up to 6.5 (Blandizzi et al., 1999) to 14 days (although only at 20% of the initial concentration) (Gomi et al., 2007) after administration.

Azithromycin is not metabolized in the cytochrome P450 system and is therefore safe in patients with mild to moderate hepatic impairment, without the need for alterations in dosing (Westphal, 2000, Mazzei et al., 1993). The main route of drug elimination is the excretion of the drug in faeces in an unchanged form with urinary clearance being minimal (Zuckerman et al., 2009).

### 1.2.2 Antimicrobial action of azithromycin

The macrolides fall into the group of bacteriostatic antimicrobials. Their mode of action is by reversibly binding to domain V of 23S ribosomal RNA (rRNA) of the 50s subunit of the bacterial ribosome, inhibiting RNA-dependent protein synthesis (Hansen et al., 1999).

More recent work has found that azithromycin also has bactericidal effects on some *Porphyromonas aeruginosa* strains, but only when they were in the stationary phase not whilst in the exponential growth phase. It is thought that azithromycin displaces $Mg^{2+}$ ions from the phospholipid moiety of lipopolysaccharide, thereby destabilizing the outer membrane (Imamura et al., 2005). Further research in this area however showed that the bactericidal effect might rely on a two-step process (Kohler et al., 2007). To determine the effect directly on the ribosome, the access of azithromycin to the ribosome in *P. aeruginosa* was blocked, which prevented bacterial death even in the stationary phase. As a result it was suggested that in the stationary phase, azithromycin does displace $Mg^{2+}$ from the outer membrane, but instead of causing cell lysis by membrane disruption, uses this process to gain access and in a second step results in cell death by interrupting protein synthesis (Kohler et al., 2007).

Furthermore it has been found that macrolide antibiotics have anti-inflammatory and immune-modulatory functions, which will be discussed under a separate heading.

Bacterial resistance to macrolides is achieved by altering the binding site on the ribosome or by active efflux. Prevention of binding to the ribosome is achieved by methylation of an adenine residue in domain V of the 23S rRNA. This is thought to be mediated through the erythromycin ribosome methylase gene, which prevents binding of macrolides to domain V resulting in high level resistance with this gene either being induced or constitutionally expressed (Zuckerman et al., 2009).

Efflux is achieved by the macrolide efflux genes (*mel* and *mef)* which are specific for 14- and 15-membered macrolides (Ambrose et al., 2005). It has been found that these genes are up regulated, when bacteria are exposed to drug concentrations below MIC. Once stimulated, activation of these genes results in the expression of active proton pumps which can clear the microorganism of the macrolide (Ambrose et al., 2005).

In the past decade there has been a significant increase in the amount of bacterial antibiotic resistance to macrolide antibiotics. For example, approximately 80-100% of *Streptococcus pneumoniae* in Japan are thought to be resistant to macrolides (Niki et al., 2009), 50% in France, but only 6.9-8.8% in Scandinavia (Riedel et al., 2007). The large variation in resistant strains in these studies was linked to the amount of antibiotics which were routinely prescribed in the different countries (Riedel et al., 2007).

### 1.2.3 Spectrum of activity

Azithromycin has a similar spectrum of activity to erythromycin, although it was found to have significantly improved potency against gram-negative organisms. It is particularly useful against *Haemophilus influenza* and *Neisseria gonorrhoeae*, being 4 times as potent as erythromycin, twice as potent against *Branhamella catarrhalis*, *Campylobacter* and *Legionella spp* and is as equally effective against *Chlamydia spp* as erythromycin (Retsema et al., 1987). It is also of effective against *streptococci* and *staphylococci*, although not effective against Methicillin-resistant Staphylococcus aureus (MRSA) (Zuckerman et al., 2009) (Table 1).

**Table 1.** List of common pathogens susceptible to azithromycin

| **Name of organism** | **Reference** |
|---|---|
| Haemophilus influenza<br>Neisseria gonorrhoeae<br>Branhamella catarrhalis<br>Campylobacter sp.<br>Legionella sp | (Retsema et al., 1987) |
| Haemophilus ducreyi<br>Ureaplasma urealyticum<br>Chlamydia trachomatis<br>Mycoplasma hominis<br>Streptococci<br>Staphylococci<br>E. coli<br>Salmonella spp<br>Yersinia enterocolitica<br>Shigella spp | (Peters et al., 1992) |

Considering this fairly broad spectrum of activity, azithromycin is used for a variety of infections. It is found to be an effective antibiotic in upper airway infections, tonsillitis and pharyngitis (Zuckerman et al., 2009),as well as lower respiratory tract infection (Panpanich et al., 2008) and community acquired pneumonia (Yanagihara et al., 2009a). However due to the possibility of contracting the macrolide resistant *S. pneumonia* strain of pneumonia it has been recommended to only use azithromycin for this purpose in conjunction with additional β-

lactam antibiotics such as penicillin (Mandell et al., 2007). At present, macrolide antibiotics are especially used in pulmonary conditions that are thought to arise due to a combination of inflammatory disregulation (the immune modulatory functions are discussed under a separate heading) and microbial infection. Azithromycin is therefore often used in patients with Cystic Fibrosis (CF) and diffuse panbronchiolitis (DPB) as well as adult asthma and chronic obstructive pulmonary disease (COPD) where it was found to significantly improve both morbidity and mortality (Altenburg et al., 2010b, Albert et al., 2011).

Azithromycin has also been found to be highly effective in sexually transmitted diseases and urinary tract infections. It has thus been recommended for conditions ranging from Chlamydia infections, gonorrhea, ureteritis, and prostatitis (Workowski et al., 2002). More recently some interest has been shown in the possible of azithromycin in the field of periodontics, although this will be discussed under a separate heading. The improved spectrum of activity when compared to erythromycin is at least partially due to the somewhat remarkable characteristics in terms of cell penetration and intracellular concentration of the drug. In the case of macrolide resistant bacteria, it was found that the increased MIC needed for antibiotics to be effective against the bacteria, are thus easily achieved in the highly concentrated intracellular environment when microorganisms are phagocytized (Yanagihara et al., 2009a) as it has been found to be effective against pathogens which are moderately to highly resistant like *Streptococcus pneumoniae* (Yanagihara et al., 2009b, Hoffman et al., 2003).

Not surprisingly, due to the high intracellular concentrations, macrolides have also been used against intracellular pathogens (Chico et al., 2008).

### 1.2.4 Adverse events

The use of macrolide antibiotics has been linked to some adverse events, although they are considered some of the safest antibiotics available (Araujo and Demoly, 2008). The most common adverse effects in patients receiving macrolide therapy are gastrointestinal complaints. Overall adverse effect rates of 7-26% for erythromycin and 6-27% for azithromycin have been reported for children (Principi and Esposito, 1999), although in most cases when gastrointestinal tract symptoms occurred, it was not necessary to discontinue treatment (Altenburg et al., 2010b, Principi and Esposito, 1999). Some of the other reported side effects related to macrolide usage are rashes (0.5-6%) and hepatotoxicity associated with a transient increase in liver enzymes (Principi and Esposito, 1999). As mentioned earlier, these side effects are generally worse for erythromycin than for any of the other 14 and 15 ringed macrolides and seem to be dose dependent, with more side effects present when higher

dosages (above 1.5 g/day) are prescribed (Addy and Martin, 2004). Some of the more severe, albeit rare, side effects associated with macrolides are ototoxicity and cardiotoxicity.

### 1.2.5 Cardiotoxicity

There have been reports of cardiotoxicity associated with the administration of certain drugs, including macrolides. Most data on the occurrence of cardiotoxicity has been presented in case studies and present a prolongation of the QT interval and Torsades de pointes (TdP). TdP is a rare polymorphic ventricular tachycardia, usually following QT prolongation which is caused by altered cardiac repolarization and is usually associated with syncope, but may result in death (Owens, 2004). The macrolides most associated with cardiotoxicity are erythromycin, especially when administered via IV, and clarithromycin. Little evidence exists showing the incidence of TdP associated with macrolide usage, although a large cohort study in 2004 showed a 2-fold increased risk of sudden death from cardiac causes among patients currently using erythromycin (Ray et al., 2004). It is thought that macrolides have two main pathways in which they affect cardiac rhythm, firstly by having an intrinsic effect, and secondly by affecting the P450 pathway. When erythromycin is prescribed concurrently with other P450 inhibiting medications, it was found that there was a 5-fold increase in the incidence of cardiac sudden death (Ray et al., 2004).This seems to be especially true for a certain group of patients who seemed predisposed to TdP, which includes patients with the following characteristics: female gender, older than 80 years of age, history of heart disease, use of QT prolonging medication, reduced drug elimination, hypokalemia/hypomagnesaemia, prolonged QT interval before therapy, bradycardia and genetic predisposition (Owens, 2004). Although there have been reports associating azithromycin with the development of TdP, it appears as though most occurrences are associated with erythromycin, and as such azithromycin is considered a low risk drug (Owens, 2004). Recently much attention has been focused on the cardiotoxicity of azithromycin in the popular media, following the publication of an observational study in the New England Journal of Medicine. Results from this study showed that patients with known cardiac disease were at an increased risk of cardiovascular death, following a 5 day course of azithromycn (Ray et al., 2012). However a clear limitation of this study was the fact that it was an observational study, which makes interpreting the results difficult.

### 1.2.6 Ototoxicity

Ototoxicity associated with macrolide use is described as being reversible, sensorineural, and bilateral hearing loss involving the lower speech frequencies and can be associated with

low grade tinnitus (Principi and Esposito, 1999). Symptoms usually appear within the first days of drug administration and recovery of hearing generally begins almost immediately once the medication is stopped. It is thought that macrolide associated ototoxicity results from excessive drug serum concentrations. In a study that conducted hearing tests on 45 patients receiving IV erythromycin, it was found that 21% of patients receiving erythromycin developed impaired hearing. The paper also listed decreased total systemic clearance and reduced renal function with development of hearing loss in the patient group (Swanson et al., 1992). Similarly, studies on guinea pigs have shown that dosages of 45 mg/kg of oral azithromycin had ototoxic effects (Uzun et al., 2001) and that topical application of azithromycin led to middle ear damage (Pawlowski et al., 2010). There have been no randomized controlled trials dealing with the ototoxic effect of azithromycin to date, although a search of online databases (PubMed and Scopus) rendered several case reports in which ototoxicity was associated both with IV and oral administration of azithromycin. Hearing loss has also been reported in HIV positive patients treated for *Mycobacterium avium* (MAC) infection with azithromycin 600 mg/day for up to 46 weeks. Of the 46 patients enrolled in the study, 17% developed transient bilateral hearing loss that resolved by 4.9 weeks of discontinuing the medication (Tseng et al., 1997). Similar results were observed by others (Brown et al., 1997) who also found a relationship between the administered dose and incidence of side effects. In conclusion, macrolide antibiotics are able to cause hearing loss that is almost always reversible and apparently this occurs only when they are administered at high dosages. The incidence of ototoxicity during low-dose, long term macrolide use is unknown, but probably negligible (Altenburg et al., 2010b).

## 1.3 Azithromycin and its antimicrobial and immune modulatory functions: A brief overview

It has been known for over 20 years that macrolide antibiotics, including azithromycin have immune modulatory properties, which were discovered after treating patients suffering from panbronchiolitis with erythromycin. Until then, this lung condition, which had both a bacterial and inflammatory component, had a 10 year survival of only 10-20%, which was increased to over 90% when treated with macrolides (Fujii et al., 1995). Since then, a significant amount of information has been gathered which shows that macrolides have both an antimicrobial as well as anti-inflammatory effect. Because of this they have been used as maintenance treatment for various chronic inflammatory conditions and they modulate their effects through a number of different mechanisms (Figure 2, adapted from Altenburg et al., 2010a). These can be subdivided into factors that either affect the pathogen directly, or

immune modulatory effects that alter the host response. In an attempt not to distract from the research topic, the effect of azithromycin on cilliary function, epithelial tight junctions and sputum production have not been included in the following discussion.



**Figure 2.** Schematic representation of immune modulatory function of azithromycin (adapted from Altenburg et al. 2010)

1. Attenuation of biofilm function
2. Suppression of bacterial quorum sensing
3. Decrease in bacterial adherence
4. Loss of flagellar mobility
5. Reduced production of bacterial toxins
6. Consolidation of epithelial tight junctions
7. Inhibition of proinflammatory cytokines produced by bacteria, neutrophils, Eosinophil's and epithelial cells
8. Reduction of neutrophil chemotaxis
9. Stimulation of neutrophil degranulation
10. Accelerated neutrophil apoptosis
11. Down regulation of adhesion molecules
12. Stimulation of phagocytosis of by macrophages
13. Reduction of T cell numbers and T cell migration
14. Modulation of dendritic cell function

### 1.3.1 The effect of azithromycin on biofilms and pathogens

Most of the information available of the effects of azithromycin and the other macrolides on bacteria and biofilms has been obtained from models dealing with lung infections and pathogens commonly encountered in upper and lower respiratory tract infections. These studies are presented here in order create an overview of the mechanisms of action of the macrolides. Regrettably very few studies deal with the application of azithromycin in periodontics, which at present makes deductions of its relevance difficult. The information that does directly relate to solely the use of azithromycin in periodontics is discussed under a separate heading.

### 1.3.2 Biofilm

Biofilms present a major challenge to antimicrobial therapy as they protect bacteria from antibiotics via several mechanisms. The latest definition of a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, which are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. This is opposed to “nonbiofilm” populations, which include colonies of bacteria growing on the surface of agar, which behave like planktonic cells “stranded” on a surface and exhibit none of the inherent resistance characteristics of true biofilms (Donlan and Costerton, 2002, Marsh et al., 2011). Microbial resistance in the biofilm is mainly due to one of three factors, namely:

- Decreased growth
- Protection by the matrix and
- Changes in phenotype.

Further evidence has recently come to light regarding efflux pumps, which remove toxins and antibiotics from bacterial cells, and that genetic information regarding these can be shared via plasmids (Li and Nikaido, 2009, Hall-Stoodley and Stoodley, 2009).

Recently, it has been found that macrolide antibiotics seem to have the ability to inhibit biofilm formation (Starner et al., 2008) although they do not seem to be able to destroy the microbial biofilm entirely (Norrington et al., 2008). One mechanism by which this is thought to happen is the reduction of gene products necessary for biofilm formation (Wagner et al., 2005). Similarly, it was found that when sub MIC concentrations of azithromycin were added to developing *P. aeruginosa* biofilms, biofilm formation was retarded, although not entirely prevented. However, once established, the mature biofilm was not found to be susceptible to

sub MIC concentrations of azithromycin (Gillis and Iglewski, 2004). Another method of biofilm inhibition that has been demonstrated is the inhibition of the formation of polysaccharides. It has been shown that azithromycin has the potential to inhibit the formation of guanosine diphospho-D-mannose dehydrogenase (Mitsuya et al., 2000) which has been found to play an important role in alginate production essential for the biofilm scaffold (Tatnell et al., 1994).

### 1.3.3 Quorum sensing

Bacterial quorum sensing is the process by which bacteria communicate with each other after reaching a critical number and which allows them to react or adapt to their environment. This is especially important in the biofilm ecology. With increasing biomass of the biofilm, bacteria start producing auto inducers such as LUXI/LUXR. These inducers function by switching certain genes (Lux-1 and Lux-2 gene) on and off which can significantly affect growth and virulence of the biofilm (Shao and Demuth, 2010). It has also been found that the auto inducers produced by certain bacteria can affect other species, which allows for a mechanism through which different species can communicate with each other. An example of this can be seen in the communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Riedel et al., 2001). It has since been found that one autoinducer involved in this process is N-acyl homoserine lactone although its exact role is still not clear. Another autoinducer that has been identified is autoinducer-2 (Shao and Demuth, 2010) which has been found to at least partially regulate the formation of leukotoxin (Shao and Demuth, 2010) as well as iron absorption (Fong et al., 2001). As a result it was found that bacteria with deficient lux-s genes, produce smaller colonies when grown in biofilms compared to wild type bacteria and it was thus concluded that autoinducer-2 was essential for biofilm growth (Shao et al., 2007). Autoinducer-2 also seems to play a role in the growth regulation of multicultural biofilms and has been found to be widely distributed among gram positive and negative bacteria where the secretion of autoinducer-2 by one organism can influence another. Unsurprisingly, reduced levels of autoinducer-2 resulted in reductions in multi species biofilm mass. Therefore, it can be concluded that there is substantial evidence that autoinducer-2 plays an important role in quorum sensing and biofilm development (Shao and Demuth, 2010). However, some bacteria can have more than one quorum sensing system to regulate gene expression. For example, *P. aeruginosa* has at least two quorum sensing systems to regulate gene expression, *Las* and *Rhl*. The *Las* system uses AHLs as an autoinducer, whereas the *Rhl* system uses Nbutanoyl- homoserine lactone. The cooperation of the *Las* and *Rhl*

systems controls a wide range of gene expression for production of pyocyanin, exotoxin A, chitinase and others enzymes (Raffa et al., 2005).

Quorum sensing has been found to be suppressed by azithromycin (Kohler et al., 2010, Tateda et al., 2007, Tateda et al., 2004, Hoffmann et al., 2007), which is thought to occur through the suppression of Quorum Sensing (QS) genes (Kohler et al., 2010). Some of the earlier work showed that both the *las* and *rhl* systems, which regulate QS, are inhibited by sub-MIC concentrations of azithromycin (Tateda et al., 2001). Further *in vitro* experiments found that azithromycin had the ability to reduce protease, elastase and chitinase production as well as QS, which were found to be dose dependent (Hoffmann et al., 2007). It appears that even at sub inhibitory concentrations of 300 times less than the minimum inhibitory concentration (MIC) for Azithromycin, a reduction in protease, elastase, and chitinase is achieved in *Pseudomonas aeruginosa*. At concentrations 100 times below MIC it was found that 71% of the genes involved in QS were suppressed, although this was found to reduce if the concentration of azithromycin was reduced (Skindersoe et al., 2008).

### 1.3.4 Bacterial adhesion

Bacterial adhesion plays an important role in the colonization of surfaces by bacteria, both inside and outside the mouth. Particularly in succession of bacteria in the plaque ecology, bacterial adhesion and coaggregation have been identified as some of the most important factors (Marsh, 2003). Macrolides have been found to reduce bacterial adhesion for several different bacterial strains, resulting in reduced biofilm formation even at very low concentrations in a dose dependent relationship (Schreiber and Szewzyk, 2008). The adherence of *Pseudomonas aeruginosa* to collagen (type IV) was also evaluated, and it was once again found that there was a reduction in adherence of bacteria to the substratum (Tsang et al., 2003). In particular, clarithromycin was found to be highly effective in reducing bacterial adhesion, as even at half MIC, adhesion of *Klebsiella pneumonia* to bronchial epithelial cells could be reduced by 80%, although this antibiotic is generally not deemed active against this microorganism (Ferrara et al., 2001). The reduction of bacterial adhesion was also reported by others, who found that sub inhibitory concentrations of erythromycin resulted in decreased attachment of Streptococcus pneumoniae to epithelial respiratory cells (Lagrou et al., 2000).

It was recently found that one mechanism through which macrolides may inhibit bacterial adhesion is by down regulating bacterial expression of adhesion molecules such as fibronectin and Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM) (Wang et al., 2010) although to date more research is needed in this area.

### 1.3.5 Bacterial mobility

Bacterial motility is highly dependent on the tail like extensions, or flagella, which some bacteria use for propulsion. Motility is thought to be an important factor in bacterial virulence as it allows for movement and invasion of tissue by bacteria. Macrolide antibiotics have been found to cause partial or complete loss of bacterial motility by reducing the number of flagella on bacterial cells (Molinari et al., 1992). Erythromycin, clarithromycin, and azithromycin at sub inhibitory concentrations (sub-MICs) were found to suppress the expression flagellin, dose dependently with azithromycin showing the strongest inhibitory effect on the expression of *P. aeruginosa* flagellin (Kawamura-Sato et al., 2000). Further investigations by the same group found that even after exposure and subsequent elimination of both azithromycin and erythromycin, there was a post antibiotic effect (PAE) that was attributed to morphological and physiological changes that were induced by the macrolides. As a result bacterial motility was inhibited for up to 5 hours *in vitro* after the antibiotic had been eliminated in *Pseudomonas aeruginosa* (Kawamura-Sato et al., 2001). Results from *in vitro* studies using immunoblotting and electron micrographs have shown that sub MIC concentrations of azithromycin result in a reduction of flagellar filaments on the surface of *Salmonella enterica serovar Typhimurium*, specifically by reducing the amount of flagellin exported from bacterial cells. This is thought to be the main mechanism of suppressing flagella formation, as the drug is not involved in suppressing the synthesis of flagellin in the cell (Matsui et al., 2005).

The reduction in motility induced by azithromycin would not only result in reduced bacterial virulence but also in easier bacterial killing and phagocytosis by macrophages (Altenburg et al., 2010a).

### 1.3.6 Bacterial toxins

There are several toxins produced by bacteria, such as exotoxin A, protease, DNase, elastase and phospholipase, which are bacterial virulence factors and strongly contribute to bacterial infection. *In vitro* studies have been conducted to evaluate the efficacy of different macrolide antibiotics at sub MIC concentrations in suppressing bacterial toxins. When compared to erythromycin and clarithromycin, azithromycin has the broadest spectrum of activity and was able to strongly suppress the synthesis of elastase, protease, lecithinase and DNase as well as pyocyanin in *Porphyromonas aeruginosa*. It was also equally successful in reducing gelatinase and was once again found to suppress bacterial motility (Molinari et al.,

1993). Other investigations showed that even at sub MIC concentrations, the macrolide antibiotics, and specifically azithromycin, are able to suppress protein synthesis as assessed by measuring the heat shock proteins Gro-El. As a result it is thought that this not only makes bacteria more susceptible to stress, but also inhibits their virulence (Tateda et al., 2000). Subinhibitory concentrations of azithromycin have been found to suppress bacterial release of exotoxin A, total protease, elastase and phospholipase C production by *P. aeruginosa*, without affecting the total protein production. Results from this study also showed that although azithromycin is not the only macrolide with this effect, it is more potent than erythromycin and roxithromycin in this regard (Mizukane et al., 1994).

Another toxin that is suppressed by macrolides is pneumolysin, which has both cytotoxic and complement activation activities and thus plays an important role tissue injury and pathogenesis caused by pneumococci. Erythromycin was found to reduce the haemolytic activity of pneumolysin (Lagrou et al., 2000). It was subsequently also found that azithromycin and clarithromycin were effective in reducing pneumolysin levels both *in vitro* and leading to improved survival rates in murine *in vivo* studies (Fukuda et al., 2006).

The same research group also evaluated the effect of different macrolides on coagulase titers of Methicillin-resistant *Staphylococcus aureus* (MRSA) and showed that subinhibitory concentrations were able to reduce coagulase levels. Although it was found that telithromycin, clarithromycin and azithromycin were all capable in reducing coagulase titres, the highest reduction was achieved with clarithromycin, with azithromycin being only moderately effective (Yanagihara et al., 2008)

## 1.4 Immune modulatory functions of azithromycin

Azithromycin has been found to have an effect on both innate and adaptive immunity influencing the host immune response in several ways.

### 1.4.1 Effect on cytokines

The term cytokine is used to describe many different proteins, peptides and glycoproteins in the body that act as signaling molecules and play an exceptionally important role in the immune system by either up regulating or down regulating the inflammatory response. Cytokines represent a large and diverse family of regulators that are produced by many different cells all over the body, especially epithelial cells and macrophages, eosinophils, lymphocytes and neutrophils. Proinflammatory cytokines (for example interleukin (IL)-1, IL-2, IL-4, IL-6, IFN- γ, TNF- α, and GM-GCSF) and chemokines (such as IL-8 and RANTES)

amplify the immune response through positive feedback loops. Opposed to these pro-inflammatory cytokines, the anti-inflammatory cytokines, such as IL-10, prostaglandins, and transforming growth factor-β (TGF-β), attenuate the immune response through a negative-feedback mechanism. Most importantly, it is this balance between the pro-inflammatory and anti-inflammatory cytokines which is essential for a well-organized immune response (Kumar et al., 2007).

This discussion will focus on the effect of azithromycin has on cytokines with immune modulatory function such as the interleukins and interferons.

Broadly, the effect of macrolides on cytokines seems to be that they decrease the formation of proinflammatory cytokines while promoting the release of anti inflammatory cytokines (Culic et al., 2001). Several studies evaluating the effect macrolides, and specifically azithromycin, have on cytokine and chemokines levels and the resulting effect on inflammation have been published over the last decade. From the results of these studies, as presented in Table 2, it can be seen that azithromycin has an effect on a multitude of target cells with resulting changes in cytokine levels.

**Table 2.** The effect of azithromycin on cytokines, chemokines and acute phase proteins

| **Target** | **Effect** | **Source/origin** | **Reference** |
|---|---|---|---|
| IL-1α, IL-1β | Down regulation | Gingival crevicular fluid (GCF) | (Ho et al., 2010) |
| | Down regulation | Human mononuclear cells+ mouse monocytes stimulated with Shiga Toxin | (Ohara et al., 2002) |
| | Down regulation | Cystic Fibrosis (CF) Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2009) |
| | Down regulation | Human corneal epithelial cells | (Li et al., 2010) |
| | Down regulation | Human neutrophils | (Reato et al., 2004) |
| | Down regulation | Mice/Bronchial macrophages | (Bosnar et al., 2009) |
| | Down regulation | Macrophages from CF mice | (Meyer et al., 2009) |
| | Up regulation | Healthy humans | (Culic et al., 2002) |
| | No significant change | Mice sensitized with ovalbumin | (Beigelman et al., 2009) |
| IL-3 | Down regulation | Mice sensitized with ovalbumin | (Beigelman et al., 2009) |
| IL-4 | Down regulation | Mice sensitized with ovalbumin | (Beigelman et al., 2009) |
| | Up regulation | Splenocytes and peritonealy cells | (Ortega et al., 2004) |
| IL-5 | Down regulation | Mice sensitized with ovalbumin | (Beigelman et al., 2009) |
| | Down Regulation | Human Th-2 cells | (Lin et al., 2011) |

| | | | |
|---|---|---|---|
| IL-6 | Down regulation | Murine bronchial macrophages | (Feola et al., 2010) |
| | Down regulation | Human mononuclear cells+ mouse monocytes stimulated with Shiga Toxin | (Ohara et al., 2002) |
| | Down regulation | Human fibroblast like synovial cells stimulated with *C. trachomatis* | (Hanada et al., 2003) |
| | Down regulated but in line with reduction of infective agent, not sure if anti infective or anti inflammatory action | Hep-2-cells infected with *Chlamydia pneumoniae* | (Kutlin et al., 2002) |
| | Down regulation | Human neutrophils | (Reato et al., 2004) |
| | Down regulation | Healthy humans | (Culic et al., 2002) |
| | Down regulation | Mice/Bronchial macrophages | (Bosnar et al., 2009) |
| | Down regulation | Human monocytes+ exposed to Mustard gas | (Gao et al., 2010) |
| | Down regulation | Cystic Fibrosis (CF) mice infected with *P. aeruginosa* | (Tsai et al., 2009) |
| | Down regulation | Bronchial epithelial cells stimulated with IFN or LPS | (Murphy et al., 2008) |
| | No change | Cystic Fibrosis (CF) and normal epithelial cells | (Cigana et al., 2007) |
| | No significant change | Mouse stimulated with LPS | (Ivetic Tkalcevic et al., 2006) |
| IL-8 | Down regulation | GCF | (Ho et al., 2010) |
| | Up regulation at low concentration (4 mg/kg) Down regulation at high concentration (400 mg/kg) | Human blood leukocytes and alveolar macrophages | (Kurdowska et al., 2001) |
| | Down regulated but in line with reduction of infective agent | Hep-2-cells infected with *Chlamydia pneumoniae* | (Kutlin et al., 2002) |
| | Down regulation | Airway epithelial cells | (Cigana et al., 2006) |
| | Down regulation | Human neutrophils | (Reato et al., 2004) |
| | Down regulation | Humans with COPD serum levels | (Parnham et al., 2005) |
| | Down regulation | Human corneal epithelial cells | (Li et al., 2010) |
| | Down regulation | Human monocytes+ exposed to Mustard gas | (Gao et al., 2010) |
| | Down regulation at high concentration (500-1000 ng/ml) | Humans with Chronic obstructive pulmonary disease (COPD) | (Hodge et al., 2006) |
| | Bi-phasic response (↑↓) | Healthy humans | (Culic et al., 2002) |
| | Bi-phasic response (↑↓) | Human bronchial epithelial cells | (Shinkai et al., 2006) |
| | No significant change in levels | Human neutrophils | (Koch et al., 2000) |
| | No significant change in levels | Sputum samples from humans after ozone inhalation | (Criqui et al., 2000) |

| | | | |
|---|---|---|---|
| | Down regulation at concentration below MIC but up regulation at high concentration | Human bronchial smooth muscle cells | (Vanaudenaerde et al., 2007) |
| | Down regulation only in some “responders” | Post lung transplant humans | (Verleden et al., 2006) |
| | Up regulation (only in cells stimulated with LPS) | Airway epithelial cells | (Ribeiro et al., 2009) |
| IL-10 | Down regulation | Murine bronchial macrophages | (Feola et al., 2010) |
| | Down regulation | Cystic Fibrosis (CF) Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2009) |
| | No Change | Human neutrophils | (Reato et al., 2004) |
| | Up regulated | Non CF epithelial cells | (Gavilanes et al., 2009) |
| | Up regulation | Bronchial epithelial cells stimulated with IFN or LPS | (Murphy et al., 2008) |
| | Up regulation | Murine dendritic cells stimulated with LPS | (Sugiyama et al., 2007) |
| IL-12 | Down regulation | Macrophages | (Yamauchi et al., 2009) |
| | Down regulation | Bronchial epithelial cells stimulated with IFN or LPS | (Murphy et al., 2008) |
| | No significant change | Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2004) |
| IL -17 | Down regulation | Cystic Fibrosis (CF) Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2009) |
| Il-18 | Up regulation | Splenocytes and peritoneal cells | (Ortega et al., 2004) |
| TNF-α | Down regulation | GCF | (Ho et al., 2010) |
| | Down regulation | Human mononuclear cells+ mouse monocytes stimulated with Shiga Toxin | (Ohara et al., 2002) |
| | Down regulation at high concentration (500-1000 ng/ml) | Humans with chronic obstructive pulmonary disease (COPD) | (Hodge et al., 2006) |
| | Down regulation | CF mice with spontaneous and LPS induced inflammation | (Legssyer et al., 2006) |
| | Down regulation | Murine bronchial macrophages | (Feola et al., 2010), |
| | Down regulation | CF and normal epithelial cells | (Cigana et al., 2007) |
| | Down regulation | Human corneal epithelial cells | (Li et al., 2010) |
| | Down regulation | Human neutrophils | (Reato et al., 2004) |
| | Down regulation | Rats/Murine macrophages | (Ianaro et al., 2000) |
| | Down regulation | Human monocytes+ exposed to Mustard gas | (Gao et al., 2010) |
| | Down regulation | Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2004) |
| | Down regulation | Cystic Fibrosis (CF) Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2009) |

| | | | |
|---|---|---|---|
| | Down Regulation | Macrophages from CF Mice | (Meyer et al., 2009) |
| | Up regulation | CF epithelial cells+ Non CF epithelial cells | (Gavilanes et al., 2009) |
| | Up regulation | Healthy Humans | (Culic et al., 2002) |
| | No significant change | Mice stimulated with LPS | (Ivetic Tkalcevic et al., 2006) |
| | Down regulation only at high dosage (40 mg/kg) | Rats stimulated with caragenin | (Ianaro et al., 2000) |
| IFNγ | Up regulation | Cystic Fibrosis (CF) mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2009) |
| | No change in level | Murine bronchial macrophages | (Feola et al., 2010) |
| | No significant change | Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2004) |
| VEGF | Down regulation | GCF | (Ho et al., 2010) |
| Keratinocyte-derived chemokines (KC) | Up regulation | LPS stimulated and non stimulated CF epithelial cells | (Gavilanes et al., 2009) |
| | Down regulation | Cystic Fibrosis (CF) mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2009) |
| | Down regulation | Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2004) |
| MIP-2 | Up regulation | Non CF epithelial cells | (Gavilanes et al., 2009) |
| | Down regulation | CF mice with spontaneous and LPS induced inflammation | (Legssyer et al., 2006) |
| | No significant change | Mouse stimulated with LPS | (Ivetic Tkalcevic et al., 2006) |
| | No significant change | Mice infected with *Pseudomonas aeruginosa* | (Tsai et al., 2004) |
| MMP1, MMp3, MMP9 | Down regulation | Human corneal epithelial cells | (Li et al., 2010) |
| RANTES | Down regulation | Human corneal epithelial cells | (Li et al., 2010) |
| PGE2 | Down regulation only at high dosage (40 mg/kg) | Rats stimulated with caragenin | (Ianaro et al., 2000) |
| GM-CSF | Down regulation | Mice/Bronchial macrophages | (Bosnar et al., 2009) |
| CCL2 | Down regulation | Mice/Bronchial macrophages | (Bosnar et al., 2009) |
| | Down regulation | Macrophages from CF mice | (Meyer et al., 2009) |
| CRP | Down regulation | Humans with COPD serum levels | (Parnham et al., 2005) |

From studying Table 2 above, it becomes clear that the pharmacological effects of azithromycin on the various cytokines are very complex. It appears that host modulation is dependent on dosage (Ianaro et al., 2000, Hodge and Michalowicz, 2001, Kurdowska et al., 2001) and that the immune modulation has a different effect on different target cells. Some studies also seem to indicate that there are temporal differences in terms of host modulatory

function (Culic et al., 2002, Shinkai et al., 2006, Shinkai et al., 2008). It was found that the initial effect of azithromycin administration leads to an increase in IL-8 production, although if administered for periods of longer than 5 days the effect was reversed. Results also showed that in the presence of bacteria and LPS, this effect was amplified even more (Shinkai et al., 2006). The demonstration that macrolides time-dependently modulate pro-inflammatory cytokine secretion may eventually account for differences among studies.

Furthermore, it has been found that the effect clarithromycin has on cytokine production *in vitro* by neutrophils is dependent on whether or not the cells are in an activated state and the presence of bacteria (Reato et al., 2004) and can either cause production or inhibition of cytokines. Whether or not, these factors are sufficient to explain why, in human trials there are "responders" and "non responders" (Khan et al., 1999, Labro et al., 1989) to the host modulation effect of azithromycin still must be determined.

### 1.4.2 Cytokine regulation by Nuclear Factor-κβ (NFkB) and Activator Protein-1 (AP1)

It has recently been discovered that one of the main mechanisms through which azithromycin exerts its anti-inflammatory action is through the inhibition of NFkB activation (Cigana et al., 2007, Cigana et al., 2006). NFkB is a ubiquitous rapid response transcription factor in cells involved in immune and inflammatory reactions, and functions by expressing cytokines, chemokines, cell adhesion molecules, growth factors, and receptors (Baeuerle and Henkel, 1994). NFkB is a heterodimer composed of NFkB1 and RelA, which both belong to the NF-KB/Rel family of proteins. Inactive NFkB is present in the cytoplasm complexed with an inhibitory protein, IkB. NFkB is activated by a number of incoming signals from the cell surface. Released from IKB inhibition, NFkB translates into the nucleus and binds to the kB motif of the target gene (Baeuerle and Henkel, 1994, Siebenlist et al., 1994). Once binding has taken place, genetic transcriptional changes occur which can result in a variety of immunological or inflammatory reactions. Some of these include the activation and proliferation of macrophages/monocytes, B and T cells and the induction of chemokine and cytokine (such as IL1, IL6 and TNFα) release as well as cell adhesion products. NFkB was also found to play a role in growth regulation and if unregulated has been associated with dysplasia and malignancies (Baeuerle and Henkel, 1994).

Similarly, AP1 is also a transcriptional factor that is important for genetic activation and translation. As is the case with NFkB, it was found that azithromycin down regulates AP-1 expression. The AP-1 transcription factor is composed of members of the Fos, Jun and activating transcription factor (ATF) families of proteins. AP-1 converts extracellular signals in bone and immune cells so as to change the expression of specific target genes, with an AP-

1 binding site. The activity of AP-1 is modulated by interactions with other transcriptional regulators and is further controlled by upstream kinases that link AP-1 to various signal transduction pathways (Wagner and Eferl, 2005).

Through these pathways, AP-1 can affect the severity of inflammation through at least four different mechanisms: (i) by activation of cytokine production in cooperation with transcription factors of the NFAT family in different cell types; (ii) by regulation of naive T-cell differentiation into T-helper 1 (Th1) or Th2 cells; (iii) by interaction and trans-repression of the glucocorticoid receptor (GR); and (iv) by modulating the activity of the innate immune system (Wagner and Eferl, 2005).

Several investigators have now found that azithromycin has the potential to inhibit both AP-1 and NFkB in a variety of cells ranging from vascular smooth muscle (Miller et al., 2000), to epithelial cells (Cigana et al., 2007, Cigana et al., 2006), macrophages (Yamauchi et al., 2009, Iwamoto et al., 2011)and human corneal epithelial cells (Li et al., 2010). However there are other studies which found that reduction of NFkB levels could not be achieved by azithromycin (Blau et al., 2007, Morinaga et al., 2009), although NFkB levels were reduced by clarithromycin (Morinaga et al., 2009) and moxifloxacin (Blau et al., 2007). In a comparative *in vitro* study, which evaluated the anti inflammatory effect of azithromycin compared to hydrocortisone and dexamethasone, it was found that hydrocortisone was four orders of magnitude more potent in suppressing NFkB expression than azithromycin. Correspondingly dexamethasone suppression of NFkB activity was 14 times more than for the antimicrobial. Using the values of NFkB activity from this experiment, it was estimated that in terms of anti inflammatory potency, hydrocortisone was four times more potent than azithromycin (Cheung et al., 2010) and less effective than Resolvin E-1 and 15-Epi-Lipoxigenase (Navarro-Xavier et al., 2010) in its pro resolution properties.

The exact method through which azithromycin exerts anti-inflammatory effects remains uncertain, although some of the pathways have recently been identified. As azithromycin has the ability to down regulate IL-12 formation in alveolar macrophages (Murphy et al., 2008), the mechanism through which this eventuates was investigated further (Yamauchi et al., 2009). It is thought that IL-12 transcription occurs through the interaction of the IL-12p40 promoter site and certain transcription factors such as AP-1, ICSB and NFAT (Zhu et al., 2003). However, azithromycin had the ability to inhibit the binding of these transcription factors to their DNA binding sites in a dose dependent manner. Additionally it was found that azithromycin could reduce the levels of active JunB and ICSBP, which was suggested as being one of the key mechanisms in the suppression of IL-12 production by macrophages. However the mechanism for the repressing the activation (phosphorylation) of JunB and ICSB is still unclear. Another explanation for the suppression of IL-12 is through the

repression of the NFkB pathway that also acts as transcription factor for the IL-12p40 promoter site (Yamauchi et al., 2009).

A graphic representation of the possible actions of azithromycin on the binding of transcription factors to the promoter site can be seen in Figure 3.



**Figure 3.** Possible intracellular mechanisms through which azithromycin acts (Yamauchi et al., 2009)

A further theory further theory for the anti-inflammatory properties of azithromycin is that macrolides not only act on the NFkB pathway and mRNA translation and protein synthesis, but also may act further upstream (Wu et al., 2009). In an *in vitro* study evaluating the effect erythromycin had on Jurkat T cells, it was found that this macrolide had the ability to inhibit the mRNA expression of RANKL and NFkB and the expression of RANKL was correlated with NFkB (Wu et al., 2009). This finding is significant in two ways, as RANKL is not only important for the intracellular activation of NFkB but also plays an important role in the activation and differentiation of osteoclasts and has thus been linked to immune mediated diseases resulting in bone destruction such as rheumatoid arthritis and periodontitis (Leibbrandt and Penninger, 2008).

More recently, it has been suggested that down regulation of NFkB is also affected by azithromycin through the down regulating of RAC-1, a signaling G-protein, which is integral to the activation of several intracellular protein kinases.

More recent work has evaluated the effect of azithromycin on the activity and expression of the toll like receptors (TLR), especially TLR-2 and TLR-4, which are associated with the activation of NFkB. As such it was found that azithromycin has the ability to reduce both the expression of TLR-2 (Karlstrom et al., 2011) as well as TLR-4 (Maezono et al., 2011,

Iwamoto et al., 2011), with both being associated with reductions in the recruitment of neutrophils. Nevertheless, more research is needed to fully understand all processes through which azithromycin affects cell functions.

### 1.4.3 Azithromycin, macrophages and phagocytosis

Monocytes and macrophages belong to the mononuclear phagocyte system (MPS). The MPS consists of cells in the bone marrow, peripheral blood and tissues, which are highly specialized for the function of endocytosis and intracellular digestion. In connective tissue organs they are termed histiocyte. They originate from a committed stem cell in the bone marrow through a monoblast stage to form promocytes. These promocytes are capable of rapid division, giving rise to the peripheral blood monocyte. The blood monocyte is a smaller cell, is more functionally active, does not divide and forms around 4-8% of the total WBC count. Its average transit time in blood is around 32 hours. The high number of vesicles in the cytoplasm as well as ruffling of the surface membrane, some lysosomes and an active Golgi apparatus characterizes the cell. It is generally thought that all macrophages are bone marrow derived, although they acquire different structural and biochemical characteristics depending on the tissue where they are found. As such they are known as Kupfer cells when found in the liver, histiocytes in connective tissue, macrophages in bone marrow, and osteoclasts in bone. However, they all share certain general properties, most notably phagocytosis and pinocytosis of smaller molecules. The main way this is controlled is by the expression of certain receptors which bind to complement and antibody fragments on opsonised pathogens. They comprise many hydrolytic enzymes that help them to degrade materials rapidly. Most importantly, macrophages have the potential of becoming activated by external stimuli. Once activated, they increase in size, have a more ruffled surface, increased number of mitochondria, increased levels of hydrolytic enzymes and lysosomes, greater membrane activity with increased endocytosis, more active metabolism and a greater ability to kill pathogens. This state of activation can be triggered by (1) cytokines produced by other cells or (2) non immunological activation by chemicals or touching the bacterial cell wall (Kumar et al., 2007).

Activated macrophages have the potential to secrete a wide variety of products, many of which are active in inflammation with an extremely wide range of activities. Furthermore, the activity of these can be regulated by interactions between the cell and its environment, other cells, the extracellular matrix and complement as well as exogenous agents. Thus macrophages can become deactivated, which favors resolution of inflammation. They are also

long-lived and readily migrate to all tissues, thus they play an important role in innate immunity.

Some of the factors macrophages produced are:

- Proteases: such as collagenase and elastase, which degrade connective tissue and plasminogen activator, which can activate plasminogen
- Chemotactic factors for other leukocytes
- Arachidonic acid metabolites: both cyclooxygenase and lipoxygenase products, which cause vasodilatation and vascular permeability
- Reactive oxygen species
- Complement components
- Coagulation factors (e.g. factor V)
- Growth promoting factors for fibroblasts blood vessels and myeloid progenitor cells
- Cytokines (especially IL-1 and TNF)
- Other products such as Platelet activation factor (PAF) and interferon-γ

Although many of the roles of macrophages are beneficial in responding to injuries, if these are not properly regulated they can cause severe problems and are associated with several diseases. Besides its importance in inflammation and immunity, MPS are the main line of defence against bacteria in the bloodstream and serve to control the blood born dissemination or organisms and unwanted material (Kumar et al., 2007).

One of the most important roles of macrophages in the immune system is the presentation of antigens, especially to T-cells. As T-cells cannot be activated by soluble antigens, presentation of processed, membrane bound antigens by macrophages or other antigen presenting cells is essential for cell mediated immunity. The processed cell is presented with class II histocompatibility antigens to CD4 T helper cells. They also produce a variety of soluble factors including IL-1, which plays a role in T and B-cell differentiation. Macrophages also lyse tumor cells by secreting their proteolytic enzymes and play a very important role in cell-mediated immunity (Taylor et al., 2005).

Besides altering cytokine expression as discussed earlier (Meyer et al., 2009, Ianaro et al., 2000), azithromycin also has the ability to modulate macrophage function.

In early *in vitro* studies it was found that the addition of erythromycin to cultured monocytes which had been isolated from blood induced the differentiation of monocytes to macrophages (Keicho et al., 1994). The increased differentiation was found to be linked to erythromycin in a dose dependent manner, with 100 μg/ml increasing the number of macrophages that attached to the culture plate by 570%, compared to a 140% increase achieved by a dosage of 1 μg/ml. The addition of Granulocyte macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF) was found to have a

synergistic effect with erythromycin and lead to an even higher differentiation rate. Furthermore it was found that the addition of erythromycin also increased $H_2O_2$ production in macrophages (Keicho et al., 1994). More recent work focused on achieving similar results with 12 ringed macrolides, which were specially designed to have minimal antimicrobial effects. Results from this study once again showed that macrolides are able to promote the differentiation to macrophages (Yoshida et al., 2005). Not only does the differentiation to macrophages increase, it was also found that azithromycin has the ability to induce changes in macrophage phenotype, resulting in a shift from M1 to M2 phenotype (Navarro-Xavier et al., 2010). Classic activation of macrophages (M1) by IFNγ, gives rise to a cell that actively secretes inflammatory cytokines and chemokines, phagocytosis and kills pathogens, and initiates the adaptive immune response. Opposed to this, macrophages which are stimulated with IL-4 /IL-13 (M2 cells) display a distinctly different pattern of activation. The later play a role in directing the Th2 humoral response, allergic and parasitic responses and the coordination of repair following an inflammatory reaction (Murphy et al., 2008). Although more critical research is needed to confirm this, it could be one reason for the reduction in inflammatory cytokine production by macrophages noted after azithromycin administration. Supporting this theory are recent findings showing that, if azithromycin was administered to mice, it increased the number of CD11b macrophages, which are thought to play a role in directing the immune response and in this study was associated with a reduction in neutrophil infiltration (Feola et al., 2010).

Besides macrophage differentiation, several studies have focused on the changes in macrophage function that are induced by azithromycin. As such it was found that low doses of azithromycin (500 ng/ml) have the ability to significantly improve the phagocytosis of apoptotic neutrophils and epithelial cells *in vitro* (Hodge et al., 2006). To confirm the relevance in a more clinical scenario, monocytes from patients with COPD were obtained after azithromycin administration. Results once again showed an increase in the phagocytosis of apoptotic neutrophils and epithelial cells (Hodge et al., 2008). It appears as though the stimulated increase in phagocytosis is unique to macrolides with a 14 or 15 member ring, as it was found that only erythromycin and azithromycin but not clarithromycin (a 16 membered ring) could enhance the phagocytosis of apoptotic neutrophils (Yamaryo et al., 2003). Furthermore it was found that *in vitro* macrolides (including azithromycin) significantly improved efferocytosis and phagocytic and chemotactic properties as well as decreased the pro inflammatory cytokine Il-8, TNF-α and IL-6 by up to 75% and reduced nitric oxide synthase (iNOS) formation in macrophage which had been exposed to sulfur mustard (Gao et al., 2010).

It appears as though azithromycin does not only affect phagocytosis but also pinocytosis, or fluid endocytosis, a non-specific process in which cells absorb small amounts of extra cellular fluid. The main difference between pinocytosis and receptor mediated endocytosis is that the later is dependent on receptors binding to a specific molecule which is then engulfed. Interestingly, it was found that although there was a dose dependent inhibition of pinocytosis this did not negatively affect phagocytosis (Tyteca et al., 2002). Further work by the same group found that azithromycin has the ability to change the structure of the cell membrane, which can lead to rearrangement of plasma membrane receptors. Once again it was shown that although there was a retarding effect of pinocytosis and fusion of endosomes with lysozomes, this did not affect bulk endocytosis (Tyteca et al., 2003). This work shows that azithromycin directly interacts with phospholipids and modifies biophysical properties of the cell membrane in living cells (Tyteca et al., 2003). A further study supporting the interaction of azithromycin with phagocytic process, investigated the effect of the drug on auto phagocytosis in macrophages. In the *in vitro* investigation, it was found that azithromycin reduced lysosomal acidification during long-term administration, resulting in a reduction of auto phagocytosis that was linked to an increase in *Mycobacterium abscessus,* an intracellular pathogen, infection in mice with cystic fibrosis (Renna et al., 2011).

### 1.4.4 Azithromycin and polymorphonuclear leukocytes

Neutrophils are terminally differentiated cells rich in cytoplasmic granules which contain a lobulated chromatin dense nucleus with no nucleolus. Four types of cytosolic granules have been characterized, which contain various receptors, enzyme components, and antimicrobial proteins. Neutrophils mature in the bone marrow before being released into the circulation, spending only 4 to 10 hours before marginating and entering tissue pools, where they survive for 1 to 2 days. Senescent neutrophils are thought to undergo apoptosis prior to removal by macrophages. This stops disintegration *in vivo*, which would otherwise expel their cytotoxic contents into the extracellular milieu, and may also play a role in terminating inflammatory responses. Neutrophils are produced in human bone marrow at the rate of 1011 cells per day. This is controlled by two colony-stimulating factors (CSFs),namely granulocyte (G-CSF) and granulocyte-macrophage (GM-CSF), that direct the production and differentiation of bone marrow progenitor cells and can increase the rate of neutrophil differentiation by as much as 10-fold during states of stress and infection. CSFs also amplify the activities of various neutrophil functions *in vitro* (Smith, 1994).

During the inflammatory response, chemotactic factors generated by infectious agents themselves, as well as those released as a result of their initial contact with phagocytes and

other components of the immune system, signal the recruitment of additional neutrophils to sites of infection and/or injury. The sequence of these leukocyte events can be divided into:

- Margination: In normally flowing blood, the red and white cells within vessels are confined to the centre, leaving a relatively cell free layer of plasma in contact with the vessel wall. If lamellar flow is interrupted, this will lead to margination of the cells, which allows contact of the neutrophils with the endothelium.
- Rolling: During rolling, the leukocytes roll along the endothelial surface and interact with specific selectins (L-, P- and E-selectin) and integrins.
- Adhesion: Following rolling, the neutrophils adhere to the endothelium. Leukocyte adhesion is dependent on interaction of specific adhesion molecules on the surface of both neutrophils as well as vascular endothelium. Some of these are leukocyte integrins, such as Intracellular adhesion molecule 1 (ICAM1) and Vascular Cell Adhesion Molecule-1 (VCAM1), expressed by endothelial cells. The surface expression of the receptors is enhanced by inflammatory and chemical mediators that result in increased adhesivity.
- Emigration and Chemotaxis: Following adhesion, leukocytes move slightly along the surface and insert pseudopods into the junction. They once again bind specific receptors (ICAM and VCAM), which allows them to crawl through between the intercellular junction (Ley et al., 2007).

This sequence is shown below in Figure 4.

**Figure 4.** Neutrophil chemotaxis and diapedesis (Ley et al., 2007).

As with monocytes, azithromycin also has an effect on other phagocytic cells, namely polymorphonuclear leukocytes or neutrophils. This effect was highlighted by a recent study (Banche 2010), which evaluated the efficacy of erythromycin in killing *Streptococcus pyogenes*. What was remarkable about the findings of this study was that *S. pyogenes*, although it has specific defences to protect it, was readily ingested by neutrophils. However once inside the cell, the pathogen was successful at evading intracellular killing and after 30 minutes in culture could no longer be destroyed by neutrophils. As erythromycin is unstable in the acidic intracellular space of neutrophils it is also ineffective at killing the bacteria, especially, as was the case in the present study, when the pathogen is resistant to erythromycin. Nevertheless, when erythromycin was added to neutrophils in culture, intracellular killing of *S. pyogenes* continued to occur past 30 minutes. As neither the antimicrobial agent nor the neutrophils were able to destroy *S. pyogenes* alone in this study, it suggests a synergistic effect between macrolides and neutrophils, which was present even at ¼ MIC (Banche et al., 2010). The following section will focus on the different mechanisms through which macrolides and neutrophils interact.

*1.4.4.1 Adhesion molecules*

The adhesion of leukocytes to the vascular endothelial wall plays an important role in the inflammatory cascade and diapedesis, a process that is dependent on the interaction of adhesion molecules.

Results obtained from *in vitro* studies show that erythromycin increases the expression of ICAM, VCAM, E-selectin and L-selectin in endothelial cells, although only at concentrations of 100 mg/l. PECAM-1 on the other hand, is down-regulated by lower concentrations of

erythromycin (30 mg/l) with no changes occurring in the expression of the integrins (Kilic et al., 2006). The increased *in vitro* expression of ICAM-1 was also noted aterythromycin concentrations of 6250 mg/L although no changes in E-selectin were noted (Lanbeck et al., 2004). Opposed to these findings, other studies have reported that the macrolide roxithromycin down regulated ICAM-1 and E-selectin expressed by endothelial cells that have been stimulated with TNF-α (Akamatsu et al., 2001).

*Ex vivo* studies of neutrophils from patients with chronic sinusitis, showed that exposing peripheral blood neutrophils to roxithromycin and clarithromycin, leads to decreased expression of L-selectin and integrin α1 (Mac-1) (Enomoto et al., 2002).

In earlier clinical studies it was found that in healthy humans azithromycin led to a significant down regulation of VCAM after 24 hours, lasting for 28 days. E-selectin followed a similar pattern, although this did not reach statistical significance (Culic et al., 2002). However, others found that in patients with coronary artery disease (CAD) and *Chlamydia pneumoniae* infection, there was no statistical difference in VCAM-1, ICAM-1 and E-selectin between the azithromycin and placebo group (Semaan et al., 2000). In contrast, Hillis et al. (2004) showed that a 5 day azithromycin course for patients recovering from an acute coronary syndrome reduced serum levels of ICAM-1. Furthermore, a different group found that in patients with CAD and *C. pneumoniae* treated with azithromycin there was a decrease in E-selectin after weeks of treatment (Parchure et al., 2002).

Animal studies have shown similar results as it was found that erythromycin pretreatment (30 mg/kg) reduced levels of mRNA and expression of the adhesion molecules P-selectin, E-selectin, VCAM-1 and ICAM-1 which were upregulated in rats after LPS stimulation (Sanz et al., 2005). These results were supported in a more recent study, which once again showed a down regulation of E-selectin and ICAM-1 in mice after LPS stimulation (Bosnar et al., 2009). When the adhesion of *ex vivo* peripheral neutrophils obtained from rats treated with erythromycin was assessed it was found that although there was no change in ICAM-1 expression, there was a decreased adhesion to endothelial cells. However, when neutrophils were obtained from exudate in the same experiment, no reduction in neutrophil adhesion could be achieved (Enomoto et al., 2003).

From the available literature it becomes clear that azithromycin can alter adhesion molecule expression that will have a direct effect on neutrophil infiltration. However, because these adhesion molecules are induced by both LPS and proinflammatory cytokines, such as TNF-α and IL-1β, the observed inhibition may be indirect, through effects of macrolides on cytokines (Bosnar et al., 2009).

*1.4.4.2 Oxidative burst*

Reactive oxygen species (ROS) are reactive molecules containing an oxygen atom. They are very small molecules that include oxygen ions and peroxides and can be either inorganic or organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress, ROS levels can increase dramatically, which can result in significant damage to cell structures. They cause tissue damage by a variety of different mechanisms which include DNA damage, lipid peroxidation, and protein damage, including gingival hyaluronic acid and proteoglycans and oxidation of important enzymes (Chapple, 1997).

The oxygen derived free radicals (e.g. superoxide, hydroxyl radicals) as well as hydrogen peroxide and hypochlorus acid, are of particular interest in periodontal disease. ROS are normal byproducts in cellular metabolism and are thus found in many cells undergoing respiratory bursts (the sudden release of ROS, especially in neutrophils and macrophages). The oxygen consumption of cells during the oxidative burst may be 10-20 times higher than normal in areas of inflammation. These are either produced by specific enzymes (myeloperoxidase and NADPH) designed to produce free radicals or as a result of metabolic pathways, although sometimes auto-oxidation of small particles may also produce free radicals. The release of relatively large volumes of free radicals in areas of inflammation can lead to significant localized tissue damage (Bartold and Narayanan, 1998).

It thus becomes clear that the suppression of ROS could be a valuable approach in limiting tissue destruction and the amplification of inflammation.

A reduction in ROS has been found to occur in several studies evaluating the effect azithromycin on the respiratory burst. A reduction in the amount of superoxide formation was thus noted in unstimulated neutrophils from both healthy and diabetic patients *ex vivo.* Results further demonstrated that azithromycin dramatically reduced superoxide levels in neutrophils from healthy and diabetic patients, after the neutrophils had been stimulated with zimosan or phorbolmyristate acetate (PMA). A further finding was that generally patients with poorly controlled diabetes had higher levels of ROS at baseline and that a larger reduction in ROS levels occurred after azithromycin administration, compared to the control (Hand et al., 2007). The same group also had similar suppression of superoxide formation by other weak basic members of the macrolide group such as roxithromycin (Hand et al., 1990) and dirithromycin (Hand and Hand, 1993).

Other reports suggest that during the first 4 days after administration of azithromycin to neutrophils cultured from patients with COPD there was a measurable increase in the

respiratory burst compared to neutrophils from patients whom had received placebo. However, after 11 days it was found that this relationship reversed, with significantly less superoxide forming in neutrophils of the azithromycin group (Parnham et al., 2005).

Similarly, azithromycin, but not clarithromycin, increased myeloperoxidase (MPO) concentrations immediately after it was administered to mice. This proinflammatory activity in azithromycin corresponds to a biphasic effect reported previously (Culic et al., 2002, Shinkai et al., 2006). Azithromycin (500 mg/day for 3 days) initially stimulated blood neutrophil degranulation and oxidative burst in healthy volunteers. However, similar to the cytokine response discussed previously, there was a delayed reduction of the oxidative burst, and an increase in apoptosis of neutrophils (Culic et al., 2002).

Changes in the oxidative burst are not limited to neutrophils alone (Hall et al., 2002). The respiratory burst of cultured THP-1 monocytes was assessed after they were stimulated with zymosan, LPS or latex beads. Similar to the neutrophils, a biphasic response was noted. Initially there was an increase in the respiratory burst, NO and H2O2 production. Simultaneously, during the first hour azithromycin increased the protective enzymes (superoxide dismutase) that were activated for the absorption of free radicals. Subsequently there was a disappearance of free radicals which resulted in a reduction of lipid peroxidation (Hall et al., 2002).

Anderson *et al* (1996) reported a dose-related reduction in superoxide production and suggested that macrolide antibiotics stabilize the membrane of neutrophils. Additional studies by Carevic and Djokic (1988) have shown that azithromycin blocks the release of lysosomal enzymes both *in vitro* and in synovial fluid of arthritic rats. Wenisch et al (1996) reported similar reductions in PMN functions after a single oral dose of azithromycin at 20 mg/kg as well as decreased phagocytosis of *Escherichia coli*. Other investigators, however, have observed no reduction of these parameters in a variety of phagocytes collected from humans (Herrera-Insua et al., 1997, Silvestri et al., 1995, Pascual et al., 1995). These conflicting observations of the cellular functions changes induced by azithromycin may be time dependent, dose/concentration-dependent, or dependent upon the manner in which the phagocytic cells are handled (Hall et al., 2002).

One of the proposed mechanisms through which macrolides suppress ROS formation in granulocytes is by inhibiting the phospholipase D-phosphatic acid phosphohydrolase pathway in activated granulocytes, leading to a decreased level of diradylglycerol production and, as a result, to reduced superoxide production (Perry et al., 1992).

*1.4.4.3 Apoptosis*

Excessive extravasation of neutrophils and their uncontrolled death at the site of inflammation result in the local release of proteolytic enzymes, reactive oxygen species, and proinflammatory mediators, which intensify inflammatory responses and cause self-amplifying tissue injury (Stockley, 2006). This inflammatory environment is characterized by the accumulation of various mediators, including the potent neutrophil chemokines, cytokines and leukotrienes (Canetti et al., 2001, Stockley, 2006).

Under homeostatic conditions, neutrophils are inactivated and removed from the site of inflammation by phagocytosis following apoptosis (Squier et al., 1995, Cohen et al., 1992, Stockley, 2006). If this phagocytic process or neutrophil apoptosis is inhibited, severe inflammation will continue which will ultimately result in local tissue destruction (Stockley, 2006). During apoptosis, the destruction of intracellular organelles occurs while the cell plasma membrane integrity is preserved, hence preventing the release of proinflammatory and histolytic compounds into the surrounding milieu. One of the mechanisms through which apoptosis is induced is through caspase-3, which catalyses the specific cleavage of proteins and nuclear DNA, which then result in apoptosis (Daigle and Simon, 2001). Caspase-3 also inhibits the release of prosurvival and proinflammatory mediators by cleaving the transcription factor NFkB (Levkau et al., 1999, Kang et al., 2001, Barkett et al., 1997, Daigle and Simon, 2001). Reports have demonstrated that inhibition of NFkB in granulocytes accelerates apoptosis in a caspase-3-dependent manner (Ward et al., 1999).

Apoptotic cell death has been noted in unstimulated neutrophils collected from healthy humans and incubated in the presence of azithromycin. It was found that azithromycin could induce apoptosis at lower concentrations and over a shorter period of time than erythromycin. Surprisingly, the effect of increased apoptosis disappeared however, when the neutrophils were incubated with azithromycin but co-stimulated with *S. pneumoniae* lysate although the reason for this could not be determined (Koch et al., 2000).

In *in vivo* studies conducted on mice which were intranasaly challenged with LPS, it was found that azithromycin and clarithromycin almost completely blocked LPS-induced GM-CSF generation. In the same study, GM-CSF production by macrophages *in vitro* was also inhibited (Bosnar et al., 2009). GM-CSF is a survival factor for neutrophils, and its neutralization by anti-GM-CSF antibodies suppresses LPS-induced neutrophilic inflammation in lungs (Bozinovski et al., 2004). Thus, macrolides, by inhibiting GM-CSF, could reduce neutrophil lifespan, increase apoptosis, and subsequently reduce their number in LPS-challenged lungs (Bosnar et al., 2009). Similar, this would also explain why the number of apoptotic granulocytes in blood of healthy volunteers was significantly increased after 3-day

dosing with azithromycin (500 mg/day p.o.) (Culic et al., 2002). These results were supported by findings showing a decreased neutrophil survival *in vitro* through inhibition of GM-CSF release from activated human airway epithelial cells (Yamasawa et al., 2004).

Azithromycin also has an effect on apoptosis in other cells. The number of apoptotic bronchial epithelial cells in bleomycin induced lung injury was found to be significantly lower in mice that had been pretreated with macrolides. The results showed that the highest reduction in apoptosis occurred after roxithromycin and clarithromycin administration, followed by azithromycin (Kawashima et al., 2002). Data collected from bronchial epithelial cells of patients with COPD treated at the Royal Adelaide Hospital showed that there was a reduction in the number of cells undergoing apoptosis in patients taking azithromycin (Hodge et al., 2008). Furthermore it was found *ex vivo* that azithromycin significantly increased apoptosis in activated human lymphocytes from healthy humans (Kadota et al., 2005). Others also reported similar findings and it was proposed that increased apoptosis in human lymphocytes occurred via the Fas-Fas ligand pathway (Ishimatsu et al., 2004) and a combination of upregulation in caspase-3 and Fas Ligand (Jun et al., 2003). The same group also evaluated the Bcl-cL and BAX pathways that are involved in apoptosis. The Bcl-2 protein family regulates one of the steps in a conserved apoptosis pathway. Among the members of this family, Bcl-2 and Bcl-xL act as inhibitors of apoptosis, whereas Bax and Bad act as promoters of apoptosis (Yang and Korsmeyer, 1996).Results indicated that azithromycin also affected these proteins and that this down regulation was associated with enhanced apoptosis in lymphocytes (Mizunoe et al., 2004).

A further possible mechanism for the increased neutrophil apoptosis associated with macrolides has been demonstrated using tulathromycin, a 15 ringed triamilide macrolide (Fischer et al., 2011). In this *in vivo* study it was demonstrate that the caspase-3-dependent pro-apoptotic effects of tulathromycin are associated with the inhibition of NFkB phosphorylation, reduction of the nuclear translocation of the NFkB p65 subunit, and decreases in mRNA levels of CXCL8 (Fischer et al., 2011). The link between NFkB and apoptosis has also been demonstrated in *in vitro* studies, where it was found that erythromycin inhibited RANKL and NFkB expression that was related to increased apoptosis in Jurkat T-cells (Wu et al., 2009). Similarly it was found that telithromycin affected NFkB expression which resulted in increased apoptosis in both a macrophage and murine epithelial lung cell line (Leiva et al., 2008).

*1.4.4.4 Degranulation*

If triggered, in an attempt to destroy the invading pathogen, neutrophils will become activated and readily release granules containing cytotoxic enzymes, such as elastase, in a process called neutrophil degranulation or exocytosis. Some studies seem to suggest that the macrolides posses the ability to upregulate or stimulate neutrophil degranulation (Culic et al., 2001).

In early *in vitro* studies different macrolides (erythromycin, azithromycin, roxithromycin, clarithromycin, spiramicin, rokitamycin, oleandomycin and dirithromycin) were added to neutrophils obtained from healthy volunteers and neutrophil degranulation was assessed. It was found that the 14 and 15 membered macrolides could increase neutrophil release of lysozyme and β-glucuronidase. Degranulation was also found to be reliant on the concentration in a time dependent manner, such that lower concentrations of the various drugs needed longer periods of time to stimulate degranulation (Abdelghaffar et al., 1996).

Similar to what was found in relation to ROS, it was found that within 2 to 24 hours after azithromycin administration, there was increased neutrophil degranulation in neutrophils isolated from healthy humans (Culic et al., 2002). Comparable results were obtained in a different *ex vivo* study, which evaluated the effect erythromycin when administered intravenously had on degranulation of peripheral blood neutrophils obtained from 6 healthy humans. Findings showed that non-stimulated neutrophil degranulation did not increase, but the drug did have an effect once the neutrophils were exposed to *Streptococcus pneumoniae*. The effects of erythromycin were maximal 4 hours after infusion was discontinued, when Bactericidal/Permeability-Increasing Protein (BPI) concentrations were 107.6±33.5% of those before erythromycin infusion, Human Neutrophil Elastase (HNE) 134.7±34.8% and Human Lactoferrin 205.9±55.9% (Schultz et al., 2000). Slight, but yet statistically significant increase in granule exocytosis was also induced by HMR 3647, a synthetic erythromycin A derivative in the ketolide family (Vazifeh et al., 1998). As opposed to the findings above, others results show that macrolides were able to inhibit neutrophil elastase, which was thought to contribute to its anti-inflammatory effect (Gorrini et al., 2001).

*1.4.4.5 Reaction to chemo attractants*

It appears as though not only the production of chemotactic factors is reduced by the macrolides (Bosnar et al., 2009, Meyer et al., 2009), but also the reaction of neutrophils to chemo attractants. As such it was found that *in vitro*, neutrophils which had been incubated in the presence of clarithromycin had a reduced chemotactic activity (Kadir et al., 2000).

Chemotaxis of neutrophils collected from humans was also found to be reduced in Gel-filtration chromatography which was done on neutrophils collected from humans who had been exposed to erythromycin (Oda et al., 1994).

This reduced reaction to chemokines by neutrophils is likely to be one of the reasons why reduced numbers of neutrophils have been found in lungs infected or challenged lungs after macrolide administration (Bosnar et al., 2009, Legssyer et al., 2006).

### 1.4.5 Effect of azithromycin on adaptive immunity

As can be seen from the literature summarized above, it is clear that macrolides have an immune modulatory function, especially in regards to innate immunity. Nevertheless, there are studies that demonstrate that macrolides can also modulate aspects of cellular or adaptive immunity, resulting in reductions in inflammation. The levels of lymphocytes in lungs from patients with diffuse panbronchiolitis who were treated with long term macrolides, showed reduced levels of cytotoxic T- lymphocyte infiltration (Kawakami et al., 1997). This effect is at least partially induced by the increased apoptosis in lymphocytes (Ishimatsu et al., 2004, Mizunoe et al., 2004, Kadota et al., 2005). Moreover, through the down regulation of cytotoxic (CD8) T-lymphocytes, azithromycin was found to increase the number of CD4 T-helper cells. These lymphocytes play a pivotal role in directing the immune response and their increased number was associated with down regulation of neutrophils in the same experiment (Feola et al., 2010).

Dendritic cells are the most important antigen-presenting cells and play a central role in the initiation and regulation of immune responses. Sugiyama et al. (2007) demonstrated that clarithromycin and azithromycin modulate the function of dendritic cells (DC). Both drugs significantly increased the intensity of CD80 on DC, but not CD86 and CD40. CD80 and CD87 are known as members of the B7 family, which is expressed on a variety of antigen-presenting cells including DC, Langerhans cells, activated macrophages and B cells and are thought to play a role in immune regulation and T-cell differentiation (Wang and Chen, 2004).

Additionally, it has been found that macrolides also have an inhibitory effect on the release of cytokines by lymphocytes. T-helper cells which were collected from healthy and chronic rhinositis patients were treated with roxithromycin and cytokine levels were evaluated. Although it was found that roxithromycin had no effect on IL-2 and IFNγ levels, it was found to reduce both IL-4 and IL-5 release in both stimulated and unstimulated lymphocytes (Asano et al., 2001a). In a different experiment the same group found that roxithromycin had the ability to reduce the proliferation of splenic B-lymphocytes in mice although no effect on T-

lymphocytes was noted. Another finding in the same study was that roxithromycin could also suppress the expression of CD40, CD80 and CD86 on B-lymphocytes that was associated with significant suppression of cell activation by antigenic stimulation (Asano et al., 2001b). On the contrary, an early *in vivo* study in healthy volunteers showed a small but significant positive effect of azithromycin on the proliferative B cell response of stimulated lymphocytes (Tomazic et al., 1993).

Similarly, *in vitro* studies, have demonstrated a marked reduction in T-cell proliferation after erythromycin administration. Interestingly it was found that the suppression of differentiation of blast cells to T-lymphocytes was only achieved if erythromycin was administered 48 hours after IL-2 stimulation. In cases where the blast cells were pretreated with azithromycin and then stimulated with IL-2, no inhibitory effect was noted (Keicho et al., 1993). However it has been suggested that these results could not be directly translated to the *in vivo* situation. As such, in an experiment evaluating lymphocyte viability and function, it was found that although azithromycin had the ability to reduce lymphocyte proliferation, this was not always the case when the drug was administered to mice. It was found that although some inhibition in lymphocyte proliferation occurred when azithromycin was administered in low dosages, this effect was lost when higher dosages of the drug were administered, presumably due to compensatory mechanisms (Ortega et al., 2002).

Recently it has been concluded that azithromycin has a pro-resolution effect on inflammation although to what extent was not clear, as it could not reduce CD3 expressing T-lymphocytes (Navarro-Xavier et al., 2010). In this murine study, it was found that azithromycin had a pro resolving effect on zymosan induced peritoneal inflammation by reducing the numbers of infiltrating neutrophils and macrophages.

## 1.5 Azithromycin and periodontal disease

Periodontitis is a multi-factorial disease, which although initiated by plaque, manifests as a result of tissue destruction mediated through the resulting inflammatory and immune responses (Kornman, 2008). Hence, it could be argued that an agent with antimicrobial action, as well as anti-inflammatory or immune modulatory functions, could be a valuable tool in limiting the destruction of the periodontal tissues. As previously discussed, azithromycin is readily distributed in the periodontal tissues, especially in areas of inflammation (Blandizzi et al., 1999) and alters cytokine levels in the GCF (Ho et al., 2010) thus clearly being bioavailable for interactions with both the host and pathogen. Adding to this, it has recently been shown that GCF concentrations of azithromycin are higher and more stable in GCF, when compared to serum levels and is thus more readily available for the treatment of

periodontitis (Lai et al., 2011). A recent extension of this study showed that there was no reduction of the GCF concentration of azithromycin with increased GCF flow, supporting its efficacy as antimicrobial when used in periodontitis cases (Jain et al., 2012).

Unfortunately there are only a limited number of studies which have dealt with the application of azithromycin in the management of periodontal disease. These studies have recently been summarized in a narrative review, specifically relating to the use of azithromycin in the treatment of periodontitis (Hirsch et al., 2012).

In probably one of the most promising reports, azithromycin was found to improve several parameters significantly in a double blinded, randomized placebo controlled clinical trial (Smith et al., 2002). In this study 46 patients were treated either with the antimicrobial (500 mg azithromycin once a day for 3 days) or placebo, administered 2 weeks after the commencement of oral hygiene instructions and mechanical debridement. Unfortunately, further information is not given on the nature of mechanical debridement. Results obtained showed that by week 22 the percentage of pockets that had remained deeper than 5 mm in the azithromycin group was significantly less (5.6%) compared to the placebo group (23.3%). Additionally it was found that for pockets which were initially deeper than 4 mm, the test group had less sites >3 mm (21.6%) after 22 weeks than the control group (44.3%). The other parameters, which were also found to be favourable for azithromycin, were fewer pockets failing to improve in probing depth (A, 6.6%; C, 21.6%) and fewer continuing to bleed on probing (A, 46.9%; C, 55.6%) when compared with the control group. Pocket depths initially measured at 4 to 5 mm or 6 to 9 mm also showed lower mean pocket depths in the patients on azithromycin at weeks 6, 10 and 22, with all measurement reaching statistical significance (Smith et al., 2002).

The Smith et al paper (2002) on the clinical improvements obtained using azithromycin was published on the back of an earlier report which evaluated microbiological improvements in the same experiment (Sefton et al., 1996). In agreement with the clinical results, it was found that there were significantly less black-pigmented anaerobes and spirochetes 6 weeks after azithromycin administration compared to the control. Microbiological counts at 22 weeks showed that although there were still significantly less spirochetes in the test group compared to control, there was not a statistical difference between the groups in relation to black pigmented anaerobes (Sefton et al., 1996). Similar reductions in black pigmented anaerobes were confirmed in others studies (Haffajee et al., 2007, Gomi et al., 2007b) although it was found that instead of a true reduction in total bacterial numbers, the reductions in black pigmented anaerobes rather represented a significant shift in the bacterial ecology (Gomi et al., 2007b). The long term clinical significance of these findings is also not clear, as it was found that although an initial difference in bacterial profile was present between

subjects who had taken azithromycin compared to those who did not, there was no statistically significant difference after 12 months (Haffajee et al., 2007). Results from *in vitro* studies specifically showed that azithromycin is highly effective against *Porphyromonas gingivalis* (Pajukanta, 1993) as well as *Aggregatibacteractinomyces comitans (A.a.)* (Pajukanta et al., 1992). More recently, it was found that in a biofilm model of three different strains of *P. gingivalis*, azithromycin was effective even in sub-MIC concentrations on significantly reducing biofilm viability, a finding that not only demonstrates its effectiveness on *P. gingivalis* but also its effectiveness against protected microbial biofilm populations (Maezono et al., 2011).

When evaluated for its efficacy to reduce bacterial numbers in periodontal abscesses without mechanical debridement, it was found that azithromycin was equally as effective as augmentin 500 mg p.o. in reducing the numbers of several periodontal pathogens such as *P. gingivalis, P. intermedia, B. forsythus, P. micros* and *F. nucleatum* (Herrera et al., 2000a). It should be remembered that mechanical debridement should generally form a part of the treatment of periodontal abscesses as it allows for drainage as well as the removal of calculus or excess tissue (Herrera et al., 2000b, Kareha et al., 1981).

Clinical improvements were also noted in other studies. One of these (Haffajee et al., 2007) assessed 96 patients over a one year period for gingival redness, bleeding on probing (BOP), suppuration, pocket depth (PD) and clinical attachment level (CAL). Patients were divided into 4 groups; namely scaling and root planning (SRP) only, SRP with 500 mg azithromycin p.o. once daily for 3 days, metronidazole 250 mg t.i.d. for 14 days or 20 mg doxycycline (SDD) for 3 months. Results showed that in general, patients receiving adjunct treatment exhibited greater clinical improvement compared to the SRP control, with the highest reduction in CAL found in the metronidazole group. Interestingly, 3 months after the initial phase, the time point at which SDD was stopped, an increase in mean pocket depth was noted in the SDD and SRP alone groups. It was also found that subjects with deeper pockets (>6 mm) showed statistically significant greater pocket reductions in the azithromycin and metronidazole groups, that maintained a statistically significant difference after 12 months and approached significance after 6 months. A further interesting observation from the results presented in the study is that although metronidazole and azithromycin as adjuncts resulted in the biggest reduction in PD, metronidazole and SDD performed best in terms of CAL. As such the only reasonable explanation for the difference between PD and AL observed is that patients receiving azithromycin generally had more recession compared to the other groups. (Haffajee et al., 2007).

However, it should be noted that results obtained in the aforementioned study showed only modest improvements in clinical parameters beyond that achieved by SRP alone. The major

reason for this finding was that although subjects entering the study had at least eight sites with PD >4 mm, the mean BPD and AL values were quite low, suggesting that most of the subjects had mild to moderate levels of periodontitis. Both the systematic reviews (Haffajee et al., 2003, Herrera et al., 2002) evaluating the effects of systemically administered antibiotics suggested that antibiotics provided greater benefit in subjects with more periodontal disease and at deeper periodontal sites.

More recently, a further study was conducted on patients with moderate chronic periodontitis harbouring *P. gingivalis* (Oteo et al., 2010), although the extent of periodontal destruction was only 29%, and thus localized (Armitage, 1999). In this blinded randomised placebo controlled study, patients received mechanical subgingival debridement over the course of 2 visits within a 7-day period with adjunctive azithromycin administered (500 mg p.o. for 3 days) at the last visit. It was found that the test group had significantly better improvements in terms of CAL and PPD. In terms of microbiological parameters, it was also noted that in the test group *P. gingivalis* decreased significantly after 1, 3 and 6 months, as did other pathogens such as *T. forsythia* and *A.a.* However due to the fact that several patients were lost to follow up over the 6-month duration of the study, there were only 15 patients available for follow up in the test group and 11 in the control group. Furthermore, the effect of smoking in the results could not be properly assessed and results from this study should be interpreted with caution (Oteo et al., 2010). As opposed to the above results, a recent placebo controlled randomized controlled trial found that there was no significant improvement in clinical or microbiological parameters compared to the control when azithromycin was administered to patients with chronic periodontitis in conjunction with subgingival root debridement over a 1 year period (Sampaio et al., 2011). A possible reason for this may be that exceptionally good results were achieved in the control group, due to the thorough mechanical debridement that was performed by experienced clinicians over a treatment period of up to 7 hours. Nevertheless, a more recent study evaluating the effect of adjunctive administration of azithromycin to after non-surgical periodontal therapy showed that both azithromycin and placebo groups demonstrated similar improvements in all clinical parameters as well as MMP-8 levels in GCF (Han et al., 2012). This finding was supported by another publication from the same group, once again showing similar outcomes in MMP-8 levels and bacterial counts between patients treated with adjunctive azithromycin or placebo. However, in this study, a significant improvement in terms of pocket depth and number of deep sites was noted in the azithromycin group at one month (Emingil et al., 2012).

When studying bacterial susceptibility to antimicrobials, it should be kept in mind that bacterial co-cultures and biofilms generally are more resistant. Indeed this was shown to be the case for co-cultures of *A.a., Capnocytophaga ochracea (Co),* and *Parvimonas micra (Pm*)

(previously *Peptostreptococcus micros*) which showed increased resistance to azithromycin, when compared to pure or monocultures alone (Mouratidou et al., 2011).

The effect of adjunct administration of azithromycin in chronic periodontitis was not only assessed, but also the effect it has in patients with localized aggressive periodontitis (Haas et al., 2008). In total, 26 patients were assessed in a double blind randomized placebo controlled trial and received subgingival root debridement (SRD) in multiple visits with or without adjunct azithromycin, which was administered at the end of mechanical debridement. Results once again showed that the biggest reduction in PD was achieved in deeper pockets and the greatest amount of change occurred during the first 3 months. Nonetheless from 6 to 12 months post-operatively, the group treated with azithromycin showed a continued improvement in CAL as well as pocket depth, whereas the control group demonstrated a trend to increase both parameters (Haas et al., 2008). A recent extension of this study evaluated the microbial profile of patients with AgP that were either treated with SRD and azithromycin or SRD and placebo in a randomized controlled trial using checkerboard-DNA-DNA-hybridization. It was found that there was no difference over a 12 month period between patients treated with azithromycin compared to placebo between any of the microbial complexes that were analysed (Haas et al., 2012). Although this study consisted of a small study population (n=24) this finding is interesting in that clinical improvements were noted in AgP patients treated with azithromycin (Haas et al., 2008)in the absence of microbiological improvements (Haas et al., 2012).

Smokers also seemed to respond well to adjunct treatment with azithromycin, and once again it was found that the largest reduction in PD occurred in deeper pockets. In this case the study population consisted of 31 subjects who had the equivalent smoke exposure to 5 pack years. Adding to the improvement in clinical parameters, Benzoyl-DL-arginine-naphthylamide (BANA) sampling also improved 10 fold, compared to the control after, over a 6 month period (Mascarenhas et al., 2005).

It should however be noted that there were statistically significant differences at base line between the test and control group, and the azithromycin dosage of 250 mg p.o. once a day for 5 days did not correspond to the other studies, thus making comparisons to other studies difficult (Mascarenhas et al., 2005).

A different double blind randomized placebo controlled study was conducted on smokers, which focused on clinical improvement following pocket elimination surgery (apically repositioned flap with osseous recontouring). The data presented suggested that surgical treatment of moderate and deep periodontal pockets in heavy smokers improved CAL gain and PD reduction as well as education of BOP. Nevertheless, the adjunctive administration of systemic azithromycin to surgical treatment did not improve overall PD reduction or CAL

gain, compared to sites that received surgical treatment only. For teeth that were treated non-surgically, the systemic administration of azithromycin furthermore yielded significant gains in overall CAL compared to baseline, whereas placebo did not. Although it would require more research to confirm these results, the authors also found that the addition of AZM during periodontal surgery in heavy smokers promoted rapid wound healing, as well as reduced short term gingival inflammation as measured according to a wound healing index. It was also found that azithromycin resulted in less plaque formation within 3 months although no explanation for this could be presented (Dastoor et al., 2007).

Azithromycin was also evaluated as an adjunct to full mouth debridement. When the effect of full mouth debridement with additional azithromycin was compared to quadrant SRP without adjunct antibiotics only, it was found that the group receiving the full mouth debridement in conjunction with azithromycin performed better in terms of clinical and microbiological parameters compared to the control (Gomi et al., 2007b). Unfortunately, due to the different protocols used between the test and control groups, it becomes hard to attribute the improvement to either only full mouth debridement or azithromycin. Adding to this, recently published systematic reviews have not found any significant benefits from the FMD protocol over the normal quadrant root planning protocol (Matthews, 2009, Lang et al., 2008) although both protocols are probably equally effective in reducing periodontal disease. Furthermore, a recent meta-analysis by the Cochrane collaboration reported that there is a small beneficial effect delivered by full mouth debridement when compared to the control, but that these improvements were only small and more research is needed in order to draw conclusions regarding real clinical benefits (Eberhard et al., 2008).

In order to negate the limitations in study design, a further study was therefore conducted more recently by the same group to evaluate the effect staged mechanical debridement had if performed during the effective half life of azithromycin, compared to full mouth debridement. It was once again found that both staged SRP and full mouth debridement led to statistically significant improvements in terms of AL and PD as well as improvements in the microbiological profile compared to baseline values. However, there was no significant difference between the test and control group (Yashima et al., 2009). These results thus fitted with the findings of the mentioned systematic reviews (Matthews, 2009, Lang et al., 2008). A further deduction that could be made when considering the earlier study (Gomi et al., 2007a), is once again the importance of administering adjunct antibiotics in conjunction with mechanical therapy to achieve the best clinical result. The value of mechanical therapy was also demonstrated in recent randomized clinical trial, evaluating the added benefit of adjunct azithromycin to non surgical debridement for the treatment of peri-implant mucositis. In total 48 patients were treated either with debridement alone or with debridement and azithromycin

and it was found that there was no added benefit of the adjunct use of the antimicrobial (Hallstrom et al., 2012). However, this should be seen in the context of the known efficacy of mechanical therapy alone in the treatment of perimucositis, which may be sufficient to resolve local inflammation (Heitz-Mayfield et al., 2011, Maximo et al., 2009, Renvert et al., 2008).

Local applications of azithromycin have also been studied (Pradeep et al., 2008). In this study eighty patients were either treated with SRD only or with SRD with adjunct azithromycin which was prepared as a PLGA in *situ gel* at a concentration of 0.5% and placed into the gingival sulcus after debridement.

For both clinical parameters (CAL and PD) there was a significant improvement from baseline, as well as a significant difference between groups at 3 months. In terms of microbiological parameters used, there was also a significant improvement from baseline and between the groups (Pradeep et al., 2008). However, in this study, the differences in reductions in PD (0.4 mm) and CAL (0.6 mm) at the 3 month time point are so small that one could question the clinical relevance. Furthermore the trial was neither placebo controlled nor blinded which leaves results at risk for bias. A similar study was done by the same research group on patients with well-controlled type II diabetes and moderate to advanced periodontitis. In this study, 63 patients were randomly assigned to receiving non-surgical therapy with either local application of azithromycin gel or placebo gel. Once again small differences were noted between the azithromycin and placebo groups (Agarwal et al., 2012). Although this study was of better design compared to the earlier study, by inclusion of a randomization protocol, results were reported as mean reduction in pocket depth, making interpretation of the data difficult. Furthermore, although the patients were blinded as to the adjunctive use of azithromycin or placebo, it is unclear if the treating clinician was blinded or not, leaving the study open for bias.

## 1.6 Azithromycin and gingival enlargement

It has been found that azithromycin is effective in reducing fibrosis and as a result significantly improved the respiratory function of mice with bleomycin induce lung fibrosis. It was found that the pretreatment of mice with azithromycin led to reductions in fibroblast proliferation as well as collagen type I deposition. The authors attributed this effect at least in part to the immune modulatory function of azithromycin, which was found to not only reduce neutrophil infiltration but also increase TH2 and TH17 cell numbers. Although these results are interesting in terms of prevention of lung fibrosis, the author suggested further work would be needed to asses if azithromycin could reverse or reduce fibrosis and collagen deposition during lung fibrosis and scaring (Wuyts et al., 2010).

Just as the lungs are subject to fibrosis, so are the gingivae, although the etiology and pathogenesis undoubtedly differ. Gingival enlargement is a well documented side effect of over 20 drugs which can be grouped into anticonvulsants, immunosuppressant's and calcium channel blockers (Dongari-Bagtzoglou (2004) adapted by Clementini et al. (2008) (Table 3)).

**Table 3.** Estimated prevalence of gingival enlargement in relation to medication (Clementini et al., 2008).

**Table 3: Estimated prevalence of Drug-Associated Gingival Enlargement according to the most frequently reported prevalence rates (Informational Paper: Drug-Associated Gingival Enlargement. *J periodontal 2004;75:1424–1431*)**

| Category | Pharmacologic Agent | Trade Name | Prevalence |
|---|---|---|---|
| **Anticonvulsants** | Phenytoin | Dilantin | 50% |
| | Sodium valproate (valproic acid) | Depakene, Depacon, Epilim, Valpro | Rare |
| | Phenobarbitone | Phenobarbital, Donnatal | <5% |
| | Vigabatrin | Sabril | Rare |
| | Carbamazepine | Tegretol | None reported |
| **Immunosuppressants** | Cyclosporin | Neoral, Sandimmune | Adults 25–30%<br>Children >70% |
| **Calcium channel blockers** | Nifedipine | Adalat, Nifecard, Procardia, Tenif | 6–15% |
| | Isradipine | DynaCirc | None reported |
| | Felodipine | Agon, Felodur, Lexxel, Plendil | Rare |
| | Amlodipine | Lotrel, Norvasc | Rare |
| | Verapamil | Calan, Covera, Isoptin, Tarka, Verelan | <5% |
| | Diltiazem | Cardizem, Dilacor, Diltiamax, Tiazac | 5–20% |

The main histological feature of gingival enlargement is the overproduction of collagen, although to date the exact pathophysiology by which this occurs has not been identified. Many factors related to cyclosporine-induced gingival overgrowth, including the serum concentration, drug dosage, age, gender, concomitant medication, and oral hygiene, have been evaluated with particular focus on the drug itself and the effect of oral hygiene. Despite the fact that gingival enlargement has been researched for over more than half a century, the pathogenesis of gingival overgrowth is not clear and treatment is still mainly focused on maintenance and improved levels of oral hygiene in conjunction with surgical removal of the overgrown tissue (Seymour, 1996).

In recent years it was found that azithromycin had the potential to reduce gingival overgrowth, although to which extent and exactly through which mechanism unclear. The first reported case of a marked reduction in gingival hyperplasia associated with the administration of azithromycin was noted in 1993, when a patient receiving cyclosporine contracted bronchitis and was administered azithromycin (Wahlstrom et al., 1995). Since then several other papers have been published with similar findings, although most of them were published in medical rather than dental journals.

It appears as the though most of the reduction in gingival enlargement occurs within the first month (Tokgoz et al., 2004), with the biggest effect during the first week (Tokgoz et al., 2004, Palomar et al., 1998) and the effect lasting up to 6 months post administration (Gomez et al., 1997) with a success rate of 89% (Citterio et al., 2001).

When compared to oral hygiene procedures alone, azithromycin together with oral hygiene measures was found to significantly reduce cyclosporine-induced gingival overgrowth over a 30 day period (Ramalho et al., 2007). A double blind randomised controlled study testing the efficacy of metronidazole and azithromycin in controlling cyclosporine-A (CSA)-induced gingival enlargement has shown that the latter was significantly more effective, although variation in results existed for different time points which could not be explained. Another interesting point in this study was that 70% of test subjects took both cyclosporine as well as calcium channel blockers (Chand et al., 2004) which could indicate that azithromycin is effective in reducing gingival enlargement caused by both of these groups of drugs.

The possibility that azithromycin is effective in not only reducing cyclosporine-induced overgrowth, but also in the reduction of gingival overgrowth associated with calcium channel blockers (Fourtounas and Vlachojannis, 2009, Hirsch, 2010)and phenytoin (Namazi et al., 2007) has recently started to receive some attention. At present only one case report exists suggesting the effectiveness of azithromycin in reducing gingival overgrowth induced by calcium channel blockers, (Hirsch, 2010) and as such, even though initial observations seem promising, much more research is currently needed to substantiate these claims.

One important point which should be mentioned is that improvement in gingival overgrowth, as presented in a systematic review on the topic, were only achieved when azithromycin was used in conjunction with oral hygiene instructions and mechanical debridement (Clementini et al., 2008). In total the review identified only five studies (Ramalho et al., 2007, Nafar et al., 2003, Chand et al., 2004, Mesa et al., 2003, Nash and Zaltzman, 1998) which had met the inclusion criteria of being randomized controlled trials with proper blinding. Results from the studies showed that only one study found no significant differences between adjunctive use of metronidazole or azithromycin (Mesa et al., 2003).

More recently, the effect on gingival overgrowth of a topical application of azithromycin in toothpaste form (85 mg azithromycin/1 g toothpaste) for one month was studied (Argani et al., 2006). Although the results from the study were promising as they showed significant decrease in gingival overgrowth, proper blinding was not used in this study, which makes interpreting results difficult. Furthermore, the repeated application of low dose antibiotics raises the question of creating an opportunity for increased microbial resistance and should thus be considered with caution.

As possible mechanism of action for the effect of azithromycin on gingival overgrowth may relate to its ability to inhibit the proliferation of fibroblasts and elevate the levels of matrixmetalloproteinases (especially MMP-2 and less MMP-1) as well as blocking the accumulation of collagen associated with gingival overgrowth (Kim 2008). This was substantiated by the finding from the same study that cyclosporin-A treated fibroblasts had up regulated collagen I and down regulated MMP-2 mRNA expression, which was blocked by azithromycin (Kim et al., 2008). However *in vivo* experiments found that there was no change in the mRNA expression of collagen I after azithromycin administration to rats with gingival hyperplasia, although a reduction in overgrowth was noted which was associated with increased phagocytic activity of fibroblasts (Paik et al., 2004). A more recent study (Condé et al., 2009) showed that the release of TGF-β, which has been associated with gingival overgrowth (Vieira Jr et al., 1999) was reduced in rats in conjunction with a reduction in fibroblast numbers as well as a reduction in inflammatory infiltrate after roxithromycin administration (Condé et al., 2009).

## 1.7 Conclusion

When reviewing the available literature, it is clear that azithromycin has anti-inflammatory or host modulatory functions in addition to its antimicrobial effects. At present, although information is available demonstrating the effect of this host modulatory potential, the mechanisms through which this occurs is still not entirely clear. Very limited information also seems to be available on the potential benefits of using azithromycin in the treatment of periodontitis, a disease marked by both inflammatory and microbial or infective components (Kornman, 2008). This is especially true for the aspect of inflammation initiated by known periodontal pathogens, such as *P. gingivalis* and those patients being more susceptible to periodontal disease. Currently, it is these patients for whom adjunctive antibiotic therapy is recommended (Heitz-Mayfield, 2009), and it may be that azithromycin could become a viable alternative to the current regime of high dosage of amoxicillin in conjunction with metronidazole, which is associated with the frequent occurrence of side effects (Winkel et al., 2001). To what degree the host modulatory function of azithromycin will benefit these patients, and why some patients seem to respond better to this effect than others (Khan et al., 1999, Labro et al., 1989), remains to be determined.

## 1.8 Aim of study

The purpose of this study was to investigate the effect of azithromycin on experimentally induced acute and chronic inflammation. Specifically effects on measures of inflammatory cell infiltrate and collagen levels induced by wound healing and inflammation associated fibrosis were assessed.

## 1.9 Hypothesis

Azithromycin has an immune modulatory effect on experimentally-induced acute and chronic inflammation in rats.

## 1.20 References


Abdelghaffar, H., Vazifeh, D. & Labro, M. T. (1996) Comparison of various macrolides on stimulation of human neutrophil degranulation *in vitro. Journal of Antimicrobial Chemotherapy* **38,** 81-93.

Addy, L. D. & Martin, M. V. (2004) Azithromycin and dentistry - A useful agent? *British Dental Journal* **197,** 141-143.

Agarwal, E., Bajaj, P., Naik, S. B. & Pradeep, A. R. (2012) Locally delivered 0.5% azithromycin, as an adjunct to non surgical treatment in chronic periodontitis with type 2 diabetes: A randomized controlled clinical trial. *Journal of Periodontology*

Akamatsu, H., Yamawaki, M. & Horio, T. (2001) Effects of roxithromycin on adhesion molecules expressed on endothelial cells of the dermal microvasculature. *Journal of Internal Medicine Research* **29,** 523-527.

Albert, R. K., Connett, J., Bailey, W. C., Casaburi, R., Cooper Jr, J. A. D., Criner, G. J., Curtis, J. L., Dransfield, M. T., Han, M. K., Lazarus, S. C., Make, B., Marchetti, N., Martinez, F. J., Madinger, N. E., McEvoy, C., Niewoehner, D. E., Porsasz, J., Price, C. S., Reilly, J., Scanlon, P. D., Sciurba, F. C., Scharf, S. M., Washko, G. R., Woodruff, P. G. & Anthonisen, N. R. (2011) Azithromycin for prevention of exacerbations of COPD. *New England Journal of Medicine* **365,** 689-698.

Al-Joburi, W., Quee, T. C., Lautar, C., Iugovaz, I., Bourgouin, J., Delorme, F. & Chan, E. C. (1989) Effects of adjunctive treatment of periodontitis with tetracycline and spiramycin. *Journal of Periodontology* **60,** 533-539.

Altenburg, J., de Graaff, C. S., van der Werf, T. S. & Boersma, W. G. (2010a) Immunomodulatory effects of macrolide antibiotics - Part 1: Biological mechanisms. *Respiration*

Altenburg, J., de Graaff, C. S., van der Werf, T. S. & Boersma, W. G. (2010b) Immunomodulatory effects of macrolide antibiotics - Part 2: Advantages and disadvantages of long-term, low-dose macrolide. Therapy *Respiration*

Ambrose, K. D., Nisbet, R. & Stephens, D. S. (2005) Macrolide efflux in Streptococcus pneumoniae is mediated by a dual efflux pump (mel and mef) and is erythromycin inducible. *Antimicrob Agents Chemotherapy* **49,** 4203-4209.

Anderson, R., Theron, A. J. & Feldman, C. (1996) Membrane-stabilizing, anti-inflammatory interactions of macrolides with human neutrophils. *Inflammation* **20,** 693-705.

Araujo, L. & Demoly, P. (2008) Macrolides allergy. *Current Pharmacological Decisions* **14,** 2840-2862.

Argani, H., Pourabbas, R., Hassanzadeh, D., Masri, M. & Rahravi, H. (2006) Treatment of cyclosporine-induced gingival overgrowth with azithromycin-containing toothpaste. *Experiments in Clinical Transplantation* **4,** 420-424.

Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* **4,** 1-6.

Asano, K., Kamakazu, K., Hisamitsu, T. & Suzaki, H. (2001a) Modulation of Th2 type cytokine production from human peripheral blood leukocytes by a macrolide antibiotic, roxithromycin, *in vitro. International Immunopharmacology* **1,** 1913-1921.

Asano, K., Suzuki, M., Shimane, T. & Suzaki, H. (2001b) Suppression of co-stimulatory molecule expressions on splenic B lymphocytes by a macrolide antibiotic, roxithromycin. *in vitro. International Immunopharmacology* **1,** 1385-1392.

Baeuerle, P. A. & Henkel, T. (1994) Function and activation of NF-κB in the immune system. *Annual Review of Immunology* **12,** 141-179.

Banche, G., Tullio, V., Allizond, V., Mandras, N., Roana, J., Scalas, D., El Fassi, F., D'Antico, S., Cuffini, A. M. & Carlone, N. (2010) Synergistic effect of erythromycin on polymorphonuclear cell antibacterial activity against erythromycin-resistant phenotypes of Streptococcus pyogenes. *Int J Antimicrobial Agents* **36,** 319-323.

Barkett, M., Xue, D., Horvitz, H. R. & Gilmore, T. D. (1997) Phosphorylation of IkappaB-alpha inhibits its cleavage by caspase CPP32 *in vitro. Journal of Biology and Chemistry* **272,** 29419-29422.

Bartold, P. & Narayanan, A. (1998) *Biology of the Periodontal Connective Tissue*. Chicago: Quintessence Publishing Co.

Bartold, P. M., Cantley, M. D. & Haynes, D. R. (2010) Mechanisms and control of pathologic bone loss in periodontitis. *Periodontology 2000* **53,** 55-69.

Beigelman, A., Gunsten, S., Mikols, C. L., Vidavsky, I., Cannon, C. L., Brody, S. L. & Walter, M. J. (2009) Azithromycin attenuates airway inflammation in a noninfectious mouse model of allergic asthma. *Chest* **136,** 498-506.

Bermudez, L. E., Inderlied, C. & Young, L. S. (1991) Stimulation with cytokines enhances penetration of azithromycin into human macrophages. *Antimicrobial Agents Chemotherapy* **35,** 2625-2629.

Birkedal-Hansen, H. (1993) Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research* **28,** 500-510.

Blandizzi, C., Malizia, T., Lupetti, A., Pesce, D., Gabriele, M., Giuca, M. R., Campa, M., Del Tacca, M. & Senesi, S. (1999) Periodontal tissue disposition of azithromycin in patients affected by chronic inflammatory periodontal diseases. *Journal of Periodontology* **70,** 960-966.

Blau, H., Klein, K., Shalit, I., Halperin, D. & Fabian, I. (2007) Moxifloxacin but not ciprofloxacin or azithromycin selectively inhibits IL-8, IL-6, ERK1/2, JNK, and NF-kappaB activation in a cystic fibrosis epithelial cell line. *Am J Physiol Lung Cell Molecular Physiology* **292,** L343-352.

Borrell, L. N., Burt, B. & Taylor, G. W. (2005) Prevalence and trends in periodontitis in the USA: from NHANES III to the NHANES, 1988 to 2000. *Journal of Dental Research* **84,** 924-930.

Bosnar, M., Bosnjak, B., Cuzic, S., Hrvacic, B., Marjanovic, N., Glojnaric, I., Culic, O., Parnham, M. J. & Erakovic Haber, V. (2009) Azithromycin and clarithromycin inhibit lipopolysaccharide-induced murine pulmonary neutrophilia mainly through effects on macrophage-derived granulocyte-macrophage colony-stimulating factor and interleukin-1beta. *J Pharmacology Experimental Therapy* **331,** 104-113.

Bosnar, M., Kelneric, Z., Munic, V., Erakovic, V. & Parnham, M. J. (2005) Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin, and cethromycin. *Antimicrob Agents Chemotherapy* **49,** 2372-2377.

Bozinovski, S., Jones, J., Beavitt, S. J., Cook, A. D., Hamilton, J. A. & Anderson, G. P. (2004) Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. *Am J Physiol Lung Cell Molecular Physiology* **286,** L877-885.

Brook, I., Lewis, M. A., Sandor, G. K., Jeffcoat, M., Samaranayake, L. P. & Vera Rojas, J. (2005) Clindamycin in dentistry: more than just effective prophylaxis for endocarditis? *Oral Surg Oral Med Oral Pathology Oral Radiology Endodontics* **100,** 550-558.

Brown, B. A., Griffith, D. E., Girard, W., Levin, J. & Wallace, R. J., Jr. (1997) Relationship of adverse events to serum drug levels in patients receiving high-dose azithromycin for mycobacterial lung disease. *Clinical Infectious Diseases* **24,** 958-964.

Burt, B. (2005) Position paper: epidemiology of periodontal diseases. *Journal of Periodontology* **76,** 1406-1419.

Caffesse, R. G., Sweeney, P. L. & Smith, B. A. (1986) Scaling and root planing with and without periodontal flap surgery. *Journal of Clinical Periodontology* **13,** 205-210.

Canetti, C., Silva, J. S., Ferreira, S. H. & Cunha, F. Q. (2001) Tumour necrosis factor-alpha and leukotriene B (4) mediate the neutrophil migration in immune inflammation. *British of Journal Pharmacology* **134,** 1619-1628.

Carevic, O. & Djokic, S. (1988) Comparative studies on the effects of erythromycin A and azithromycin upon extracellular release of lysosomal enzymes in inflammatory processes. *Agents Actions* **25,** 124-131.

Carlier, M. B., Zenebergh, A. & Tulkens, P. M. (1987) Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. *Journal of Antimicrobial Chemotherapy* **20 Suppl B,**47-56.

Chand, D. H., Quattrocchi, J., Poe, S. A., Terezhalmy, G. T., Strife, C. F. & Cunningham, R. J. (2004) Trial of metronidazole vs. azithromycin for treatment of cyclosporine-induced gingival overgrowth. *Paediatric Transplantation* **8,** 60-64.

Chapple, I. L. (1997) Reactive oxygen species and antioxidants in inflammatory diseases. *Journalof Clinical Periodontology* **24,** 287-296.

Cheung, P. S., Si, E. C. & Hosseini, K. (2010) Anti-inflammatory activity of azithromycin as measured by its NF-KB inhibitory activity. *Ocular Immunology and Inflammation* **18,** 32-37.

Chico, R. M., Pittrof, R., Greenwood, B. & Chandramohan, D. (2008) Azithromycin-chloroquine and the intermittent preventive treatment of malaria in pregnancy. *Malaria Journal* **7,** 255.

Cigana, C., Assael, B. M. & Melotti, P. (2007) Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells. *Antimicrobial Agents for Chemotherapy* **51,** 975-981.

Cigana, C., Nicolis, E., Pasetto, M., Assael, B. M. & Melotti, P. (2006) Anti-inflammatory effects of azithromycin in cystic fibrosis airway epithelial cells. *Biochemistry Biophysiology Research Communications* **350,** 977-982.

Citterio, F., Di Pinto, A., Borzi, M. T., Scata, M. C., Foco, M., Pozzetto, U. & Castagneto, M. (2001) Azithromycin treatment of gingival hyperplasia in kidney transplant recipients is effective and safe. *Transplant Proceedings* **33,** 2134-2135.

Clementini, M., Vittorini, G., Crea, A., Gualano, M. R., Macri, L. A., Deli, G. & La Torre, G. (2008) Efficacy of AZM therapy in patients with gingival overgrowth induced by Cyclosporine A: a systematic review. *BMC Oral Health* **8,** 34.

Cohen, J. J., Duke, R. C., Fadok, V. A. & Sellins, K. S. (1992) Apoptosis and programmed cell death in immunity. *Annual Reviews of Immunology* **10,** 267-293.

Condé, S. A. P., Bastos, M. G., Vieira, B. J. & Aarestrup, F. M. (2009) Down-regulation of transforming growth factor beta-2 expression is associated with the reduction of cyclosporin induced gingival overgrowth in rats treated with roxithromycin: An experimental study. *BMC Oral Health* **9**

Criqui, G. I., Solomon, C., Welch, B. S., Ferrando, R. E., Boushey, H. A. & Balmes, J. R. (2000) Effects of azithromycin on ozone-induced airway neutrophilia and cytokine release. *European Respiratory Journal* **15,** 856-862.

Culic, O., Erakovic, V. & Parnham, M. J. (2001) Anti-inflammatory effects of macrolide antibiotics. *European Journal of Pharmacology* **429,** 209-229.

Culic, O., Erakovic, V., Cepelak, I., Barisic, K., Brajsa, K., Ferencic, Z., Galovic, R., Glojnaric, I., Manojlovic, Z., Munic, V., Novak-Mircetic, R., Pavicic-Beljak, V., Sucic, M., Veljaca, M., Zanic-Grubisic, T. & Parnham, M. J. (2002) Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects. *European Journal of Pharmacology* **450,** 277-289.

Cutress, T., Powell, R. & Ball, M. (1982) Differing profiles of periodontal disease in two similar South Pacific island populations. *Community Dentistry and Oral Epidemiology* **10,** 193-203.

Daigle, I. & Simon, H. U. (2001) Critical role for caspases 3 and 8 in neutrophil but not eosinophil apoptosis. *International Archives of Allergy and Immuno*logy **126,** 147-156.

Dastoor, S. F., Travan, S., Neiva, R. F., Rayburn, L. A., Giannobile, W. V. & Wang, H. L. (2007) Effect of adjunctive systemic azithromycin with periodontal surgery in the

treatment of chronic periodontitis in smokers: a pilot study. *Journal of Periodontology* **78,** 1887-1896.

Dongari-Bagtzoglou, A. (2004) Drug-associated gingival enlargement. *Journal of Periodontology* **75,** 1424-1431.

Donlan, R. M. & Costerton, J. W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *ClinicalMicrobiological Reviews* **15,** 167-193.

Drisko, C. H. (2001) Nonsurgical periodontal therapy. *Periodontology 2000* **25,** 77-88.

Eberhard, J., Jepsen, S., Jervoe-Storm, P. M., Needleman, I. & Worthington, H. V. (2008) Full-mouth disinfection for the treatment of adult chronic periodontitis. *Cochrane Database Systematic Review*CD004622.

Emingil, G., Han, B., Ozdemir, G., Tervahartiala, T., Vural, C., Atilla, G., Baylas, H. & Sorsa, T. (2012) Effect of azithromycin, as an adjunct to nonsurgical periodontal treatment, on microbiological parameters and gingival crevicular fluid biomarkers in generalized aggressive periodontitis. *Journal of Periodontal Research*

Enomoto, F., Andou, I., Nagaoka, I. & Ichikawa, G. (2002) Effect of new macrolides on the expression of adhesion molecules on neutrophils in chronic sinusitis. *Auris Nasus Larynx* **29,** 267-269.

Enomoto, F., Kin, R., Kataoka, T., Yamazaki, Y., Yokoi, H., Fujimori, M., Nagaoka, I. & Ichikawa, G. (2003) Modulation of neutrophil adhesion to vascular endothelial cells in rat experimental otitis media treated with a macrolide. *Auris Nasus Larynx* **30,** 247-251.

Feola, D. J., Garvy, B. A., Cory, T. J., Birket, S. E., Hoy, H., Hayes, D., Jr. & Murphy, B. S. (2010) Azithromycin alters macrophage phenotype and pulmonary compartmentalization during lung infection with Pseudomonas. *Antimicrobial Agents Chemotherapy* **54,** 2437-2447.

Ferrara, A., Dos Santos, C. & Lupi, A. (2001) Effect of different antibacterial agents and surfactant protein-A (SP-A) on adherence of some respiratory pathogens to bronchial epithelial cells. *International Journal of Antimicrobial Agents* **17,** 401-405.

Fiese, E. F. & Steffen, S. H. (1990) Comparison of the acid stability of azithromycin and erythromycin A. *Journal of Antimicrobial Chemotherapy* **25 Suppl A,** 39-47.

Fischer, C. D., Beatty, J. K., Zvaigzne, C. G., Morck, D. W., Lucas, M. J. & Buret, A. G. (2011) Anti-Inflammatory benefits of antibiotic-induced neutrophil apoptosis: tulathromycin induces caspase-3-dependent neutrophil programmed cell death and inhibits NF-kappaB signaling and CXCL8 transcription. *Antimicrob Agents Chemother* **55,**338-348.

Fong, K. P., Chung, W. O., Lamont, R. J. & Demuth, D. R. (2001) Intra- and interspecies regulation of gene expression by Actinobacillus actinomycetemcomitans LuxS. *Infections and Immunology* **69,** 7625-7634.

Foulds, G., Luke, D. R., Teng, R., Willavize, S. A., Friedman, H. & Curatolo, W. J. (1996) The absence of an effect of food on the bioavailability of azithromycin administered as tablets, sachet or suspension. *Journal of Antimicrobial Chemotherapy* **37 Suppl C,** 37-44.

Foulds, G., Shepard, R. M. & Johnson, R. B. (1990) The pharmacokinetics of azithromycin in human serum and tissues. *Journal of Antimicrobial Chemotherapy* **25 Suppl A,**73-82.

Fourtounas, C. & Vlachojannis, J. G. (2009) Gingival hyperplasia and calcium channel blockers. *Journal of Clinical Hypertension (Greenwich)* **11,** 111.

Fujii, T., Kadota, J., Kawakami, K., Iida, K., Shirai, R., Kaseda, M., Kawamoto, S. & Kohno, S. (1995) Long term effect of erythromycin therapy in patients with chronic Pseudomonas aeruginosa infection. *Thorax* **50,** 1246-1252.

Fukuda, Y., Yanagihara, K., Higashiyama, Y., Miyazaki, Y., Hirakata, Y., Mukae, H., Tomono, K., Mizuta, Y., Tsukamoto, K. & Kohno, S. (2006) Effects of macrolides on pneumolysin of macrolide-resistant Streptococcus pneumoniae. *European Respiration J* **27,** 1020-1025.

Gao, X., Ray, R., Xiao, Y., Ishida, K. & Ray, P. (2010) Macrolide antibiotics improve chemotactic and phagocytic capacity as well as reduce inflammation in sulfur mustard-exposed monocytes. *Pulmonary Pharmacological Therapy* **23,** 97-106.

Gavilanes, X., Huaux, F., Meyer, M., Lebecque, P., Marbaix, E., Lison, D., Scholte, B., Wallemacq, P. & Leal, T. (2009) Azithromycin fails to reduce increased expression of neutrophil-related cytokines in primary-cultured epithelial cells from cystic fibrosis mice. *Journal of Cystic Fibrosis* **8,** 203-210.

Giamarellos-Bourboulis, E. J. (2008) Macrolides beyond the conventional antimicrobials: a class of potent immunomodulators. *International Journal of Antimicrobial Agents* **31,** 12-20.

Gillis, R. J. & Iglewski, B. H. (2004) Azithromycin retards Pseudomonas aeruginosa biofilm formation. *Journal of Clinical Microbiology* **42,** 5842-5845.

Gladue, R. P. & Snider, M. E. (1990) Intracellular accumulation of azithromycin by cultured human fibroblasts. *Antimicrobial Agents and Chemotherapy* **34,** 1056-1060.

Gladue, R. P., Bright, G. M., Isaacson, R. E. & Newborg, M. F. (1989) *In vitro* and *in vivo* uptake of azithromycin (CP-62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection. *Antimicrobial Agents and Chemotherapy* **33,** 277-282.

Gomez, E., Sanchez-Nunez, M., Sanchez, J. E., Corte, C., Aguado, S., Portal, C., Baltar, J. & Alvarez-Grande, J. (1997) Treatment of cyclosporin-induced gingival hyperplasia with azithromycin. *Nephrology and Dialysis Transplantation* **12,** 2694-2697.

Gomi, K., Yashima, A., Iino, F., Kanazashi, M., Nagano, T., Shibukawa, N., Ohshima, T., Maeda, N. & Arai, T. (2007a) Drug concentration in inflamed periodontal tissues after systemically administered azithromycin. *Journal of Periodontology* **78,** 918-923.

Gomi, K., Yashima, A., Nagano, T., Kanazashi, M., Maeda, N. & Arai, T. (2007b) Effects of full-mouth scaling and root planing in conjunction with systemically administered azithromycin. *Journal of Periodontology* **78,** 422-429.

Gorrini, M., Lupi, A., Viglio, S., Pamparana, F., Cetta, G., Iadarola, P., Powers, J. C. & Luisetti, M. (2001) Inhibition of human neutrophil elastase by erythromycin and flurythromycin, two macrolide antibiotics *American Journal of Respiration and Cellular Molecular Biology* **25,** 492-499.

Greenstein, G. (1992) Periodontal response to mechanical non-surgical therapy: a review. *Journal of Periodontology* **63,** 118-130.

Haas, A. N., de Castro, G. D., Moreno, T., Susin, C., Albandar, J. M., Oppermann, R. V. & Rosing, C. K. (2008) Azithromycin as an adjunctive treatment of aggressive periodontitis: 12-months randomized clinical trial. *Journal of Clinical Periodontology* **35,** 696-704.

Haas, A. N., Silva-Boghossian, C. M., Colombo, A. P., Susin, C., Albandar, J. M., Oppermann, R. V. & Rosing, C. K. (2012) Adjunctive azithromycin in the treatment of aggressive periodontitis: Microbiological findings of a 12-month randomized clinical trial. *Journal of Dentistry* **40,** 556-563.

Haffajee, A. D., Socransky, S. S. & Gunsolley, J. C. (2003) Systemic anti-infective periodontal therapy. A systematic review. *Annals of Periodontology* **8,** 115-181.

Haffajee, A. D., Torresyap, G. & Socransky, S. S. (2007) Clinical changes following four different periodontal therapies for the treatment of chronic periodontitis: 1-Year results. *Journal of Clinical Periodontology* **34,** 243-253.

Hall, I. H., Schwab, U. E., Ward, E. S., Butts, J. D., Wolford, E. T. & Ives, T. J. (2002) Disposition and intracellular activity of azithromycin in human THP-1 acute monocytes. *International Journal of Antimicrobial Agents* **20,** 348-360.

Hallstrom, H., Persson, G. R., Lindgren, S., Olofsson, M. & Renvert, S. (2012) Systemic antibiotics and debridement of peri-implant mucositis. A randomized clinical trial. *Journal of Clinical Periodontology* **39** 574-581.

Hall-Stoodley, L. & Stoodley, P. (2009) Evolving concepts in biofilm infections. *Cellular Microbiology* **11,** 1034-1043.

Han, B., Emingil, G., Ozdemir, G., Tervahartiala, T., Vural, C., Atilla, G., Baylas, H. & Sorsa, T. (2012) Azithromycin as an adjunctive treatment of generalized severe chronic periodontitis: Clinical, microbiological and biochemical parameters. *Journal of Periodontology*

Hanada, H., Ikeda-Dantsuji, Y., Naito, M. & Nagayama, A. (2003) Infection of human fibroblast-like synovial cells with Chlamydia trachomatis results in persistent infection and interleukin-6 production. *Microbial Pathology* **34,** 57-63.

Hand, W. L. & Hand, D. L. (1993) Interactions of dirithromycin with human polymorphonuclear leukocytes. *Antimicrobial Agents of Chemotherapy* **37,** 2557-2562.

Hand, W. L. & Hand, D. L. (2001) Characteristics and mechanisms of azithromycin accumulation and efflux in human polymorphonuclear leukocytes. *International Journal of Antimicrobial Agents* **18,** 419-425.

Hand, W. L., Hand, D. L. & King-Thompson, N. L. (1990) Antibiotic inhibition of the respiratory burst response in human polymorphonuclear leukocytes. *Antimicrobial Agents and Chemotherapy* **34,** 863-870.

Hand, W. L., Hand, D. L. & Vasquez, Y. (2007) Increased polymorphonuclear leukocyte respiratory burst function in type 2 diabetes. *Diabetes Research in Clinical Practice* **76,** 44-50.

Hansen, L. H., Mauvais, P. & Douthwaite, S. (1999) The macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23S ribosomal RNA. *Molecular Microbiology* **31,** 623-631.

Heitz-Mayfield, L. J. (2009) Systemic antibiotics in periodontal therapy. *Australian Dental Journal* **54 Suppl 1,** S96-101.

Heitz-Mayfield, L. J., Salvi, G. E., Botticelli, D., Mombelli, A., Faddy, M. & Lang, N. P. (2011) Anti-infective treatment of peri-implant mucositis: a randomised controlled clinical trial. *Clinical Oral Implants Research* **22** 237-241.

Herrera, D., Roldan, S. & Sanz, M. (2000b) The periodontal abscess: a review. *Journal of Clinical Periodontology* **27,** 377-386.

Herrera, D., Roldan, S., O'Connor, A. & Sanz, M. (2000a) The periodontal abscess (II). Short-term clinical and microbiological efficacy of 2 systemic antibiotic regimes. *Journal of Clinical Periodontology* **27,** 395-404.

Herrera, D., Sanz, M., Jepsen, S., Needleman, I. & Roldan, S. (2002) A systematic review on the effect of systemic antimicrobials as an adjunct to scaling and root planing in periodontitis patients. *Journal of Clinical Periodontology* **29 Suppl 3,** 136-159; discussion 160-132.

Herrera-Insua, I., Perez, P., Ramos, C., Martinez, P., Gomez-Lus, M. L. & Prieto, J. (1997) Synergistic effect of azithromycin on the phagocytic killing of Staphylococcus aureus by human polymorphonuclear leukocytes. *European Journal of Clinical Microbiology and Infectious Diseases* **16,** 13-16.

Hillis, G. S., Pearson, C. V., Harding, S. A., Sutherland, S., Ludlam, C. A., Marioni, J. C., Prescott, R. J., Fox, K. A. & Flapan, A. D. (2004) Effects of a brief course of azithromycin on soluble cell adhesion molecules and markers of inflammation in survivors of an acute coronary syndrome: A double-blind, randomized, placebo-controlled study. *American Heart Journal* **148,** 72-79.

Hirsch, R. (2010) Periodontal healing and bone regeneration in response to azithromycin *Australian Dental Journal* **55,** 193-199.

Hirsch, R., Deng, H. & Laohachai, M. N. (2012) Azithromycin in periodontal treatment: more than an antibiotic. *Journal of Periodontal Research* **47** 137-148.

Hirschfeld, L. & Wasserman, B. (1978) A long-term survey of tooth loss in 600 treated periodontal patients. *Journal of Periodontology* **45,** 225-237.

Ho, W., Eubank, T., Leblebicioglu, B., Marsh, C. & Walters, J. (2010) Azithromycin decreases crevicular fluid volume and mediator content. *Journal of Dental Research* **89,** 831-835.

Hodge, P. & Michalowicz, B. (2001) Genetic predisposition to periodontitis in children and young adults. *Periodontology 2000* **26,** 113-134.

Hodge, S., Hodge, G., Brozyna, S., Jersmann, H., Holmes, M. & Reynolds, P. N. (2006) Azithromycin increases phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages. *European Respiratory Journal* **28,** 486-495.

Hodge, S., Hodge, G., Jersmann, H., Matthews, G., Ahern, J., Holmes, M. & Reynolds, P. N. (2008) Azithromycin improves macrophage phagocytic function and expression of mannose receptor in chronic obstructive pulmonary disease. *American Journal of Respiration and Critical Care Medicine* **178,** 139-148.

Hoffman, H. L., Klepser, M. E., Ernst, E. J., Petzold, C. R., Sa'adah, L. M. & Doern, G. V. (2003) Influence of macrolide susceptibility on efficacies of clarithromycin and azithromycin against Streptococcus pneumoniae in a murine lung infection model. *Antimicrobial Agents and Chemotherapy* **47,** 739-746.

Hoffmann, N., Lee, B., Hentzer, M., Rasmussen, T. B., Song, Z., Johansen, H. K., Givskov, M. & Hoiby, N. (2007) Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of Pseudomonas aeruginosa and attenuates chronic P. aeruginosa lung infection in Cftr (-/-) mice. *Antimicrobial Agents and Chemotherapy* **53,** 3677-3687.

Ianaro, A., Ialenti, A., Maffia, P., Sautebin, L., Rombola, L., Carnuccio, R., Iuvone, T., D'Acquisto, F. & Di Rosa, M. (2000) Anti-inflammatory activity of macrolide antibiotics. *Journal of Pharmacology and Experimental Therapy* **292,** 156-163.

Imamura, Y., Higashiyama, Y., Tomono, K., Izumikawa, K., Yanagihara, K., Ohno, H., Miyazaki, Y., Hirakata, Y., Mizuta, Y., Kadota, J. I., Iglewski, B. H. & Kohno, S. (2005) Azithromycin exhibits bactericidal effects on Pseudomonas aeruginosa through interaction with the outer membrane. *Antimicrobial Agents and Chemotherapy* **49,** 1377-1380.

Ishimatsu, Y., Kadota, J., Iwashita, T., Nagata, T., Ishii, H., Shikuwa, C., Kaida, H., Mukae, H. & Kohno, S. (2004) Macrolide antibiotics induce apoptosis of human peripheral lymphocytes *in vitro*. *International Journal of Antimicrobial Agents* **24,** 247-253.

Ivetic Tkalcevic, V., Bosnjak, B., Hrvacic, B., Bosnar, M., Marjanovic, N., Ferencic, Z., Situm, K., Culic, O., Parnham, M. J. & Erakovic, V. (2006) Anti-inflammatory activity of azithromycin attenuates the effects of lipopolysaccharide administration in mice. *European Journal of Pharmacology* **539,** 131-138.

Iwamoto, S., Kumamoto, T., Azuma, E., Hirayama, M., Ito, M., Amano, K., Ido, M. & Komada, Y. (2011) The effect of azithromycin on the maturation and function of murine bone marrow-derived dendritic cells. *Clinical and Experimental Immunology* **166,** 385-392.

Jain, N., Lai, P. C. & Walters, J. D. (2012) Effect of gingivitis on azithromycin concentrations in gingival crevicular fluid. *Journal of Periodontology*

Jun, Y. T., Kim, H. J., Song, M. J., Lim, J. H., Lee, D. G., Han, K. J., Choi, S. M., Yoo, J. H., Shin, W. S. & Choi, J. H. (2003) *In vitro* effects of ciprofloxacin and roxithromycin on apoptosis of jurkat T lymphocytes. *Antimicrobial Agents and Chemotherapy* **47,** 1161-1164.

Kadir, T., Izzetin, F. V., Cevikbas, A., Johansson, C. B. & Clark, P. (2000) *In vitro* effects of clarithromycin on human polymorphonuclear leukocyte functions. *Chemotherapy* **46,** 198-203.

Kadota, J., Mizunoe, S., Kishi, K., Tokimatsu, I., Nagai, H. & Nasu, M. (2005) Antibiotic-induced apoptosis in human activated peripheral lymphocytes. *International Journal of Antimicrobial Agents* **25,** 216-220.

Kang, K. H., Lee, K. H., Kim, M. Y. & Choi, K. H. (2001) Caspase-3-mediated cleavage of the NF-kappa B subunit p65 at the NH2 terminus potentiates naphthoquinone analog-induced apoptosis. *Journal of Biology and Chemotherapy* **276,** 24638-24644.

Kareha, M. J., Rosenberg, E. S. & DeHaven, H. (1981) Therapeutic considerations in the management of a periodontal abscess with an intrabony defect. *Journal of Clinical Periodontology* **8,** 375-386.

Karlstrom, A., Heston, S. M., Boyd, K. L., Tuomanen, E. I. & McCullers, J. A. (2011) Toll-like receptor 2 mediates fatal immunopathology in mice during treatment of secondary pneumococcal pneumonia following influenza. *Journal of Infectious Diseases* **204,** 1358-1366.

Kawakami, K., Kadota, J., Iida, K., Fujii, T., Shirai, R., Matsubara, Y. & Kohno, S. (1997) Phenotypic characterization of T cells in bronchoalveolar lavage fluid (BALF) and peripheral blood of patients with diffuse panbronchiolitis; the importance of cytotoxic T cells. *Clinical Experiments in Immunology* **107,** 410-416.

Kawamura-Sato, K., Iinuma, Y., Hasegawa, T., Horii, T., Yamashino, T. & Ohta, M. (2000) Effect of subinhibitory concentrations of macrolides on expression of flagellin in Pseudomonas aeruginosa and Proteus mirabilis. *Antimicrobial Agents and Chemotherapy* **44,** 2869-2872.

Kawamura-Sato, K., Iinuma, Y., Hasegawa, T., Yamashino, T. & Ohta, M. (2001) Postantibiotic suppression effect of macrolides on the expression of flagellin in Pseudomonas aeruginosa and Proteus mirabilis. *Journal of Infections and Chemotherapy* **7,** 51-54.

Kawashima, M., yatsunami, J., Fukuno, Y., Nagata, M., Tominaga, M. & Hayashi, S. (2002) Inhibitory effects of 14-membered ring macrolide antibiotics on bleomycin-induced acute lung injury. *Lung* **180,** 73-89.

Keicho, N., Kudoh, S., Yotsumoto, H. & Akagawa, K. S. (1993) Antilymphocytic activity of erythromycin distinct from that of FK506 or cyclosporin A. *Journal of Antibiotics (Tokyo)* **46,** 1406-1413.

Keicho, N., Kudoh, S., Yotsumoto, H. & Akagawa, K. S. (1994) Erythromycin promotes monocyte to macrophage differentiation. *Journal of Antiibiotics (Tokyo)* **47,** 80-89.

Khan, A. A., Slifer, T. R., Araujo, F. G. & Remington, J. S. (1999) Effect of clarithromycin and azithromycin on production of cytokines by human monocytes *International. Journal of Antimicrobial Agents* **11,** 121-132.

Kilic, B., Kruse, M. & Stahlmann, R. (2006) The *in vitro* effects of quinupristin/dalfopristin, erythromycin and levofloxacin at low concentrations on the expression of different cell adhesion molecules on the surface of endothelial cells (Eahy926). *Toxicology* **218,** 30-38.

Kim, J. Y., Park, S. H., Cho, K. S., Kim, H. J., Lee, C. K., Park, K. K., Choi, S. H. & Chung, W. Y. (2008) Mechanism of azithromycin treatment on gingival overgrowth. *Journal of Dental Research* **87,** 1075-1079.

Koch, C. C., Esteban, D. J., Chin, A. C., Olson, M. E., Read, R. R., Ceri, H., Morck, D. W. & Buret, A. G. (2000) Apoptosis, oxidative metabolism and interleukin-8 production in human neutrophils exposed to azithromycin: effects of Streptococcus pneumoniae. *Journal of Antimicrobial Chemotherapy* **46,** 19-26.

Kohler, T., Dumas, J. L. & Van Delden, C. (2007) Ribosome protection prevents azithromycin-mediated quorum-sensing modulation and stationary-phase killing of Pseudomonas aeruginosa. *Antimicrobial Agents in Chemotherapy* **51,** 4243-4248.

Kohler, T., Perron, G. G., Buckling, A. & van Delden, C. (2010) Quorum sensing inhibition selects for virulence and cooperation in Pseudomonas aeruginosa. *PLoS Pathology* **6,** e1000883.

Kornman, K. S. (2008) Mapping the pathogenesis of periodontitis: A new look. *Journal of Periodontology* **79,** 1560-1568.

Kumar, V., Abbas, A. & Fausto, N. (2007) *Robins basic pathology*. Philadelphia: Saunders.

Kurdowska, A., Noble, J. M. & Griffith, D. E. (2001) The effect of azithromycin and clarithromycin on ex vivo interleukin-8 (IL-8) release from whole blood and IL-8 production by human alveolar macrophages. *Journal of Antimicrobial Chemotherapy* **47,** 867-870.

Kutlin, A., Roblin, P. M. & Hammerschlag, M. R. (2002) Effect of prolonged treatment with azithromycin, clarithromycin, or levofloxacin on Chlamydia pneumoniae in a continuous-infection Model. *Antimicrobial Agents and Chemotherapy* **46,** 409-412.

Labro, M. T., el Benna, J. & Babin-Chevaye, C. (1989) Comparison of the in-vitro effect of several macrolides on the oxidative burst of human neutrophils. *Journal of Antimicrobial Chemotherapy* **24,** 561-572.

Lagrou, K., Peetermans, W. E., Jorissen, M., Verhaegen, J., Van Damme, J. & Van Eldere, J. (2000) Subinhibitory concentrations of erythromycin reduce pneumococcal adherence to respiratory epithelial cells *in vitro. Journal of Antimicrobial Chemotherapy* **46,** 717-723.

Lai, P. C., Ho, W., Jain, N. & Walters, J. D. (2011) Azithromycin concentrations in blood and gingival crevicular fluid after systemic administration. *Journal of Periodontology* **82,** 1582-1586.

Lanbeck, P., Odenholt, I. & Riesbeck, K. (2004) Dicloxacillin and erythromycin at high concentrations increase ICAM-1 expression by endothelial cells: a possible factor in the pathogenesis of infusion phlebitis. *Journal of Antimicrobial Chemotherapy* **53,** 174-179.

Lang, N. P., Tan, W. C., Krahenmann, M. A. & Zwahlen, M. (2008) A systematic review of the effects of full-mouth debridement with and without antiseptics in patients with chronic periodontitis. *Journal of Clinical Periodontology* **35,** 8-21.

Langley, J. M., Halperin, S. A., Boucher, F. D. & Smith, B. (2004) Azithromycin is as effective as and better tolerated than erythromycin estolate for the treatment of pertussis. *Pediatrics* **114,** e96-101.

Legssyer, R., Huaux, F., Lebacq, J., Delos, M., Marbaix, E., Lebecque, P., Lison, D., Scholte, B. J., Wallemacq, P. & Leal, T. (2006) Azithromycin reduces spontaneous and induced inflammation in DeltaF508 cystic fibrosis mice. *Respiratory Research* **7,** 134.

Leibbrandt, A. & Penninger, J. M. (2008) RANK/RANKL: regulators of immune responses and bone physiology. *Annals of the New York Academy of Science* **1143,** 123-150.

Leiva, M., Ruiz-Bravo, A. & Jimenez-Valera, M. (2008) Effects of telithromycin in *in vitro* and *in vivo* models of lipopolysaccharide-induced airway inflammation. *Chest* **134,** 20-29.

Levkau, B., Scatena, M., Giachelli, C. M., Ross, R. & Raines, E. W. (1999) Apoptosis overrides survival signals through a caspase-mediated dominant-negative NF-kappa B loop. *Nature Cell Biology* **1,** 227-233.

Ley, K., Laudanna, C., Cybulsky, M. I. & Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Natural Reviews of Immunology* **7,** 678-689.

Li, D. Q., Zhou, N., Zhang, L., Ma, P. & Pflugfelder, S. C. (2010) Suppressive effects of azithromycin on zymosan-induced production of proinflammatory mediators by human corneal epithelial cells. *Invest Ophthalmology Visual Science* **51,** 5623-5629.

Li, X. Z. & Nikaido, H. (2009) Efflux-mediated drug resistance in bacteria: an update. *Drugs* **69,** 1555-1623.

Lin, S. J., Lee, W. J., Liang, Y. W., Yan, D. C., Cheng, P. J. & Kuo, M. L. (2011) Azithromycin inhibits IL-5 production of T helper type 2 cells from asthmatic children. *International Archives of Allergy and Immunology* **156,** 179-186.

Löe, H., Anerud, A., Boysen, H. & Morrison, E. (1986) Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *Journal of Clinical Periodontology* **13,** 431-445.

Maezono, H., Noiri, Y., Asahi, Y., Yamaguchi, M., Yamamoto, R., Izutani, N., Azakami, H. & Ebisu, S. (2011) Antibiofilm effects of azithromycin and erythromycin on Porphyromonas gingivalis. *Antimicrobial Agents and Chemotherapy* **55,** 5887-5892.

Mandell, G. L. & Coleman, E. (2001) Uptake, transport, and delivery of antimicrobial agents by human polymorphonuclear neutrophils. *Antimicrobial Agents and Chemotherapy* **45,** 1794-1798.

Mandell, L. A., Wunderink, R. G., Anzueto, A., Bartlett, J. G., Campbell, G. D., Dean, N. C., Dowell, S. F., File, T. M., Jr., Musher, D. M., Niederman, M. S., Torres, A. & Whitney, C. G. (2007) Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clinical Infectious Diseases* **44 Suppl 2,** S27-72.

Marsh, P. D. (2003) Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub- and supragingival environment. *Oral Diseases* **9 Suppl 1,** 16-22.

Marsh, P. D., Moter, A. & Devine, D. A. (2011) Dental plaque biofilms: communities, conflict and control. *Periodontology 2000* **55,** 16-35.

Mascarenhas, P., Gapski, R., Al-Shammari, K., Hill, R., Soehren, S., Fenno, J. C., Giannobile, W. V. & Wang, H. L. (2005) Clinical response of azithromycin as an adjunct to non-surgical periodontal therapy in smokers. *Journal of Periodontology* **76,** 426-436.

Matsui, H., Eguchi, M., Ohsumi, K., Nakamura, A., Isshiki, Y., Sekiya, K., Kikuchi, Y., Nagamitsu, T., Masuma, R., Sunazuka, T. & Omura, S. (2005) Azithromycin inhibits the formation of flagellar filaments without suppressing flagellin synthesis in Salmonella enterica serovar typhimurium. *Antimicrob Agents and Chemotherapy* **49,** 3396-3403.

Matthews, D. C. (2009) No difference between full-mouth and quadrant-wise treatment of chronic periodontitis. *Evid Based Dentistry* **10,** 17.

Maximo, M. B., de Mendonca, A. C., Renata Santos, V., Figueiredo, L. C., Feres, M. & Duarte, P. M. (2009) Short-term clinical and microbiological evaluations of peri-implant diseases before and after mechanical anti-infective therapies. *Clinical Oral Implants Research* **20** 99-108.

Mazzei, T., Surrenti, C., Novelli, A., Crispo, A., Fallani, S., Carla, V., Surrenti, E. & Periti, P. (1993) Pharmacokinetics of azithromycin in patients with impaired hepatic function. *Journal Antimicrobial Chemotherapy* **31 Suppl E,**57-63.

McFall, W. T., Jr. (1982) Tooth loss in 100 treated patients with periodontal disease. A long-term study. *Journal of Periodontology* **53,** 539-549.

Mesa, F. L., Osuna, A., Aneiros, J., Gonzalez-Jaranay, M., Bravo, J., Junco, P., Del Moral, R. G. & O'Valle, F. (2003) Antibiotic treatment of incipient drug-induced gingival overgrowth in adult renal transplant patients. *Journal of Periodontal Research* **38,** 141-146.

Meyer, M., Huaux, F., Gavilanes, X., van den Brule, S., Lebecque, P., Lo Re, S., Lison, D., Scholte, B., Wallemacq, P. & Leal, T. (2009) Azithromycin reduces exaggerated cytokine production by M1 alveolar macrophages in cystic fibrosis. *American Journal of Respiratory and Cellular Molecular Biol*ogy **41,** 590-602.

Miller, S. A., Selzman, C. H., Shames, B. D., Barton, H. A., Johnson, S. M. & Harken, A. H. (2000) Chlamydia pneumoniae activates nuclear factor kappaB and activator protein 1 in human vascular smooth muscle and induces cellular proliferation. *Journal of Surgical Research* **90,** 76-81.

Mitsuya, Y., Kawai, S. & Kobayashi, H. (2000) Influence of macrolides on guanosine diphospho-D-mannose dehydrogenase activity in Pseudomonas biofilm. *Journal of Infectious Chemotherapy* **6,** 45-50.

Mizukane, R., Hirakata, Y., Kaku, M., Ishii, Y., Furuya, N., Ishida, K., Koga, H., Kohno, S. & Yamaguchi, K. (1994) Comparative *in vitro* exoenzyme-suppressing activities of azithromycin and other macrolide antibiotics against Pseudomonas aeruginosa. *Antimicrob Agents and Chemotherapy* **38,** 528-533.

Mizunoe, S., Kadota, J., Tokimatsu, I., Kishi, K., Nagai, H. & Nasu, M. (2004) Clarithromycin and azithromycin induce apoptosis of activated lymphocytes via down-regulation of Bcl-xL. *International Immunopharmacol* **4,** 1201-1207.

Molinari, G., Guzman, C. A., Pesce, A. & Schito, G. C. (1993) Inhibition of Pseudomonas aeruginosa virulence factors by subinhibitory concentrations of azithromycin and other macrolide antibiotics. *Journal of Antimicrobial Chemotherapy* **31,** 681-688.

Molinari, G., Paglia, P. & Schito, G. C. (1992) Inhibition of motility of Pseudomonas aeruginosa and Proteus mirabilis by subinhibitory concentrations of azithromycin. *European Jpurnal of Clinical Microbiology and Infectious Disease* **11,** 469-471.

Morinaga, Y., Yanagihara, K., Miyashita, N., Seki, M., Izumikawa, K., Kakeya, H., Yamamoto, Y., Mukae, H., Yamada, Y., Kohno, S. & Kamihira, S. (2009) Azithromycin, clarithromycin and telithromycin inhibit MUC5AC induction by Chlamydophila pneumoniae in airway epithelial cells. *Pulm Pharmacol Ther* **22,** 580-586.

Mouratidou, A., Karbach, J., D'Hoedt, B. & Al-Nawas, B. (2011) Antibiotic susceptibility of cocultures in polymicrobial infections such as peri-implantitis or periodontitis: An *in vitro* model. *Journal of Periodontology* **82,** 1360-1366.

Murphy, B. S., Sundareshan, V., Cory, T. J., Hayes, D., Jr., Anstead, M. I. & Feola, D. J. (2008) Azithromycin alters macrophage phenotype. *Journal of Antimicrobial Chemotherapy* **61,** 554-560.

Nafar, M., Ataie, R., Einollahi, B., Nematizadeh, F., Firoozan, A. & Poorrezagholi, F. (2003) A comparison between the efficacy of systemic and local azithromycin therapy in treatment of cyclosporine induced gingival overgrowth in kidney transplant patients. *Transplant Proceedings* **35,** 2727-2728.

Namazi, S., Haghighi, A. B. & Karimzadeh, I. (2007) Is oral azithromycin effective for the treatment of phenytoin-induced gingival overgrowth in epileptic patients? *Medical Hypotheses* **69,** 1159-1160.

Nash, M. M. & Zaltzman, J. S. (1998) Efficacy of azithromycin in the treatment of cyclosporine-induced gingival hyperplasia in renal transplant recipients. *Transplantation* **65,** 1611-1615.

Navarro-Xavier, R. A., Newson, J., Silveira, V. L., Farrow, S. N., Gilroy, D. W. & Bystrom, J. (2010) A new strategy for the identification of novel molecules with targeted proresolution of inflammation properties. *Journal of Immunology* **184,** 1516-1525.

Niki, Y., Hanaki, H., Matsumoto, T., et al. (2009) Nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese Society of Chemotherapy in 2007: general view of the pathogens' antibacterial susceptibility. *J Infect Chemotherapy* **15,** 156-167.

Norrington, D. W., Ruby, J., Beck, P. & Eleazer, P. D. (2008) Observations of biofilm growth on human dentin and potential destruction after exposure to antibiotics. *Oral Surg Oral Medicine Oral Pathology Oral Radiology Endododontics* **105,** 526-529.

Oda, H., Kadota, J., Kohno, S. & Hara, K. (1994) Erythromycin inhibits neutrophil chemotaxis in bronchoalveoli of diffuse panbronchiolitis. *Chest* **106,** 1116-1123.

Ohara, T., Kojio, S., Taneike, I., Nakagawa, S., Gondaira, F., Tamura, Y., Gejyo, F., Zhang, H. M. & Yamamoto, T. (2002) Effects of azithromycin on shiga toxin production by Escherichia coli and subsequent host inflammatory response. *Antimicrobial Agents and Chemotherapy* **46,** 3478-3483.

Ortega, E., Escobar, M. A. & de Cienfuegos, G. A. (2002) *In vitro* and ex vivo effects of erythromycin A, azithromycin and josamycin on the splenic response to specific antigens and mitogens. *Journal of Antimicrobial Chemotherapy* **49,** 1031-1034.

Ortega, E., Escobar, M. A., Gaforio, J. J., Algarra, I. & Alvarez De Cienfuegos, G. (2004) Modification of phagocytosis and cytokine production in peritoneal and splenic murine cells by erythromycin A, azithromycin and josamycin. *Journal of Antimicrobial Chemotherapy* **53,** 367-370.

Oteo, A., Herrera, D., Figuero, E., O'Connor, A., Gonzalez, I. & Sanz, M. (2010) Azithromycin as an adjunct to scaling and root planing in the treatment of Porphyromonas gingivalis-associated periodontitis: a pilot study. *Journal of Clinical Periodontology* **37,** 1005-1015.

Owens, R. C., Jr. (2004) QT prolongation with antimicrobial agents: understanding the significance. *Drugs* **64,** 1091-1124.

Page, R. C., Offenbacher, S., Schroeder, H. E., Seymour, G. J. & Kornman, K. S. (1997) Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontology 2000* **14,** 216-248.

Paik, J. W., Kim, C. S., Cho, K. S., Chai, J. K., Kim, C. K. & Choi, S. H. (2004) Inhibition of cyclosporin A-induced gingival overgrowth by azithromycin through phagocytosis: an *in vivo* and *in vitro* study. *Journal of Periodontology* **75,** 380-387.

Pajukanta, R. (1993) *In vitro* antimicrobial susceptibility of Porphyromonas gingivalis to azithromycin, a novel macrolide. *Oral Microbiol and Immunology* **8,** 325-326.

Pajukanta, R., Asikainen, S., Saarela, M., Alaluusua, S. & Jousimies-Somer, H. (1992) *In vitro* activity of azithromycin compared with that of erythromycin against Actinobacillus actinomycetemcomitans. *Antimicrobial Agents and Chemotherapy* **36,** 1241-1243.

Palomar, R., Belart, M., Soy, D., Oppenheimer, F. & Campistol, J. M. (1998) Effectiveness and safety of azithromycin on the treatment of cyclosporine-induced gingival overgrowth. *Nephron* **79,** 101-102.

Panpanich, R., Lerttrakarnnon, P. & Laopaiboon, M. (2008) Azithromycin for acute lower respiratory tract infections. *Cochrane Database Systematic Reviews* CD001954.

Parchure, N., Zouridakis, E. G. & Kaski, J. C. (2002) Effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of Chlamydia pneumoniae infection. *Circulation* **105,** 1298-1303.

Parnham, M. J., Culic, O., Erakovic, V., Munic, V., Popovic-Grle, S., Barisic, K., Bosnar, M., Brajsa, K., Cepelak, I., Cuzic, S., Glojnaric, I., Manojlovic, Z., Novak-Mircetic, R., Oreskovic, K., Pavicic-Beljak, V., Radosevic, S. & Sucic, M. (2005) Modulation of neutrophil and inflammation markers in chronic obstructive pulmonary disease by short-term azithromycin treatment. *European Journal of Pharmacology* **517,** 132-143.

Pascual, A., Conejo, M. C., Garcia, I. & Perea, E. J. (1995) Factors affecting the intracellular accumulation and activity of azithromycin. *J Antimicrob Chemotherapy* **35,** 85-93.

Pawlowski, K. S., Si, E., Wright, C. G., Koulich, E., Hosseini, K. & Roland, P. S. (2010) Ototoxicity of topical azithromycin solutions in the guinea pig. *Archives of Otolaryngology and Head and Neck Surgery* **136,** 481-487.

Pearlman, B. A. (1993) Long-term periodontal care: a comparative retrospective survey. *Journal of Periodontology* **64,** 723-729.

Perry, D. K., Hand, W. L., Edmondson, D. E. & Lambeth, J. D. (1992) Role of phospholipase D-derived diradylglycerol in the activation of the human neutrophil respiratory burst oxidase. Inhibition by phosphatidic acid phosphohydrolase inhibitors. *Journal of Immunology* **149,** 2749-2758.

Peters, D. H., Friedel, H. A. & McTavish, D. (1992) Azithromycin. A review of its antimicrobial activity, pharmacokinetic properties and clinical efficacy. *Drugs* **44,** 750-799.

Pradeep, A. R., Sagar, S. V. & Daisy, H. (2008) Clinical and microbiologic effects of subgingivally delivered 0.5% azithromycin in the treatment of chronic periodontitis. *Journal of Periodontology* **79,** 2125-2135.

Principi, N. & Esposito, S. (1999) Comparative tolerability of erythromycin and newer macrolide antibacterials in paediatric patients. *Drug Safety* **20,** 25-41.

Raffa, R. B., Iannuzzo, J. R., Levine, D. R., Saeid, K. K., Schwartz, R. C., Sucic, N. T., Terleckyj, O. D. & Young, J. M. (2005) Bacterial communication ("quorum sensing") via ligands and receptors: A novel pharmacologic target for the design of antibiotic drugs. *Journal of Pharmacology and Experimental Therapeutics* **312,** 417-423.

Ramalho, V. L., Ramalho, H. J., Cipullo, J. P., Azoubel, R. & Burdmann, E. A. (2007) Comparison of azithromycin and oral hygiene program in the treatment of cyclosporine-induced gingival hyperplasia. *Renal Failure* **29,** 265-270.

Ray, W. A., Murray, K. T., Meredith, S., Narasimhulu, S. S., Hall, K. & Stein, C. M. (2004) Oral erythromycin and the risk of sudden death from cardiac causes. *New England. Journal of Medicine* **351,** 1089-1096.

Reato, G., Cuffini, A. M., Tullio, V., Mandras, N., Roana, J., Banche, G., Foa, R. & Carlone, N. A. (2004) Immunomodulating effect of antimicrobial agents on cytokine production by human polymorphonuclear neutrophils. *Int J Antimicrobial Agents* **23,** 150-154.

Reddy J, Africa CW & JR, P. (1986) Darkfield microscopy of subgingival plaque of an urban black population with poor oral hygiene. *Journal of Clinical Periodontology* **13,** 578-582.

Renna, M., Schaffner, C., Brown, K., Shang, S., Tamayo, M. H., Hegyi, K., Grimsey, N. J., Cusens, D., Coulter, S., Cooper, J., Bowden, A. R., Newton, S. M., Kampmann, B., Helm, J., Jones, A., Haworth, C. S., Basaraba, R. J., DeGroote, M. A., Ordway, D. J., Rubinsztein, D. C. & Floto, R. A. (2011) Azithromycin blocks autophagy and may predispose cystic fibrosis patients to mycobacterial infection. *Journal of Clinical Investigations* **121,** 3554-3563.

Renvert, S., Roos-Jansaker, A. M. & Claffey, N. (2008) Non-surgical treatment of peri-implant mucositis and peri-implantitis: a literature review. *Journal of Clinical Periodontology* **35,** 305-315.

Retsema, J., Girard, A., Schelkly, W., Manousos, M., Anderson, M., Bright, G., Borovoy, R., Brennan, L. & Mason, R. (1987) Spectrum and mode of action of azithromycin (CP-62,993), a new 15-membered-ring macrolide with improved potency against gram-negative organisms. *Antimicrobial Agents and Chemotherapy* **31,** 1939-1947.

Ribeiro, C. M., Hurd, H., Wu, Y., Martino, M. E., Jones, L., Brighton, B., Boucher, R. C. & O'Neal, W. K. (2009) Azithromycin treatment alters gene expression in inflammatory, lipid metabolism, and cell cycle pathways in well-differentiated human airway epithelia. *PLoS One* **4,** e5806.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S. & Eberl, L. (2001) N-acylhomoserine-lactone-mediated communication between Pseudomonas aeruginosa and Burkholderia cepacia in mixed biofilms. *Microbiology* **147,** 3249-3262.

Riedel, S., Beekmann, S. E., Heilmann, K. P., Richter, S. S., Garcia-de-Lomas, J., Ferech, M., Goosens, H. & Doern, G. V. (2007) Antimicrobial use in Europe and antimicrobial resistance in Streptococcus pneumoniae. *European Journal of Clinical Microbiology and Infectious Diseases* **26,** 485-490.

Rodvold, K. A., Gotfried, M. H., Danziger, L. H. & Servi, R. J. (1997) Intrapulmonary steady-state concentrations of clarithromycin and azithromycin in healthy adult volunteers. *Antimicrobial Agents and Chemotherapy* **41,** 1399-1402.

Sampaio, E., Rocha, M., Figueiredo, L. C., Faveri, M., Duarte, P. M., Gomes Lira, E. A. & Feres, M. (2011) Clinical and microbiological effects of azithromycin in the treatment of generalized chronic periodontitis: a randomized placebo-controlled clinical trial. *Journal of Clinical Periodontology*

Sanz, M. J., Nabah, Y. N., Cerda-Nicolas, M., O'Connor, J. E., Issekutz, A. C., Cortijo, J. & Morcillo, E. J. (2005) Erythromycin exerts *in vivo* anti-inflammatory activity downregulating cell adhesion molecule expression. *British Journal of Pharmacology* **144,** 190-201.

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. & Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *Journal of Clinical Investigation* **83,** 865-875.

Schreiber, F. & Szewzyk, U. (2008) Environmentally relevant concentrations of pharmaceuticals influence the initial adhesion of bacteria. *Aquatic Toxicology* **87,** 227-233.

Schultz, M. J., Speelman, P., Hack, C. E., Buurman, W. A., van Deventer, S. J. & van Der Poll, T. (2000) Intravenous infusion of erythromycin inhibits CXC chemokine production, but augments neutrophil degranulation in whole blood stimulated with Streptococcus pneumoniae. *Journal of Antimicrobial Chemotherapy* **46,** 235-240.

Sefton, A. M., Maskell, J. P., Beighton, D., Whiley, A., Shain, H., Foyle, D., Smith, S. R., Smales, F. C. & Williams, J. D. (1996) Azithromycin in the treatment of periodontal disease. Effect on microbial flora. *Journal of Clinical Periodontology* **23,** 998-1003.

Semaan, H. B., Gurbel, P. A., Anderson, J. L., Muhlestein, J. B., Carlquist, J. F., Horne, B. D. & Serebruany, V. L. (2000) The effect of chronic azithromycin therapy on soluble endothelium-derived adhesion molecules in patients with coronary artery disease. *Journal of Cardiovascular Pharmacology* **36,** 533-537.

Seymour, R. A. (1996) The pathogenesis of drug-induced gingival overgrowth. *Journal of Clinical Periodontology* **23,** 165-175.

Shao, H. & Demuth, D. R. (2010) Quorum sensing regulation of biofilm growth and gene expression by oral bacteria and periodontal pathogens. *Periodontology 2000* **52,** 53-67.

Shao, H., Lamont, R. J. & Demuth, D. R. (2007) Autoinducer 2 is required for biofilm growth of Aggregatibacter (Actinobacillus) actinomycetemcomitans. *Infections and Immunity* **75,** 4211-4218.

Shinkai, M., Foster, G. H. & Rubin, B. K. (2006) Macrolide antibiotics modulate ERK phosphorylation and IL-8 and GM-CSF production by human bronchial epithelial cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **290,** L75-L85.

Shinkai, M., Henke, M. O. & Rubin, B. K. (2008) Macrolide antibiotics as immunomodulatory medications: proposed mechanisms of action. *Pharmacological Therapeutics* **117,** 393-405.

Siebenlist, U., Franzoso, G. & Brown, K. (1994) Structure, regulation and function of NF-κB. *Annual Review of Cell Biology* **10,** 405-455.

Silvestri, M., Oddera, S., Eftimiadi, C. & Rossi, G. A. (1995) Azithromycin induces *in vitro* a time-dependent increase in the intracellular killing of Staphylococcus aureus by human polymorphonuclear leucocytes without damaging phagocytes. *Journal of Antimicrobial Chemotherapy* **36,** 941-950.

Skindersoe, M. E., Alhede, M., Phipps, R., Yang, L., Jensen, P. O., Rasmussen, T. B., Bjarnsholt, T., Tolker-Nielsen, T., Hoiby, N. & Givskov, M. (2008) Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy* **52,** 3648-3663.

Smith, J. A. (1994) Neutrophils, host defense, and inflammation: A double-edged sword. *Journal of Leukocyte Biology* **56,** 672-686.

Smith, S. R., Foyle, D. M., Daniels, J., Joyston-Bechal, S., Smales, F. C., Sefton, A. & Williams, J. (2002) A double-blind placebo-controlled trial of azithromycin as an adjunct to non-surgical treatment of periodontitis in adults: clinical results. *Journal of Clinical Periodontology* **29,** 54-61.

Socransky, S. S. & Haffajee, A. D. (2002) Dental biofilms: difficult therapeutic targets. *Periodontology 2000* **28,** 12-55.

Squier, M. K., Sehnert, A. J. & Cohen, J. J. (1995) Apoptosis in leukocytes. *Journal of Leukocite Biology* **57,** 2-10.

Starner, T. D., Shrout, J. D., Parsek, M. R., Appelbaum, P. C. & Kim, G. (2008) Subinhibitory concentrations of azithromycin decrease nontypeable Haemophilus influenzae biofilm formation and Diminish established biofilms. *Antimicrobial Agents and Chemotherapy* **52,** 137-145.

Stockley, R. A. (2006) Neutrophilic inflammation: "don't you go to pieces on me!". *European Respiratory Journal* **28,** 257-258.

Sugiyama, K., Shirai, R., Mukae, H., Ishimoto, H., Nagata, T., Sakamoto, N., Ishii, H., Nakayama, S., Yanagihara, K., Mizuta, Y. & Kohno, S. (2007) Differing effects of clarithromycin and azithromycin on cytokine production by murine dendritic cells. *Clinical Experiments in Immunology* **147,** 540-546.

Swanson, D. J., Sung, R. J., Fine, M. J., Orloff, J. J., Chu, S. Y. & Yu, V. L. (1992) Erythromycin ototoxicity: prospective assessment with serum concentrations and audiograms in a study of patients with pneumonia. *American Journal of Medicine* **92,** 61-68.

Tateda, K., Comte, R., Pechere, J. C., Kohler, T., Yamaguchi, K. & Van Delden, C. (2001) Azithromycin inhibits quorum sensing in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy* **45,** 1930-1933.

Tateda, K., Ishii, Y., Kimura, S., Horikawa, M., Miyairi, S. & Yamaguchi, K. (2007) Suppression of Pseudomonas aeruginosa quorum-sensing systems by macrolides: a promising strategy or an oriental mystery? *Journal of Infections and Chemotherapy* **13,** 357-367.

Tateda, K., Ishii, Y., Matsumoto, T., Kobayashi, T., Miyazaki, S. & Yamaguchi, K. (2000) Potential of macrolide antibiotics to inhibit protein synthesis of Pseudomonas aeruginosa: suppression of virulence factors and stress response. *Journal of Infections and Chemotherapy* **6,** 1-7.

Tateda, K., Standiford, T. J., Pechere, J. C. & Yamaguchi, K. (2004) Regulatory effects of macrolides on bacterial virulence: potential role as quorum-sensing inhibitors. *Current Pharmacoogical Desicions* **10,** 3055-3065.

Tatnell, P. J., Russell, N. J. & Gacesa, P. (1994) GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in Pseudomonas aeruginosa: evidence from metabolite studies. *Microbiology* **140 ( Pt 7),**1745-1754.

Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H. H., Brown, G. D. & Gordon, S. (2005) Macrophage receptors and immune recognition. *Annual Reviews of Immunology* **23,** 901-944.

Togami, K., Chono, S. & Morimoto, K. (2011) Distribution characteristics of clarithromycin and azithromycin, macrolide antimicrobial agents used for treatment of respiratory infections, in lung epithelial lining fluid and alveolar macrophages. *Biopharmaceutics and Drug Disposition* **32,** 389-397.

Tokgoz, B., Sari, H. I., Yildiz, O., Aslan, S., Sipahioglu, M., Okten, T., Oymak, O. & Utas, C. (2004) Effects of azithromycin on cyclosporine-induced gingival hyperplasia in renal transplant patients. *Transplant Proceedings* **36,** 2699-2702.

Tomazic, J., Kotnik, V. & Wraber, B. (1993) *In vivo* administration of azithromycin affects lymphocyte activity *in vitro. Antimicrobial Agents and Chemotherapy* **37,** 1786-1789.

Tsai, W. C., Hershenson, M. B., Zhou, Y. & Sajjan, U. (2009) Azithromycin increases survival and reduces lung inflammation in cystic fibrosis mice. *Inflammation Research* **58,** 491-501.

Tsai, W. C., Rodriguez, M. L., Young, K. S., Deng, J. C., Thannickal, V. J., Tateda, K., Hershenson, M. B. & Standiford, T. J. (2004) Azithromycin blocks neutrophil recruitment in Pseudomonas endobronchial infection. *American Journal of Respiration and Critical Care Medicine* **170,** 1331-1339.

Tsang, K. W., Ng, P., Ho, P. L., Chan, S., Tipoe, G., Leung, R., Sun, J., Ho, J. C., Ip, M. S. & Lam, W. K. (2003) Effects of erythromycin on Pseudomonas aeruginosa adherence to collagen and morphology *in vitro. European Respiratory Journal* **21,** 401-406.

Tseng, A. L., Dolovich, L. & Salit, I. E. (1997) Azithromycin-related ototoxicity in patients infected with human immunodeficiency virus. *Clinical Infectious Diseases* **24,** 76-77.

Tyteca, D., Schanck, A., Dufrene, Y. F., Deleu, M., Courtoy, P. J., Tulkens, P. M. & Mingeot-Leclercq, M. P. (2003) The macrolide antibiotic azithromycin interacts with lipids and affects membrane organization and fluidity: studies on Langmuir-Blodgett

monolayers, liposomes and J774 macrophages. *Journal of Membrany Biology* **192,** 203-215.

Tyteca, D., Van Der Smissen, P., Mettlen, M., Van Bambeke, F., Tulkens, P. M., Mingeot-Leclercq, M. P. & Courtoy, P. J. (2002) Azithromycin, a lysosomotropic antibiotic, has distinct effects on fluid-phase and receptor-mediated endocytosis, but does not impair phagocytosis in J774 macrophages. *Experimental Cell Research* **281,** 86-100.

Uzun, C., Koten, M., Adali, M. K., Yorulmaz, F., Yagiz, R. & Karasalihoglu, A. R. (2001) Reversible ototoxic effect of azithromycin and clarithromycin on transiently evoked otoacoustic emissions in guinea pigs. *Journal of Laryngology and Otology* **115,** 622-628.

Van Dyke, T. E., Lester, M. A. & Shapira, L. (1993) The role of the host response in periodontal disease progression: Implications for future treatment strategies. *Journal of Periodontology* **64,** 792-806.

Vanaudenaerde, B. M., Wuyts, W. A., Geudens, N., Dupont, L. J., Schoofs, K., Smeets, S., Van Raemdonck, D. E. & Verleden, G. M. (2007) Macrolides inhibit IL17-induced IL8 and 8-isoprostane release from human airway smooth muscle cells. *American Journal of Transplantation* **7,** 76-82.

Vazifeh, D., Preira, A., Bryskier, A. & Labro, M. T. (1998) Interactions between HMR 3647, a new ketolide, and human polymorphonuclear neutrophils. *Antimicrobial Agents and Chemotherapy* **42,** 1944-1951.

Verleden, G. M., Vanaudenaerde, B. M., Dupont, L. J. & Van Raemdonck, D. E. (2006) Azithromycin reduces airway neutrophilia and interleukin-8 in patients with bronchiolitis obliterans syndrome. *American Journal of Respiration and Critical Care Medicine* **174,** 566-570.

Vieira Jr, J. M., Noronha, I. L., Malheiros, D. M. A. C. & Burdmann, E. A. (1999) Cyclosporine-induced interstitial fibrosis and arteriolar TGF-β expression with preserved renal blood flow. *Transplantation* **68,** 1746-1753.

Wagner, E. F. & Eferl, R. (2005) Fos/AP-1 proteins in bone and the immune system. *Immunology Reviews* **208,** 126-140.

Wagner, T., Soong, G., Sokol, S., Saiman, L. & Prince, A. (2005) Effects of azithromycin on clinical isolates of Pseudomonas aeruginosa from cystic fibrosis patients. *Chest* **128,** 912-919.

Wahlstrom, E., Zamora, J. U. & Teichman, S. (1995) Improvement in cyclosporine-associated gingival hyperplasia with azithromycin therapy. *New England Journal of Medicine* **33, 2,** 753-754.

Wang, J. H., Lee, S. H., Kwon, H. J. & Jang, Y. J. (2010) Clarithromycin inhibits rhinovirus-induced bacterial adhesions to nasal epithelial cells. *Laryngoscope* **120,** 193-199.

Wang, S. & Chen, L. (2004) T lymphocyte co-signaling pathways of the B7-CD28 family. *Cellular and Molecular Immunology* **1,** 37-42.

Ward, C., Chilvers, E. R., Lawson, M. F., Pryde, J. G., Fujihara, S., Farrow, S. N., Haslett, C. & Rossi, A. G. (1999) NF-kappaB activation is a critical regulator of human granulocyte apoptosis *in vitro. Journal of Biological Chemistry* **274,** 4309-4318.

Wenisch, C., Parschalk, B., Zedtwitz-Liebenstein, K., Weihs, A., el Menyawi, I. & Graninger, W. (1996) Effect of single oral dose of azithromycin, clarithromycin, and roxithromycin on polymorphonuclear leukocyte function assessed ex vivo by flow cytometry. *Antimicrobial Agents and Chemother* **40,** 2039-2042.

Westphal, J. F. (2000) Macrolide - induced clinically relevant drug interactions with cytochrome P-450A (CYP) 3A4: an update focused on clarithromycin, azithromycin and dirithromycin. *British Journal of Clinical Pharmacology* **50,** 285-295.

Wilms, E. B., Touw, D. J. & Heijerman, H. G. (2006) Pharmacokinetics of azithromycin in plasma, blood, polymorphonuclear neutrophils and sputum during long-term therapy in patients with cystic fibrosis. *Therapeutic Drug Monitoring* **28,** 219-225.

Winkel, E. G., Van Winkelhoff, A. J., Timmerman, M. F., Van der Velden, U. & Van der Weijden, G. A. (2001) Amoxicillin plus metronidazole in the treatment of adult

periodontitis patients. A double-blind placebo-controlled study. *Journal of Clinical Periodontology* **28,** 296-305.

Workowski, K. A., Levine, W. C. & Wasserheit, J. N. (2002) U.S. Centers for Disease Control and Prevention guidelines for the treatment of sexually transmitted diseases: an opportunity to unify clinical and public health practice. *Annals of Internal Medicine* **137,** 255-262.

Wu, L., Lin, J. H., Bao, K., Li, P. F. & Zhang, W. G. (2009) *In vitro* effects of erythromycin on RANKL and nuclear factor-kappa B by human TNF-alpha stimulated Jurkat cells. *International Immunopharmacology* **9,** 1105-1109.

Wuyts, W. A., Willems, S., Vos, R., Vanaudenaerde, B. M., De Vleeschauwer, S. I., Rinaldi, M., Vanhooren, H. M., Geudens, N., Verleden, S. E., Demedts, M. G., Thomeer, M., Verbeken, E. K. & Verleden, G. M. (2010) Azithromycin reduces pulmonary fibrosis in a bleomycin mouse model. *Experimental Lung Research* **36,** 602-614.

Yamaryo, T., Oishi, K., Yoshimine, H., Tsuchihashi, Y., Matsushima, K. & Nagatake, T. (2003) Fourteen-member macrolides promote the phosphatidylserine receptor-dependent phagocytosis of apoptotic neutrophils by alveolar macrophages. *Antimicrobial Agents and Chemotherapy* **47,** 48-53.

Yamasawa, H., Oshikawa, K., Ohno, S. & Sugiyama, Y. (2004) Macrolides inhibit epithelial cell-mediated neutrophil survival by modulating granulocyte macrophage colony-stimulating factor release. *American Journal of Respiration and Cellular Molecular Biology* **30,** 569-575.

Yamauchi, K., Shibata, Y., Kimura, T., Abe, S., Inoue, S., Osaka, D., Sato, M., Igarashi, A. & Kubota, I. (2009) Azithromycin suppresses interleukin-12p40 expression in lipopolysaccharide and interferon-gamma stimulated macrophages. *International Journal of Biological Science* **5,** 667-678.

Yanagihara, K., Izumikawa, K., Higa, F., Tateyama, M., Tokimatsu, I., Hiramatsu, K., Fujita, J., Kadota, J. & Kohno, S. (2009a) Efficacy of azithromycin in the treatment of community-acquired pneumonia, including patients with macrolide-resistant Streptococcus pneumoniae infection. *Internal Medicine* **48,** 527-535.

Yanagihara, K., Izumikawa, K., Higa, F., Tateyama, M., Tokimatsu, I., Hiramatsu, K., Fujita, J., Kadota, J. I. & Kohno, S. (2009b) Efficacy of azithromycin in the treatment of community-acquired pneumonia, including patients with macrolide-resistant Streptococcus pneumoniae infection. *Internal Medicine* **48,** 527-535.

Yanagihara, K., Morinaga, Y., Nakamura, S., Seki, M., Izumikawa, K., Kakeya, H., Yamamoto, Y., Yamada, Y., Kamihira, S. & Kohno, S. (2008) Subinhibitory concentrations of telithromycin, clarithromycin and azithromycin reduce methicillin-resistant Staphylococcus aureus coagulase *in vitro* and *in vivo. Journal of Antimicrobial Chemotherapy* **61,** 647-650.

Yang, E. & Korsmeyer, S. J. (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood* **88,** 386-401.

Yashima, A., Gomi, K., Maeda, N. & Arai, T. (2009) One-stage full-mouth versus partial-mouth scaling and root planing during the effective half-life of systemically administered azithromycin. *Journal of Periodontology* **80,** 1406-1413.

Yek, E. C., Cintan, S., Topcuoglu, N., Kulekci, G., Issever, H. & Kantarci, A. (2010) Efficacy of amoxicillin and metronidazole combination for the management of generalized aggressive periodontitis. *Journal of Periodontology*

Yoshida, K., Sunazuka, T., Nagai, K., Sugawara, A., Cho, A., Nagamitsu, T., Harigaya, Y., Otoguro, K., Akagawa, K. S. & Omura, S. (2005) Macrolides with promotive activity of monocyte to macrophage differentiation. *Journal of Antibiotics (Tokyo)* **58,** 79-81.

Zhu, C., Rao, K., Xiong, H., Gagnidze, K., Li, F., Horvath, C. & Plevy, S. (2003) Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. *Journal of Biology and Chemistry* **278,** 39372-39382.

Zuckerman, J. M., Qamar, F. & Bono, B. R. (2009) Macrolides, Ketolides, and Glycylcyclines: Azithromycin, Clarithromycin, Telithromycin, Tigecycline. *Infectious Disease Clinics of North America* **23,** 997-1026.

# Chapter 2. The effect of azithromycin on acute and chronic inflammation in an *in vivo* experimental model

Key words: Azithromycin, macrolide, inflammation, rat, neutrophil, macrophage, fibrosis, lymphocyte

## 2.1 Abstract

Background: Macrolide antibiotics have been found to have both antimicrobial and anti-inflammatory properties. They may be useful adjuncts in the treatment of conditions in which both these factors play a role, such as periodontitis.

Objectives: The aim of this study was to evaluate the effect of azithromycin in a rat model of experimentally induced acute and chronic inflammation.

Material and methods: Polyurethane sponges loaded with either heat killed *Porphyromonas gingivalis* (HKPG), *Mycobacterium tuberculosis* (HKTB) or Phosphate Buffered Saline (PBS) were surgically implanted into the fore flanks of rats. To determine any acute inflammatory effects animals received azithromycin 4 days prior to surgery, while to determine the effects on chronic inflammation animals did not receive azithromycin until day 25 post operatively. The control groups did not receive azithromycin. The sponges were retrieved at days 7, 14, 21, 35 and 49, wet weights recorded and then processed for histological evaluation of acute and chronic inflammation and fibrosis. Additionally, immunohistochemical staining was used to identify macrophages using CD163 and CD68 macrophage markers at days 21 and 35. Biochemical analyses were used to determine serum levels of C-reactive protein (CRP) and hydroxyproline (HP) content in the retrieved sponges.

Results: No differences were found between wet and dry weights of sponges for any of the groups.

Acute Inflammation: A trend for lower inflammation and infiltration scores was observed for all azithromycin treated groups compared to untreated groups, although this was not statistically significant. Azithromycin reduced neutrophil infiltration in all groups, but this reduction was only significant in the acute HKTB group ($p= 0.008$). Although azithromycin reduced CD68 cell counts between treated and untreated animals in all groups, this was found to be significant only for animals with PBS sponges at day 21 as well as day 35 and TB sponges at day 35. Similarly, for CD163 it was found that although azithromycin reduced counts of CD163 positive cells, this was only significant at day 35 for PBS ($p<0.001$) and HKTB ($p<0.001$). The hydroxyproline content differed within sponges for rats with HKTB

sponges at days 7 ($p=0.001$), 14 ($p=0.04$) and 21 ($p=0.001$) as well as PBS sponge implants was at all time points ($p<0.05$) for animals that had received azithromycin, compared to control animals.

CRP levels increased between 32-58% in all groups after sponge implantation, but a significant difference was only observed between the azithromycin treated and untreated groups at day 21 for animals with PBS sponge implants.

Chronic Inflammation: Animals in this part of the study received the late (day 25) administration of azithromycin. No statistically significant effects were demonstrated on any of the measured (histological or biochemical) parameters, except the sponge hydroxyproline content, which was significantly reduced at day 35 for animals that had received HKTB ($p=0.012$) and HKPG ($p=0.025$) sponges.

Conclusions: Azithromycin has the potential to suppress neutrophil infiltration and thus modulate acute inflammation in a subcutaneous rat model for inflammation. The drug has a limited effect on macrophage infiltration and the collagen content of the resulting scar tissue, but no significant impact on serum CRP levels. Once chronic inflammation has ensued, the anti-inflammatory effect of azithromycin was less marked than effects on acute inflammation, although a tendency for reduction of inflammation was observed.

## 2.2 Introduction

Chronic periodontitis is a common disease of the oral cavity that affects around 5-20% of the adult population globally (Burt, 2005, Borrell et al., 2005) although some estimate its prevalence being as high as 60% for chronic periodontitis and up to 90% for gingivitis amongst groups with limited oral care (Page et al., 1997). Nevertheless, differences in susceptibility between individuals have been identified (Löe et al., 1986, Cutress et al., 1982, Reddy J et al., 1986). Although it has been accepted that the microbial biofilm forming on hard tissue surfaces in the oral cavity is the initiating factor (Loe H, 1965), it is thought that the bulk of tissue destruction occurs as a result of the host response (Birkedal-Hansen, 1993, Page and Schroeder, 1976, Kinane and Bartold, 2007) and is driven by inflammatory processes triggered by the bacteria as well as their products, in which neutrophils, macrophages, lymphocytes and fibroblasts play the biggest role (Van Dyke et al., 1993). As such, it has become evident that the periodontal destruction occurs as a result of the interplay between the pathogen and host response, which is influenced by several factors, including genetics and environmental risk as well as other predisposing factors (Kornman, 2008). Furthermore, it has more recently been suggested that environmental changes brought about by the response of the host to the presence of the microbial biofilm is a strong factor in the

transition to a more pathogenic micro flora, containing well known periodontal pathogens such as *P. gingivalis, T. forsythia* and *T. denticola*, which in turn may exacerbate local tissue destruction (Marsh et al., 2011, Marsh, 2003). It has thus been suggested that if the host response could be controlled, this could alter disease progression and may even prevent periodontal destruction (Kornman, 1999), which has lead to examination of several different such agents for this purpose with varying success (Bartold et al., 2010 b).

Macrolide antibiotics are examples of therapeutic agents with dual action, as they have not only bacteriostatic but also immunomodulatory properties. In particular the azalide, azithromycin, a chemical derivative of erythromycin has received much attention recently, mainly due to its longer half life, improved acid stability and excellent tissue distribution (Zuckerman et al., 2009). This is especially true for cells of the immune system where it can reach drug concentrations 800 times higher than plasma concentration (Rodvold et al., 1997) and remain at detectable levels for up to 28 days (Culic et al., 2002). Several *in vitro* (Yamauchi et al., 2009, Wenisch et al., 1996, Vanaudenaerde et al., 2007, Tyteca et al., 2003) and *in vivo* (Tsai et al., 2009, Tomazic et al., 1993, Scaglione and Rossoni, 1998, Li et al., 2010)investigations have confirmed the immune modulatory potential of azithromycin in relation to lung conditions, and found that this antimicrobial at least partially confers its effect through the suppression of the intracellular transcription factor NFkB (Cigana et al., 2006, Cigana et al., 2007) and alterations of cytokine production (Matsumura et al., 2011). This in turn is thought to be a mechanism through which it can affect recruitment, survival, function and the phenotype of cells such as neutrophils (Culic et al., 2001, Culic et al., 2002), macrophages (Murphy et al., 2008, Feola et al., 2010) and lymphocytes (Ishimatsu et al., 2004, Mizunoe et al., 2004). Furthermore, recent findings have shown that azithromycin has the ability to reduce fibrosis or scaring following lung damage (Wuyts et al., 2010), as well as gingival enlargement (Wirnsberger et al., 1998, Wahlstrom et al., 1995, Verma and Dhawan, 2005, Tokgoz et al., 2004) which are associated with increased collagen production (Dongari-Bagtzoglou, 2004).

Although several studies have evaluated the effect of adjunct azithromycin in relation to non-surgical (Smith et al., 2002, Haffajee et al., 2007, Oteo et al., 2010) and surgical periodontal therapy (Mascarenhas et al., 2005) with varying success (Sampaio et al., 2011), no studies to date have evaluated the effect azithromycin may have in terms of minimizing inflammatory changes induced by periodontal pathogens. This research project aimed to determining the efficacy of azithromycin in reducing induced inflammation using an *in vivo* experimental model of acute and chronic inflammation.

## 2.3 Materials and Methods

The animal model for inducing acute and chronic inflammation is based on previous investigations (Bartold et al. 1989). However, in our study a number of modifications were included for experimental analyses.

### 2.3.1 Preparation of non-viable bacteria

*2.3.1.1 Heat killed Porphyromonas gingivalis (HKPG)*

A live bacterial culture of *Porphyromonas gingivalis* (W50) was established for this study. To confirm purity of the culture, gram staining was performed and slides were assessed under 50X magnification (Olympus Light microscope, Olympus Optical Company, LTD, Japan), immersed in oil (Microbact Reagent Mineral Oil, Oxoid, Australia). Using an aseptic technique, bacteria were plated onto anaerobic plates (Oxoid, Australia) and were cultured under anaerobic conditions (gas composition: N2:CO2:H2 (90:5:5)) in an airtight flask placed in an incubator (Townsen & Mercar, Australia) set at 37°C. Anaerobic conditions were maintained and monitored using oxygen indicator strips (Oxoid, Australia). After 7 days, once sufficient bacterial growth had taken place, bacterial cell suspensions were prepared using sterile phosphate-buffered saline (pH 7.2) in a volume of 2.5 ml directly from the plate. Suspended bacteria were then placed in a 10 ml sterile centrifuge tube. Cell density was estimated to be greater than $10^{11}$ organisms/ml (optical density (560 nm) >5.0). The heat killed *P. gingivalis* was prepared by incubating the suspension at $60^{o}$ C maintained for 10 minutes. The absence of any viable bacteria was confirmed by streaking a sponge loaded with heat killed *P. gingivalis* over an agar plate and incubating it using the same protocol as above. Furthermore, a drop of the suspension was directly transferred onto an agar plate and streaked out, after which it was also cultured under the same conditions.

*2.3.1.2 Heat killed Mycobacterium tuberculosis (HKTB)*

Heat killed *Mycobacterium tuberculosis* was kindly donated by Associate Professor D. Haynes, Department of Pathology, Medical School North, Frome Road, Adelaide. A suspension of 0.5 mg /ml of HKTB was created using sterile PBS as the suspension medium.

### 2.3.2 Animals

108 female Dark Agouti (DA) rats with a weight range of 140-160 g were acquired through the Veterinary Services Division of SA Pathology, Adelaide. The research protocol was approved by the animal ethics committees of both the University of Adelaide (approval NoM-2010-070) and SA Pathology (approval No 148c/06). The experiment was conducted in strict accordance with the guidelines of the National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985).

### 2.3.3 Experimental groups and treatment procedures

Two equivalent sized polyurethane sponges (Dunlop Foams, Victoria, Australia) were surgically implanted into the lateral flanks of all study animals. Control animals received sponges treated with phosphate buffered saline (PBS), and experimental sponges were impregnated with either heat killed *Mycobacterium tuberculosis* (HKTB) or heat killed *Porphyromonas gingivalis* (HKPG) suspended in PBS.

A total of 36 animals received control sponges, 36 received HKTB impregnated sponges and another 36 had HKPG impregnated sponges implanted. Six animals were assigned to each time point (Day 7,14,21,35 and 49) for the time course aspect of the study and grouped according to azithromycin administration to form 6 major groups each with 3 animals.

#### *2.3.3.1 Acute inflammation: Pre-operative azithromycin treated Groups (1-3)*

Starting four days prior to surgery, azithromycin (Zithromax, Pfizer) oral suspension was reconstituted as recommended by the supplier and administered at a dosage of 20 mg/kg for three days by means of gastric gavage.

Group 1 were implanted with PBS loaded sponges (n=15), Group 2 were implanted with HKTB impregnated sponges (n=15) and Group 3 were implanted with HKPG impregnated sponges (n=15).

#### *2.3.3.2 Chronic inflammation: Post-operative azithromycin treated Groups (4-6)*

To test the effect of azithromycin on chronic inflammation an additional three groups were created. Animals in Group 4 were implanted with PBS loaded sponges (n=15), Group 5 implanted with HKTB impregnated sponges (n=15) and Group 6 implanted with HKPG

impregnated sponges (n=15). Azithromycin (Zithromax, Pfizer) oral suspension was prepared fresh following supplier recommendations and administered to test animals at a dosage of 20 mg/kg on days 25, 26 and 27 by means of gastric gavage at a stage when chronic inflammation was present.

Additionally, 3 animals for day 35 and 49 were included as controls for the respective groups, receiving the same sponges as the other animals in their group, although these animals did not receive any test medication at Days 25, 26 and 27 and thus acted as negative controls.

### 2.3.4 Sponge implantation procedure

Polyurethane sponges 7 mm in thickness were cut into 15x15 mm blocks and sterilized by autoclaving at 134°C for 5 minutes. The control sponges contained sterile 0.2 M Phosphate Buffered Saline (PBS), and the impregnated sponges had either heat killed *Mycobacterium tuberculosis (HKTB)* or heat killed *Porphyromonas gingivalis (HKPG*) which were prepared as described above. The sponges were immersed and compressed in 1 ml of suspension, followed by overnight air-drying in sterile 12-well plates at 37°C under UV light to prevent contamination and obtain a constant weight. On the day of surgery, the sponges were implanted into each foreflank with aseptic surgical technique. All the surgeries were done using the following set protocol: animals were anaesthetized using inhalation anaesthesia by 2% v/v isoflurane (Veterinary Companies of Australia, Pty. Ltd. Australia) with $O_2$ flow rates of 2 litres per minute. Once the animals were sufficiently anaesthetized, a midline dorsal incision was made, followed by blunt dissection to create two subcutaneous pouches in the area of the left and right shoulder into which one sponge was implanted on either side. After wound closure, all animals were monitored during recovery and post-operative observations using clinical record sheets were performed daily for the first 7 days.

### 2.3.5 Post-operative monitoring

All animals were kept within the animal services facilities of the Institute for Medical and Veterinary Sciences, Frome road, Adelaide. Animals were housed in their respective groups of three and provided with sufficient shelter, food and water. Post-operatively, the rats that had not been preoperatively treated with azithromycin were administered 22.7 mg/ml enrofloxacin (Baytril®, Bayer AG, Leverkusen, Germany) orally for 1 week as required by the Animal Ethics Committees. Enrofloxacin was chosen, as it is a broad spectrum antimicrobial with no reported immune modulatory action. The animals were monitored for

food and water intake, signs of wound infection and general well being using clinical record sheets and their weights recorded weekly.

### 2.3.6 Retrieval surgery

On the days of retrieval (Days 7,14,21,35 and 49) animals were anaesthetized by halothane inhalation, blood was collected by cardiac puncture and then the animals were immediately killed by $CO_2$ inhalation and cervical dislocation. The sponges were retrieved and cut in half. One half of the sponge was placed into 10% phosphate buffered formalin for seven days and the other half was weighed to obtain a wet weight and subsequently stored and dried overnight at 37°C after which the dry weight was obtained.

### 2.3.7 Histological preparation and analysis

One half of the sponge from both the left and right foreflank was placed into 10% phosphate buffered formalin at time of retrieval and allowed to fix at room temperature for seven days followed by washing in PBS and then processed for paraffin embedding. Serial paraffin sections (6 μm) were prepared and stained with haematoxylin and eosin.

For each of the rats, sections for analysis were chosen at random and evaluated at 10X magnification using a light microscope in order to assign a score for both the extent of inflammation and connective tissue infiltration. The designed scoring system for inflammation was as follows:

0= no inflammation

1= mild inflammation,

2= moderate inflammation,

3= Severe inflammation.

Similarly the scoring system for infiltration was:

1= 0-20% infiltration,

2= 20-50% infiltration,

3= 50-80% infiltration

4= 80-100% infiltration

Furthermore cell counts of neutrophils, and lymphocytes were performed in four predetermined areas of the sponge by two independent examiners, taking care not to include the fibrous capsule surrounding the sponge. In order to perform cell counts, care was taken to attempt to choose the same area on every sponge. A 1 mm counting grid was used at 40x magnification and every second line in the grid was used to count the number of neutrophils

and lymphocytes. The cell count for each of the four areas was combined to obtain a total cell count, which was deemed representative for the sponge.

### 2.3.8 Macrophage immunohistochemistry

Immunohistochemical analysis for pan macrophage marker (CD68) and the M2 phenotype marker CD163 was performed on samples obtained from animals from all groups at days 21 and 35 according to previously described methodology (Brown et al., 2009). In brief, the sponges that were fixed for histological analysis were serially sectioned at a thickness of 6 μm and mounted on glass slides. To deparafinise the sections, they were treated with xylene, followed by exposure to a graded series of ethanol solutions (100-70%). After deparafinisation, slides were placed into citrate antigen retrieval buffer (10 mM citric acid monohydrate, pH 6.0) and brought to a boil (95 to 100$^{o}$C) for 20 minutes. After cooling to room temperature the slides were washed three times in PBS. To reduce non-specific peroxidase production, all slides were incubated with 0.1% sodium azide for 20 minutes and subsequently washed three times in PBS. The sections were subsequently incubated with 1% horse serum (Vector Laboratories, Burlingame, USA) for 60 minutes at room temperature. Following incubation with the blocking serum, sections were incubated over night with either CD163 (AbD Serotec, Martinsried, Germany) at a concentration of 1/200 or CD68 (AbD Serotec, Martinsried, Germany) at a concentration on 1/800. Samples were incubated with polyclonal mouse IgG1κ2 (BD Biosciences, California, USA) or omission of primary antibody for negative controls, and for a positive control, deparafinized rat liver was used. All slides were washed three times in PBS the next day and sections were incubated for 45 minutes with biotinylated universal secondary antibody (anti-mouse IgG, Vector Laboratories, Burlingame, USA) at room temperature and subsequently washed three times in PBS. To detect the biotinylated secondary antibody, avidin-biotin complex (Vectastain ABC system, Burlingame, USA) was prepared 30 minutes prior to incubation and then applied to all samples, allowing a 45 minute incubation period. Slides were once again washed three times in PBS and 3-amino-9-ethylcarbazole (AEC) was added (AEC-substrate kit, Vector Laboratories, Burlingame, USA) after which slides were incubated for 20 minutes in the dark at room temperature to allow colour formation. All slides were subsequently washed in distilled water and counterstained with H&E and mounted using Aquatex (Merck, Darmstadt, Germany) mounting medium. For both positive and negative controls, sections were de-parafinized and antigen retrieval was performed in the same way as for the test tissue.

In order to assess macrophage infiltration for both cells expressing CD68 or CD163, a scoring system was designed according to the intensity and location of the cellular infiltrate,

staining positive for the respective primary antibody and evaluated by two blinded examiners. The chosen scoring system was:

0= minimal or no staining present

1= limited staining confined to areas adjacent to sponge matrix or in capsular connective tissue.

2= moderate staining mainly confined areas adjacent to sponge.

3= heavy infiltration of cells adjacent to sponge matrix and mild infiltration into non-adjacent connective tissue.

4= heavy infiltration of cells adjacent to sponge matrix and significant infiltration of cells into non-adjacent connective tissue.

### 2.3.9 Biochemical assays

*2.3.9.1 C-reactive protein*

Serum samples were obtained by means of centrifugation of the obtained whole blood on the day of sacrifice. All samples were allowed to clot at room temperature for 2 hours, after which they were centrifuged (Eppendorf Centrifuge 5415R) at 1300 rcf for 10 minutes at room temperature. Once centrifuged, serum was pipetted from the samples and frozen at minus 18$^{\circ}$C until required. C-reactive protein (CRP) levels were measured using Enzyme Linked Immuno Sorbent Assay (Catalog No. 2210-2-HR, Life Diagnostics, Inc, West Chester, PA, USA). Samples were prepared according to the manufacturer's recommendations. Briefly, the test sample was diluted and incubated in microtiter wells for 45 minutes. Subsequently, the wells were washed and horseradish peroxidase (HRP) conjugate was added and incubated for 30 minutes. The wells were then washed to remove unbound HRP-labeled antibodies and TMB reagent was added and incubated for 20 minutes at room temperature to allow for development of color. Colour formation was stopped by the addition of stop solution and optical density was measured at 450 nm wavelength using a photo spectrometer Bio-Tek Powerwave (Bio-Tek Instruments Inc., Vermont, USA). The optical density readings were then processed for CRP concentration determination using the KC4 software (Bio-Tek Instruments Inc., Vermont, USA).

*2.3.9.2 Hydroxyproline (HP) assay*

Hydroxyproline levels were determined by modification of the technique described by Reddy and Enwemeka (1996). All chemicals for the procedure were obtained from the Sigma Chemical Company (Sigma, Mouse). For the hydroxyproline *stock*, a solution containing 1

mg/ml of hydroxyproline (HP) was prepared in distilled water. The acetate-citrate buffer (pH 6.5) was prepared by dissolving 120 g of sodium acetate trihydrate, 46 g of citric acid, 12 ml acetic acid, and 34 g of sodium hydroxide in 800 ml of distilled water; pH was adjusted to 6.5 and brought to one litre. For the *Chloramine T reagent (0.056 M),*1.27 g of chloramine T was dissolved in 20 ml (50% n-propanol) and brought to 100 mL with acetate- citrate buffer. For *Ehrlich's reagent (1 M),* 15 g of p-dimethylaminobenzaldehyde was dissolved in n-propanol/perchloric acid (2:1 v/v) and brought to 100 mL. Since this reagent is not stable, it was prepared freshly before each assay.

### 2.3.10 Assay

Serial dilutions of standard hydroxyproline were prepared from the stock (1 mg/ml) (0.5 g/ml, 0.25 g/ml, 0.125 g/ml and 0 g/ml) and mixed gently with sodium hydroxide (2 M final concentration) in a total volume of 1 ml. For the samples, 50 mg of dried sponge were weighed on a Mettler AE260 digital balance (Greifensee, Switzerland) and placed in a 5 ml polypropylene tube containing 1 ml of NaOH (2 M final concentration). To hydrolyze the samples, they were left overnight at 120°C. Following this, all the individual samples were placed into 1.5 ml centrifuge tubes and spun at 2000 rcf at 20°C for 5 minutes in a centrifuge (Eppendorf Centrifuge, 5415R) to ensure sedimentation of any solids. Twenty microliters of the supernatant for each sample or standard was pipetted into a 96 well plate (Falcon Microtest 96 well plate, Becton Dickinson and Company, Franklin Lakes, NJ USA) and 20 µl HCL (2 M final concentration) was added to attain a neutral pH. After this, 90 µl of Chloramine T was added and the plate was placed on a well shaker (Titertek well shaker, DSG, Titertek No 541307) to ensure gentle mixing over a 20-minute period. Following this, 100 µl Ehrlich’s reagent was added to each well, mixed gently and the chromophore was developed by incubating the samples at 65°C for 10 min. Absorbance readings were measured at 540 nm wavelength using the Bio-Tek Powerwave XS and the hydroxproline concentrations determined using the dedicated KC4 software.

### 2.3.11 Statistical analysis

Independent statistical analysis was performed by a statistician (ML) from the Data Management & Analysis Centre, Discipline of Public Health, University of Adelaide, SA, 5000, Australia. A log transformation was applied to normalized data and analysis of variance was used to determine if there was an interaction between sponge treatment (PBS, HKTB and HKPGTB), drug application (no administration, pre-operative or post-operative) time point

(day) and cell counts in the time course study. Prism 5 (Graphpad Software Inc.) was also used to perform One-way ANOVA and any post hoc tests where the One-Way ANOVA showed significance. Statistical significance was accepted at $p<0.05$.

## 2.4 Results

The results are presented for the effects of azithromycin:

1. Administered 4 days pre-operatively on acute inflammation.
2. Administered post-operatively at day 25 on chronic inflammation.

### 2.4.1 The effect of azithromycin on acute inflammation

*2.4.1.1 Animal weight monitoring*

All animals recovered well after surgery and drug administration and gained weight post operatively until the time of sacrifice (Figures 5a and b).

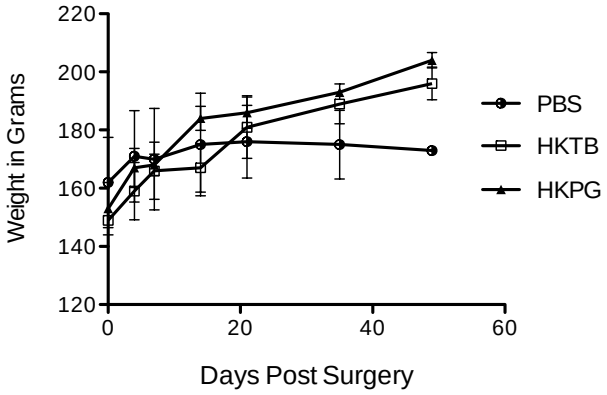



**Figure 5a.** Rat weights for pre-operatively treated azithromycin groups

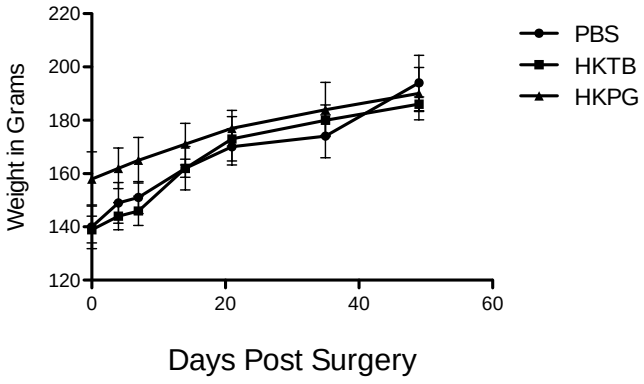



**Figure 5b.** Rat weights for untreated groups

*2.4.1.2 Sponge wet and dry weights*

There was no pattern or significant difference for the mean wet or dry weights obtained from treated and untreated animals in any of the groups, irrespective of whether azithromycin had been administered pre operatively or not (Table 4).

**Table 4.** Mean wet and dry sponge weights at different time points for the different groups.

Mean Wet Weights per group

| | **PBS Pre-op Treated** | **PBS Untreated** | **TB Pre-op Treated** | **TB-Untreated** | **PG- Pre-op Treated** | **PG Untreated** |
|---|---|---|---|---|---|---|
| **Day 7** | 0.57g ±0.055g | 0.68g±0.021g | 0.66g±0.065g | 0.435g±0.043g | 0.623g±0.032g | 0.235g±0.05g |
| **Day 14** | 0.46g±0.073g | 0.3g±0.036g | 0.51g±0.04g | 0.34g±0.052g | 0.442g±0.034g | 0.405g0.032g |
| **Day 21** | 0.38g±0.045g | 0.2g±0.037g | 0.39g±0.045g | 0.24g±0.016g | 0.317g±0.025g | 0.235g±0.05g |
| **Day 35** | 0.24g±0.031g | 0.18g±0.034g | 0.37g±0.078g | 0.27g±0.035g | 0.178g±0.024g | 0.13g±0.039g |
| **Day 49** | 0.27g±0.013g | 0.14g±0.009g | 0.26g±0.043g | 0.225g±0.038g | 0.181g±0.021g | 0.108g±0.008g |

Mean Dry Weights per group

| | **PBS Pre-op Treated** | **PBS Untreated** | **TB Pre-op Treated** | **TB Untreated** | **PG Pre-op Treated** | **PG Untreated** |
|---|---|---|---|---|---|---|
| **Day 7** | 0.08g±0.049g | 0.041g±0.04g | 0.083g±0.01g | 0.71g±0.08g | 0.106g±0.014g | 0.236g±0.360g |
| **Day 14** | 0.118g±0.04g | 0.043g±0.05g | 0.093g±0.09g | 0.056g±0.07g | 0.066g±0.005g | 0.060g±0.005g |
| **Day 21** | 0.063g±0.008g | 0.036g±0.007g | 0.073g±0.08g | 0.048g±0.004g | 0.057g±0.008g | 0.051g±0.007g |
| **Day 35** | 0.048g±0.006g | 0.04g±0.08g | 0.080g±0.06g | 0.062g±0.01g | 0.049g±0.005g | 0.036g±0.005g |
| **Day 49** | 0.057g±0.013g | 0.037±0.011g | 0.062g±0.013g | 0.06g±0.011g | 0.045±0.008g | 0.041g±0.008g |

### 2.4.2 Biochemical analysis

*2.4.2.1 C-reactive protein levels*

Azithromycin treatment did not result in a clear reduction of CRP in any of the groups ($p>0.05$). Generally, CRP values increased between 32-58% in the 14 days following surgery, after which they tended to return to baseline values by day 21 (Figure 6). When looking at increased CRP levels in the groups where azithromycin was administered pre-operatively compared to animals that did not receive the drug, students t-test analysis showed there was a significant difference between treated and untreated animals at day 21 for animals with PBS sponge implants ($p=0.024$). Furthermore difference in CRP levels in the HKPG approached significant difference at the Day 14 time point ($p=0.084$).

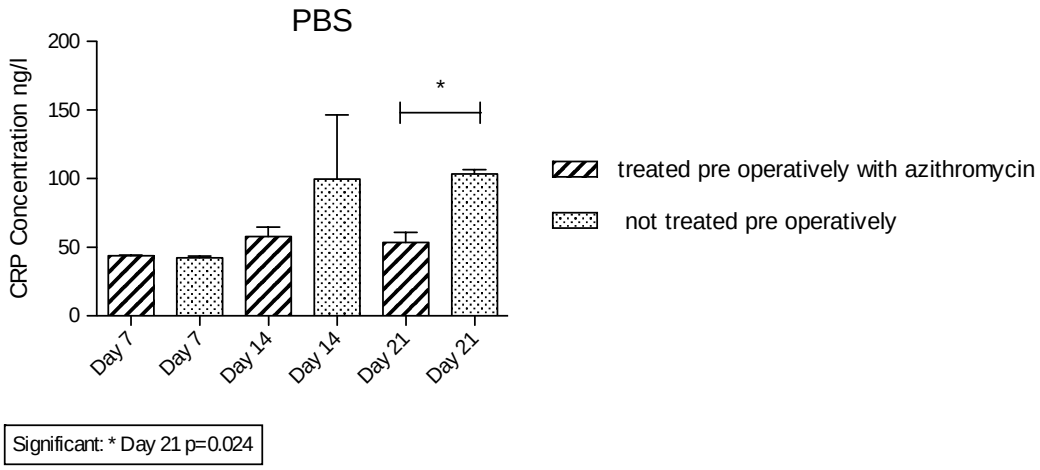



**Figure 6a.** CRP levels for pre-operatively azithromycin treated animals with PBS sponge implants compared to untreated animals

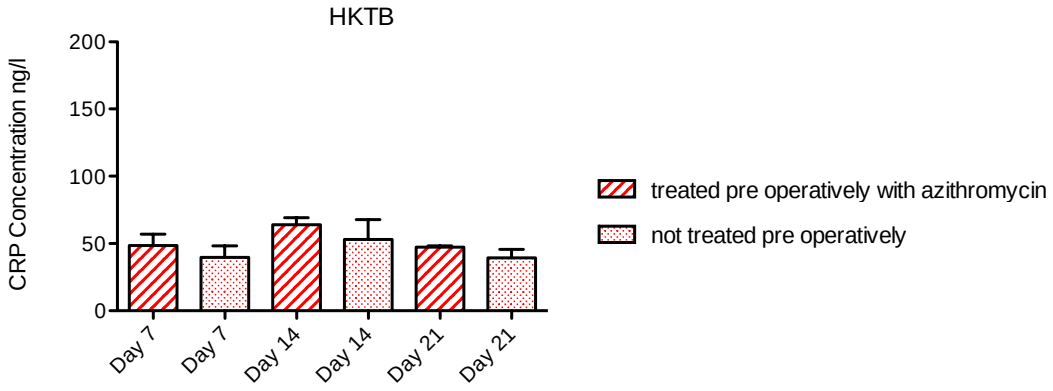



**Figure 6b.** CRP levels for pre-operatively azithromycin treated animals with HKTB sponge implants compared to untreated animals

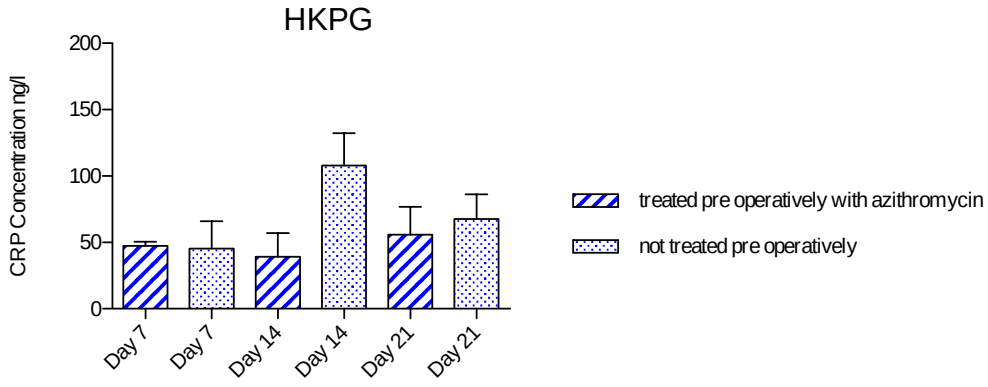



**Figure 6c.** CRP levels for pre-operatively azithromycin treated animals with HKPG sponge implants compared to untreated animal
Differences in CRP levels approaching significance at day 14 (p=0.084).

*2.4.2.2 Hydroxyproline (HP) levels*

Pre-operative azithromycin treatment in the PBS treated groups led to significantly lower hydroxyproline levels at all time points (p=0.034) (Figure 7a). The group of rats which had the HKPG impregnated sponges implanted and received azithromycin pre-operatively, also showed a tendency towards lower HP concentrations in the analysed sponges compared to their untreated counterparts although this was only significant at day 35 (p= 0.016) (Figure 7c).

Interestingly, for the HKTB group, HP analysis showed significantly lower HP levels in the sponges from animals that had not received azithromycin pre-operatively, compared to those that had at days 7 (p<0.001) and 14 (p=0.04). However at day 21 this tendency was reversed with animals that had received the drug now demonstrating lower HP levels (p=0.019). After day 21 no further significant differences could be detected at day 35 (p=0.94) and 49 (p- 0.41) between animals that had received azithromycin and those that had not (Figure 7b), although the trend remained.

Figures 7a, b and c show mean hydroxyproline concentrations for animals that had received azithromycin pre-operatively compared to animals that did not receive azithromycin. Data points plotted on the graph represent mean HP levels for animals at the day 7,14,21,35 and 49 retrievals.

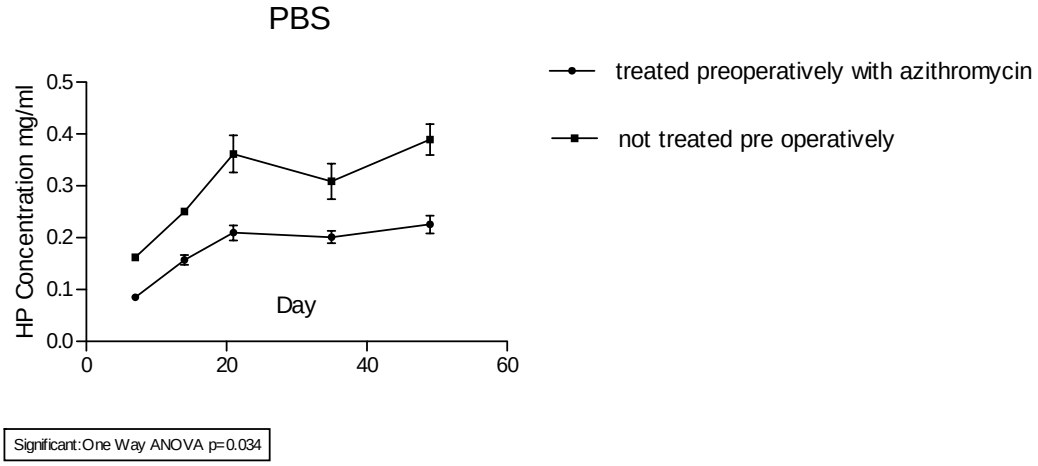



**Figure 7a.** Hydroxyproline levels in PBS sponge implant groups

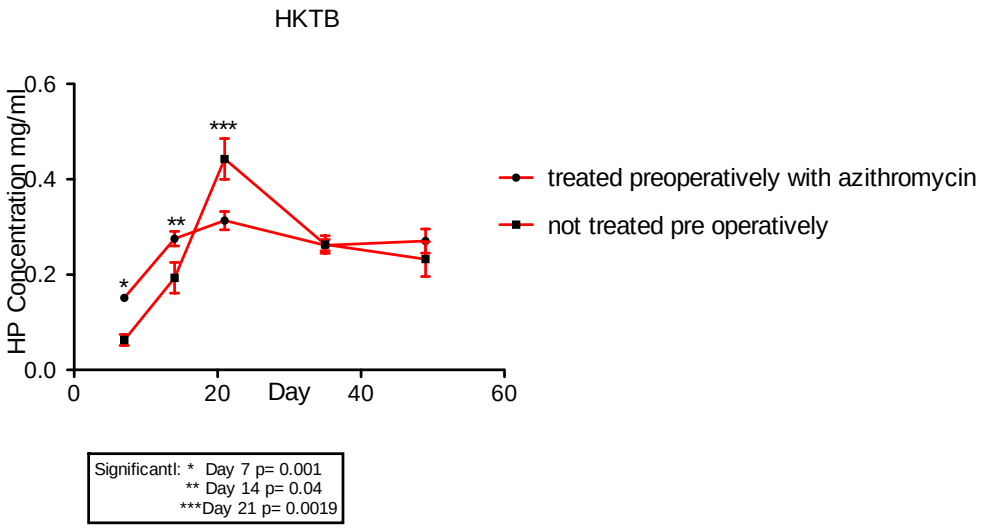



**Figure 7b.** Hydroxyproline levels HKTB sponge implant groups

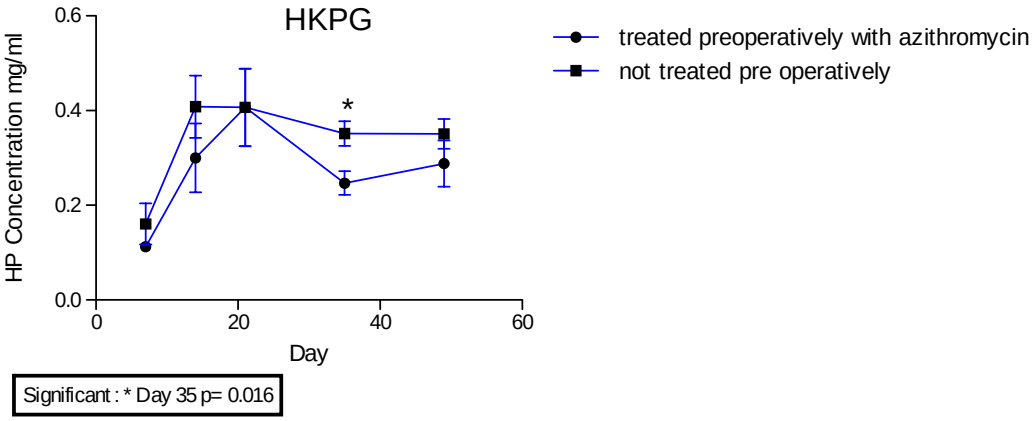
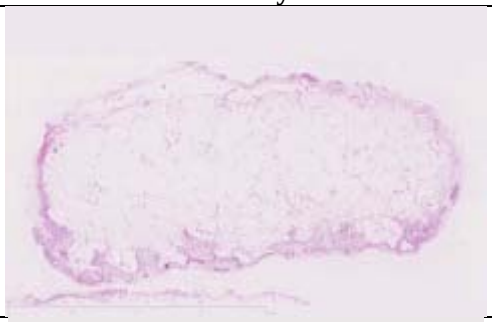
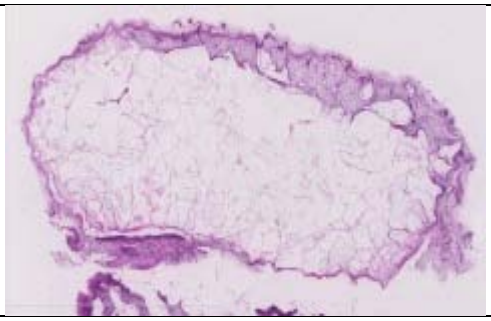
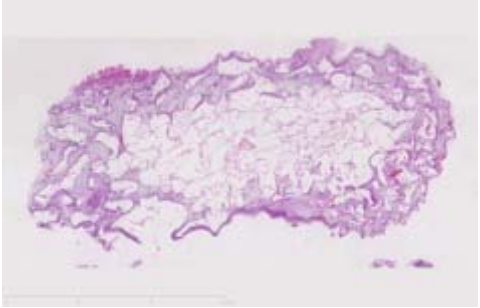
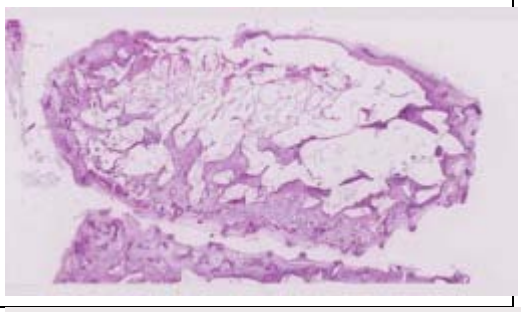
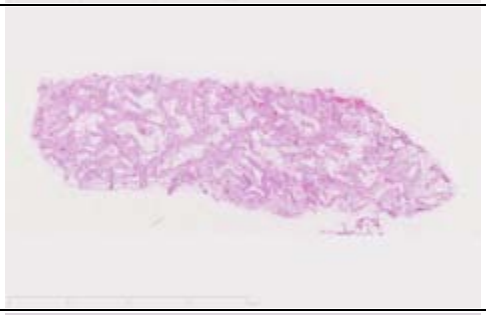
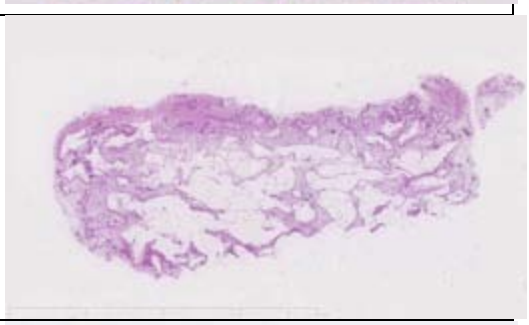
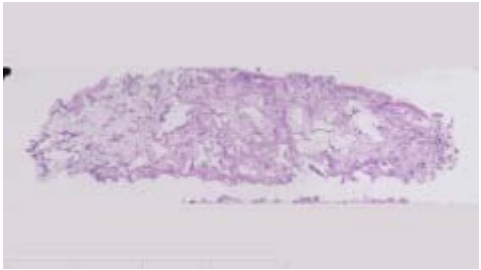
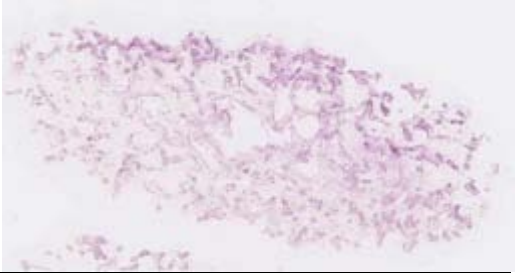
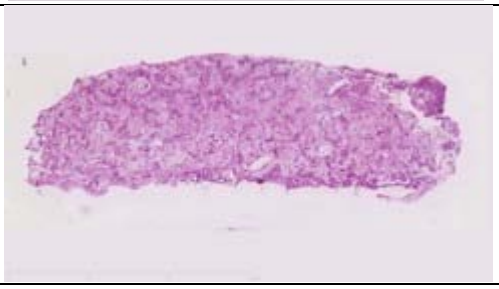
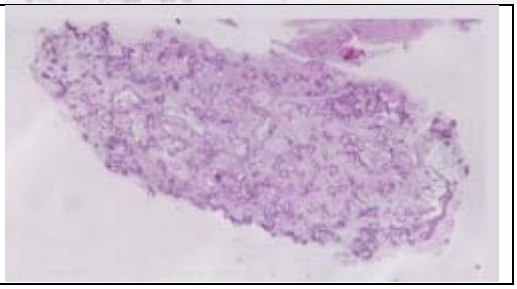


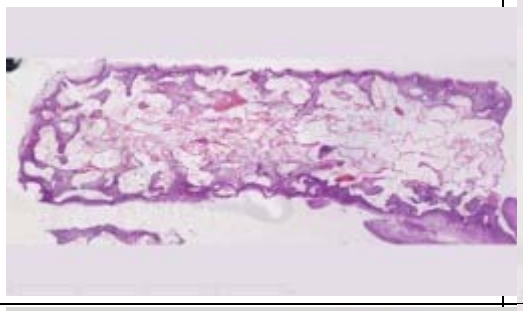
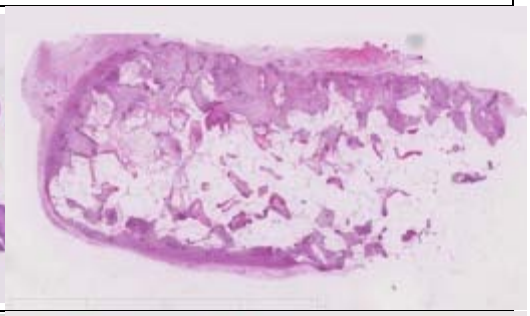
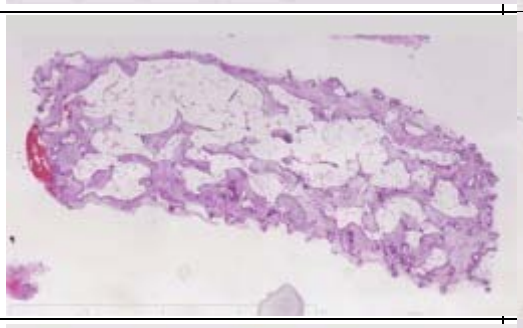
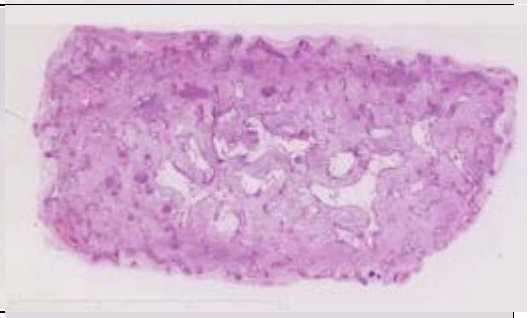
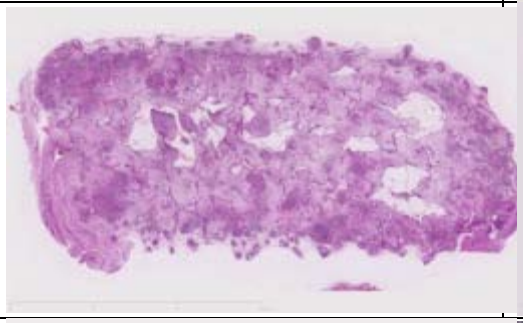
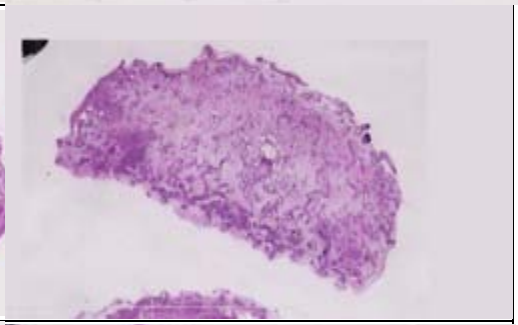
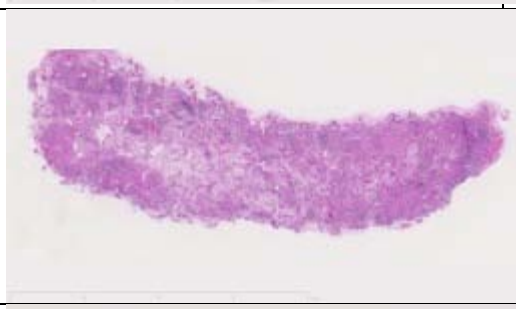
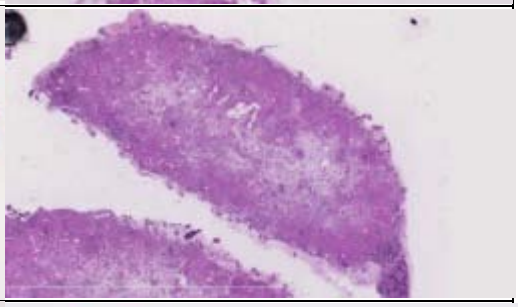
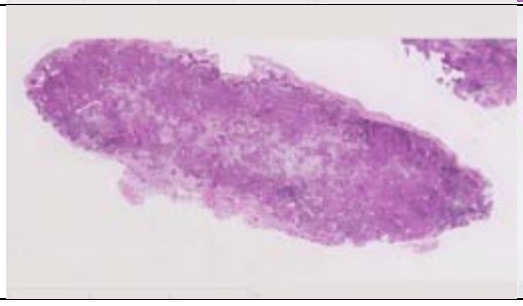
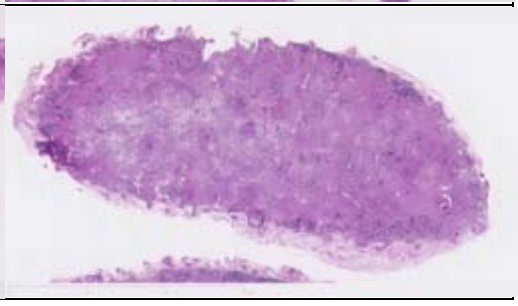

**Figure 7c.** Hydroxyproline levels HKPG sponge implant groups

### 2.4.3 Histological analysis

*2.4.3.1 Qualitative assessment*

The histological appearance of sponges obtained from animals treated pre-operatively with azithromycin compared to animals that did not receive any azithromycin in the different groups at every time point can be seen in Figure 8.

| Day | Group<br>PBS pre-operatively treated with azithromycin | Group<br>PBS not treated with azithromycin |
|---|---|---|
| 7 |  |  |
| 14 |  |  |
| 21 |  |  |
| 35 |  |  |
| 49 |  |  |

| Day | Group<br>HKTB pre-operatively treated with azithromycin | Group<br>HKTB not treated with azithromycin |
|---|---|---|
| 7 |  |  |
| 14 |  |  |
| 21 |  |  |
| 35 |  |  |
| 49 |  |  |

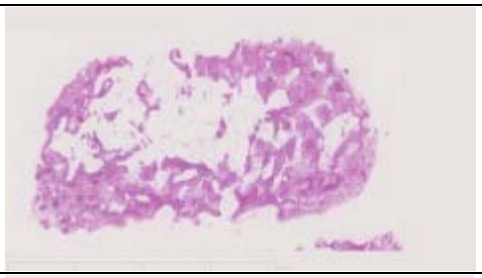
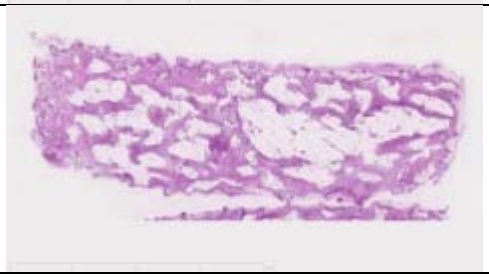
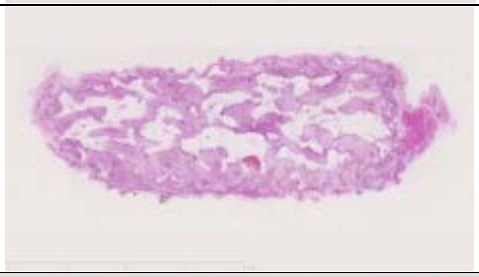
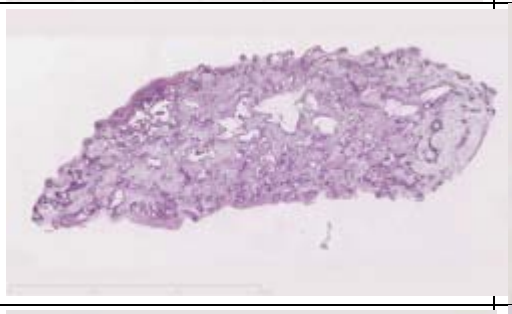
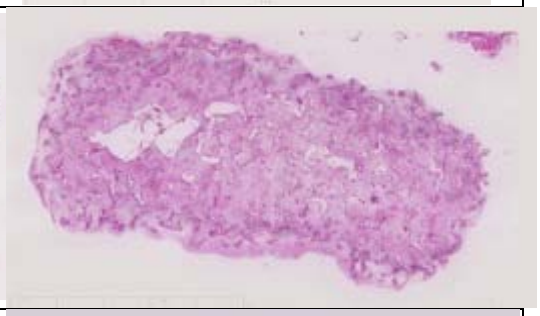
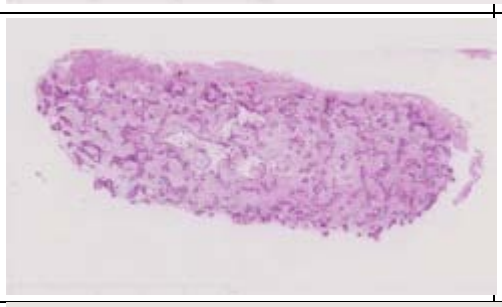
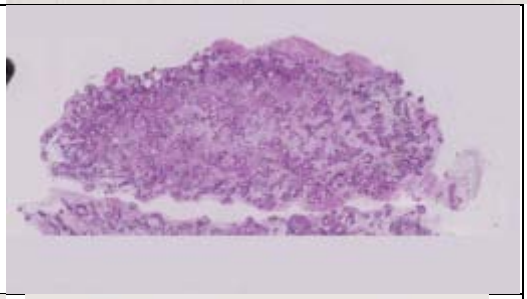
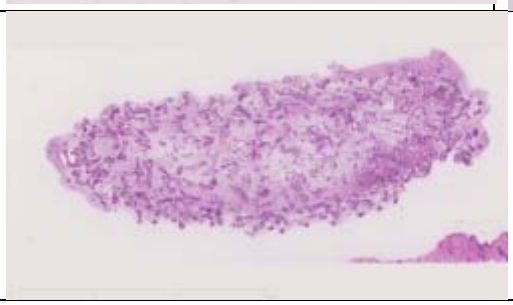
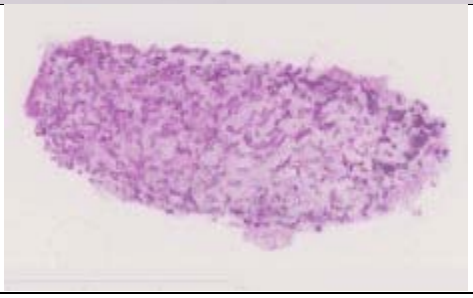
| Day | Group<br>HKPG pre-operatively treated with azithromycin | Group<br>HKPG not treated with azithromycin |
|---|---|---|
| 7 |  |  |
| 14 |  |  |
| 21 |  |  |
| 35 |  |  |
| 49 |  |  |

**Figure 8.** Histological appearance (x10 magnification) of representative sponges obtained from the different groups at all time points stained with H&E illustrating differences between animals that had received azithromycin prior to sponge implantation compared to animals that had not received azithromycin. Connective tissue and inflammatory cell infiltration can be seen commencing in the periphery and progressing towards the centre over time.

When examining the reaction induced by the different groups, it was found that there were far fewer cells in the negative control (PBS) sponges when compared to the other two groups, with the HKTB sponges eliciting the strongest reaction (HKTB>HKPG>PBS). Generally, infiltration started in the outer third of the sponge at day 7 with substantial neutrophil accumulation, progressing to two thirds of the sponge by day 21 at which point a more chronic inflammatory infiltrate was noted, marked by increasing numbers of lymphocytes and macrophages. By days 35 and 49 complete infiltration of the sponge had taken place.

In the early stages (days 7 to 14) sponges were characterized by an acute inflammatory infiltrate consisting mainly of neutrophils and fibroblasts. Over time these were replaced with increasing numbers of lymphocytes. Furthermore, from day 21 the presence of giant cells was noted adjacent to the surface of the sponge material. In some areas these appeared to be actively involved in the destruction of sponge material. Similarly, in areas adjacent to the sponge matrix, increasing numbers phagocytic cells (possibly macrophages) were noted with intracellular inclusions. An interesting observation was that at day 7 HKTB sponges from animals that did not receive pre-operative azithromycin had a particularly strong neutrophil infiltrate and very little fibroblast infiltration, although this seemed to reverse to some extent by day 14. In contrast to this, HKTB sponges retrieved from animals that received pre-operative azithromycin showed a more mature infiltrate with high numbers of fibroblasts and lower numbers of neutrophils at earlier time points.

A qualitative histological assessment was used to evaluate the inflammatory infiltration of the sponge, starting at the periphery and progressing to the centre. This infiltration was most pronounced for sponges impregnated with HKTB, followed by HKPG and lastly PBS (Figure 9a, b and c) regardless of whether or not animals had received azithromycin. Similarly, for inflammatory scores, HKTB sponges (Figure 9b) were more inflamed then HKPG (Figure 9c) followed by PBS (Figure 9a). No differences were observed for any of the groups between test animals that had received azithromycin compared to the respective counterparts that did not receive azithromycin ($p>0.05$) (Figures 9a, b and c).

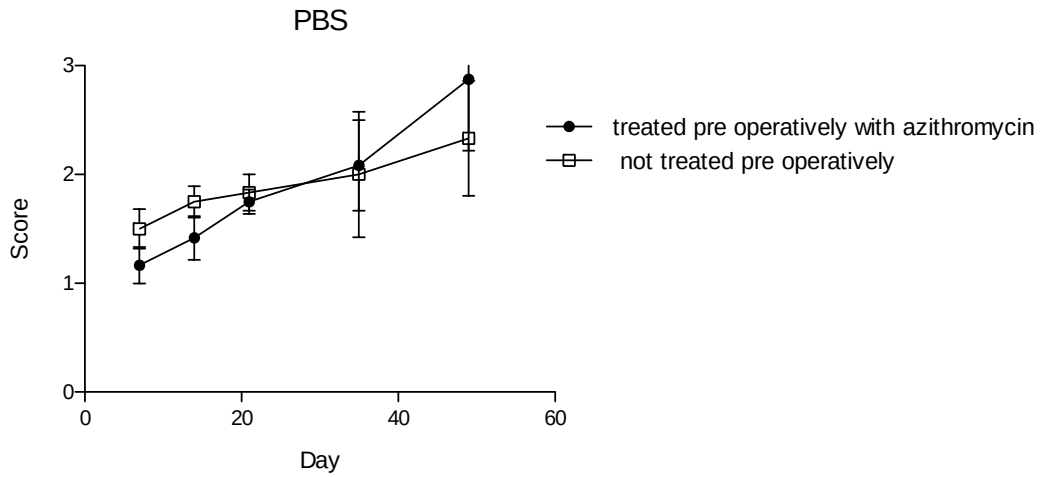



**Figure 9a.** Combined inflammation and infiltration score in the PBS sponge implanted animals that had received azithromycin compared to those that had not received azithromycin

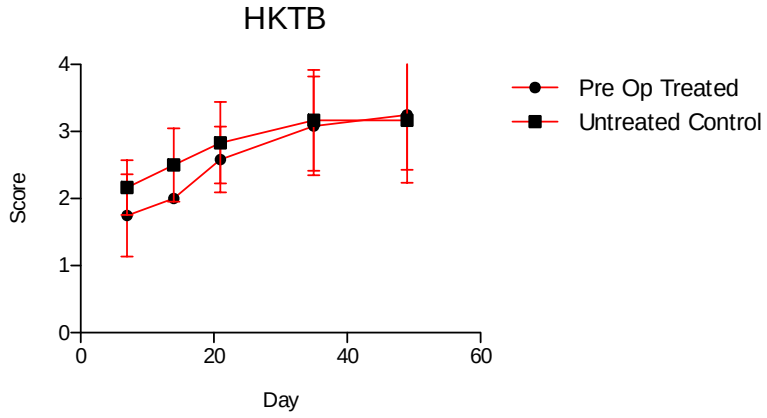



**Figure 9b.** Combined inflammation and infiltration score for the HKTB sponge implanted animals that had received azithromycin compared to those that had not received azithromycin compared to those that had not received azithromycin

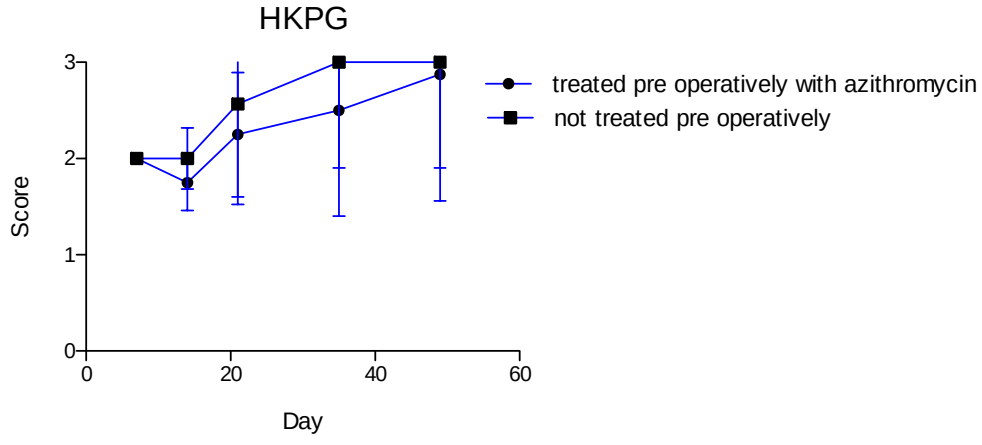



**Figure 9c.** Combined inflammation and infiltration score for the HKPG sponge implanted animals that had received azithromycin compared to those that had not received azithromycin

*2.4.3.2 Quantitative assessment*

To quantitatively assess the degree of acute and chronic inflammation and repair, differential cell counts were done for neutrophils and lymphocytes.

*2.4.3.3 Neutrophil counts*

Neutrophil counts were conducted for the evaluation of acute inflammation only. The neutrophil content of the sponges changed significantly during the time course of the study period. As such, high neutrophil counts were observed over the first 14 days, which reduced with time. A difference in the mean number of neutrophils was noted between the treatment groups by ANOVA two way analysis ($p<0.001$) in the order of HKTB>HKPG>PBS.

The pre-operative administration of azithromycin resulted ina three fold reduction in neutrophil numbers ($p=0.008$) compared to control treatment for the HKTB group (Figure 10b) with a similar trend noted for animals in the HKPG group (Figure 10c), although this was not statistically significant ($p=0.45$). Pre-operative administration of azithromycin did not have any significant effect on animals that received PBS sponges ($p>0.05$) (Figure 10a).

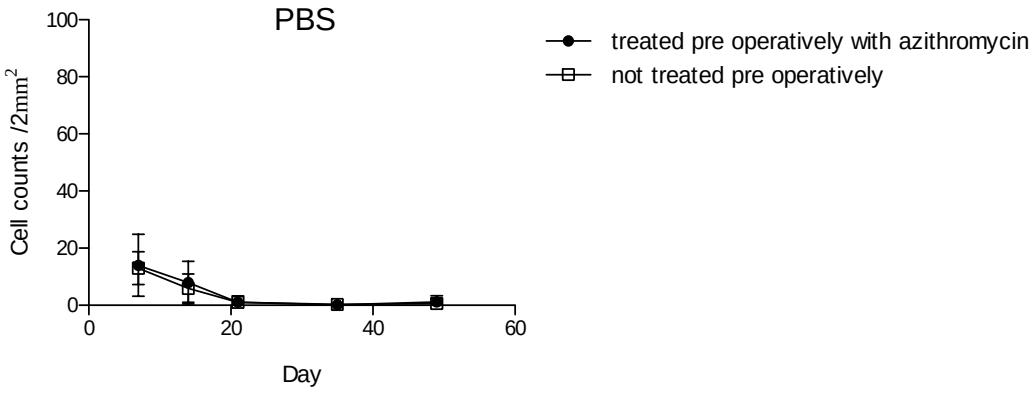



**Figure 10a.** Neutrophil counts for rats with PBS sponge implants

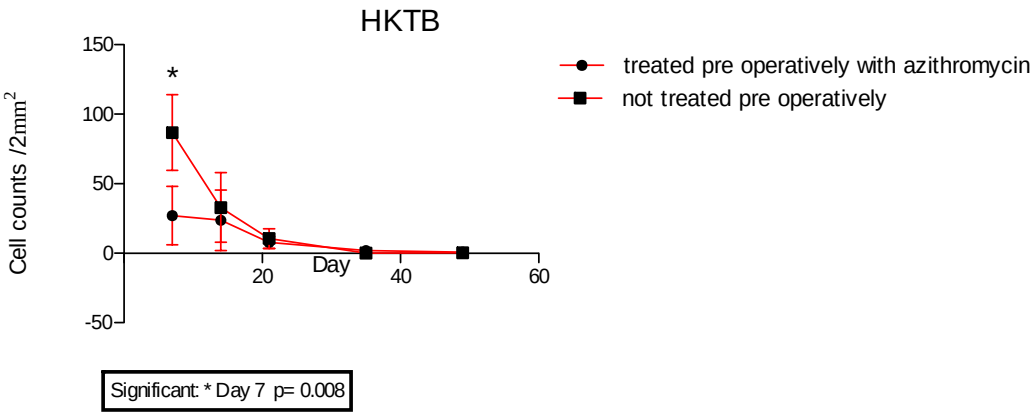



**Figure 10b**. Neutrophil counts for rats with HKTB sponges

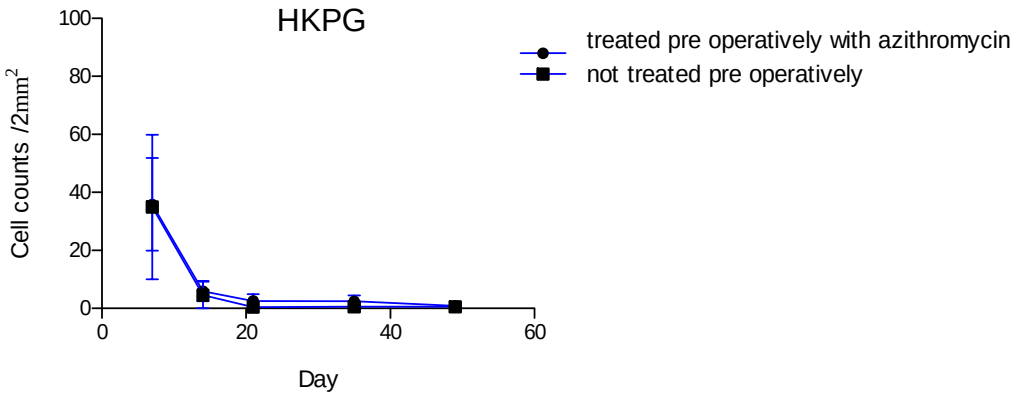



**Figure 10c.** Neutrophil counts for rats with HKPGsponges

*2.4.3.4 Lymphocyte counts*

There was a steady increase in lymphocyte numbers over the 49-day period of the experiment for all groups (Figures 11a, b and c). The lymphocyte cell counts at days 35 and 49 showed significantly higher numbers for all animals in the HKTB groups compared to the HKPG and PBS groups, irrespective of whether the animals had received azithromycin or not (one way ANOVA, $p<0.001$). Although there were higher lymphocyte counts found in the HKPG group compared to the PBS group for both treated and untreated animals, this was not statistically significantly different between these two groups ($p=0.172$). Furthermore, it was found that when intragroup lymphocyte cell count comparisons were done between animals that did and did not receive azithromycin at each of the separate time points, no significant difference was found in the PBS (Figure 11a), HKTB (Figure 11b) and HKPG (Figure 11c) groups ($p>0.05$).

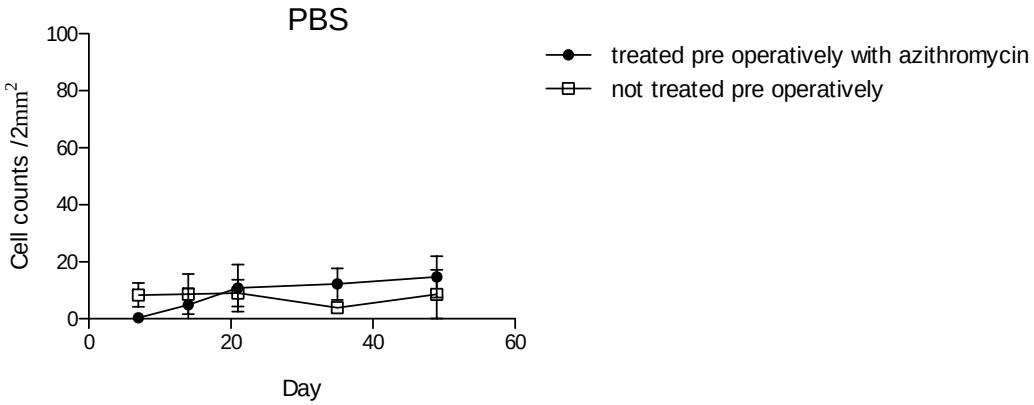



**Figure 11a.** Lymphocyte counts between pre-operatively treated animals and untreated animals in the PBS sponge implant group

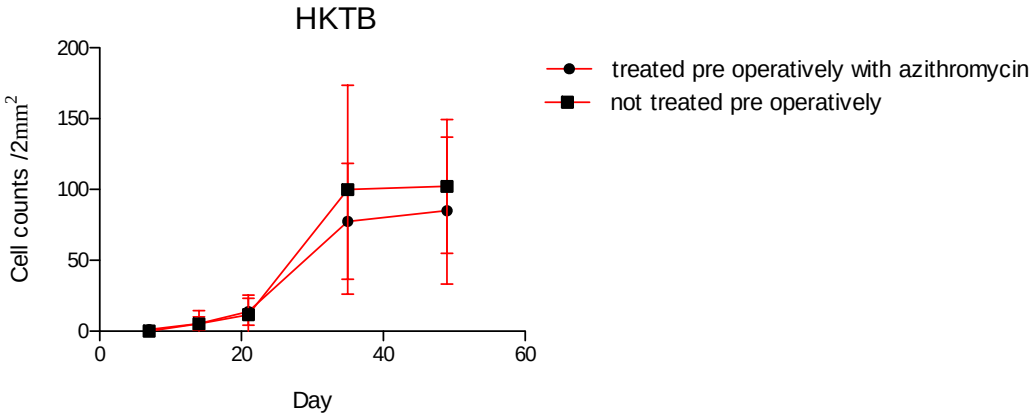



**Figure 11b.** Lympohcyte counts between pre-operatively treated animals and untreated animals in the HKTB sponge implant group

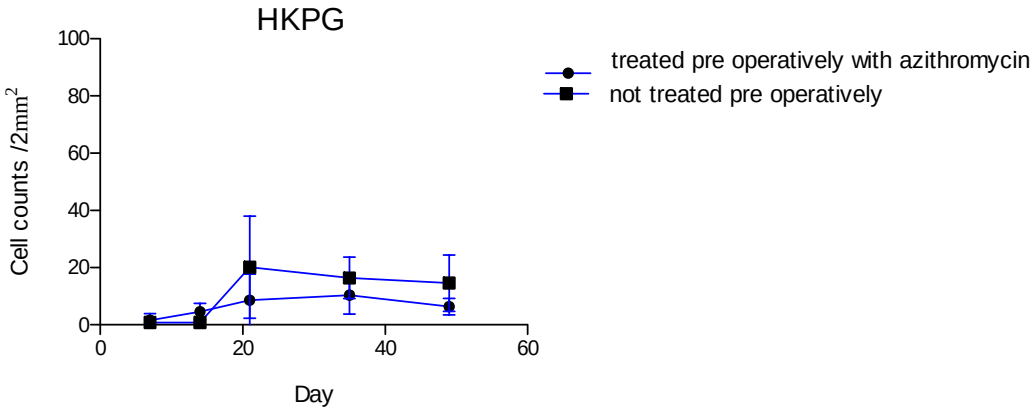



**Figure 11c.** Lympohcyte counts between pre-operatively treated animals and untreated animals in the HKPG sponge implant group

*2.4.3.5 Imunohistochemistry*

Cells of both CD163 and CD68 cell lines were identified in all slides. Scores for the amount of macrophage infiltration showed a definitive trend towards lower macrophage numbers for animals that had received azithromycin prior to treatment, compared to untreated animals in the respective groups. However, it was found that for CD68 this was only significant for animals with PBS sponges at days 21 and 35 (One way ANOVA p= 0.0028) and TB sponges at day 35 (p=0.02) (Figures 12a, b and c). Similarly, for CD163 it was found that although azithromycin reduced counts of CD163 positive cells, there was no significant difference between treated and untreated animals in any of the groups at day 21 and only significant differences at day 35 for PBS, ($p<0.001$) and HKTB ($p<0.001$) but not HKPG ($p=0.13$) (Figures 13a, b and c).

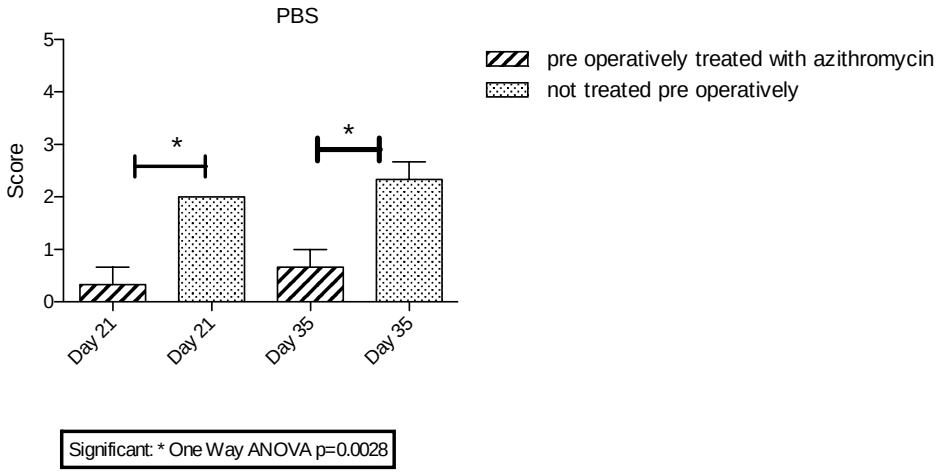



**Figure 12a.** CD 68 positive cell scores of rats with PBS sponge implants sacrificed at days 21 and 35 after sponge implantation for animals treated with azithromycin pre-operatively compared to animals that did not receive the drug

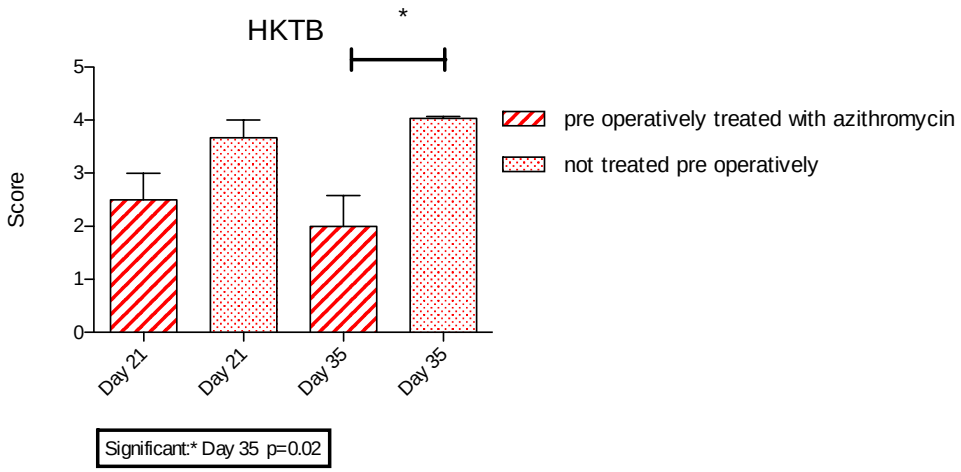



**Figure 12b.** CD 68 positive cell scores of rats with HKTB sponge implants sacrificed at days 21 and 35 after sponge implantation for animals treated with azithromycin pre-operatively compared to animals that did not receive the drug

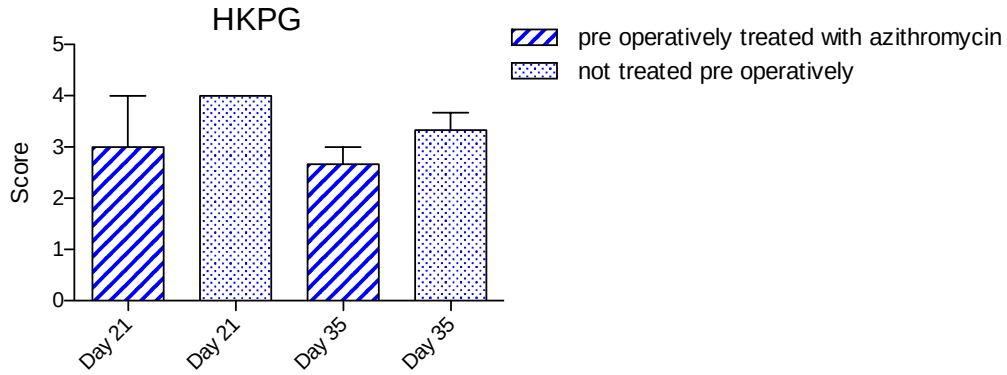



**Figure 12c.** CD 68 positive cell scores of rats with HKPG sponge implants sacrificed at days 21 and 35 after sponge implantation for animals treated with azithromycin pre-operatively compared to animals that did not receive the drug

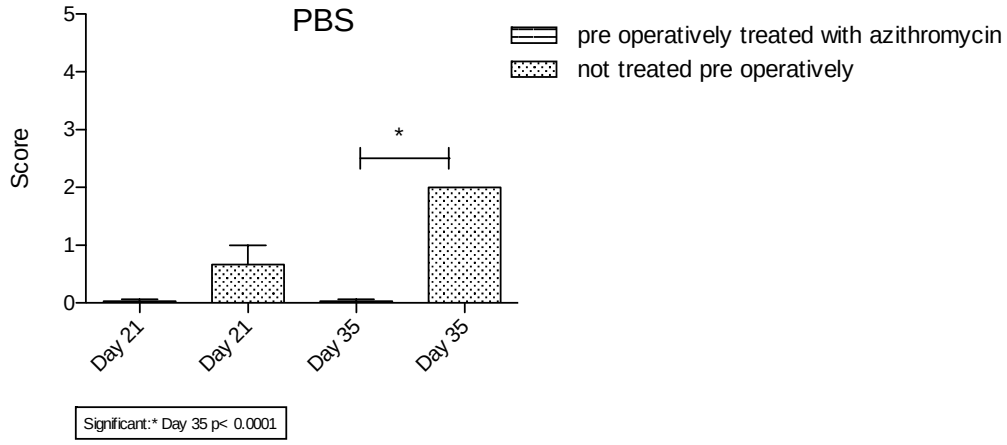



**Figure 13a.** CD 163 positive cell scores of rats with PBS sponge implants sacrificed at days 21 and 35 after sponge implantation for animals treated with azithromycin pre-operatively compared to animals that did not receive the drug

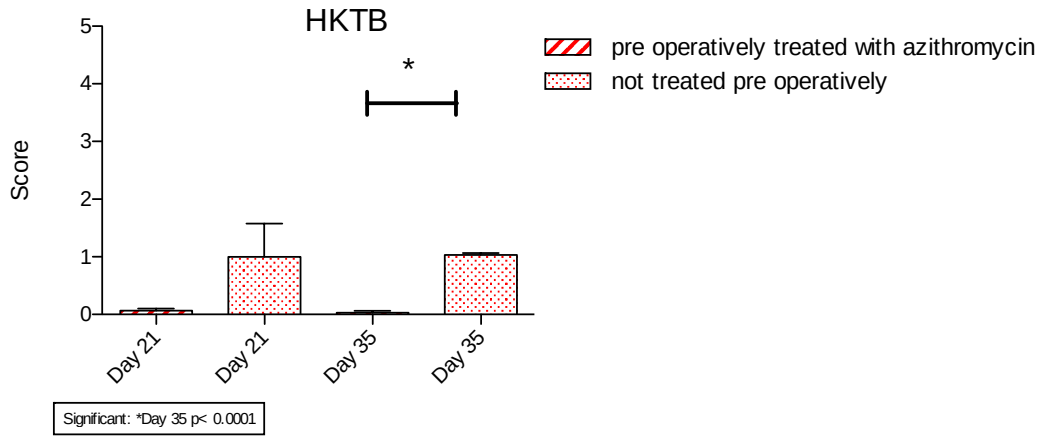



**Figure 13b.** CD 163 positive cell scores of rats with HKTB sponge implants sacrificed at days 21 and 35 after sponge implantation for animals treated with azithromycin pre-operatively compared to animals that did not receive the drug

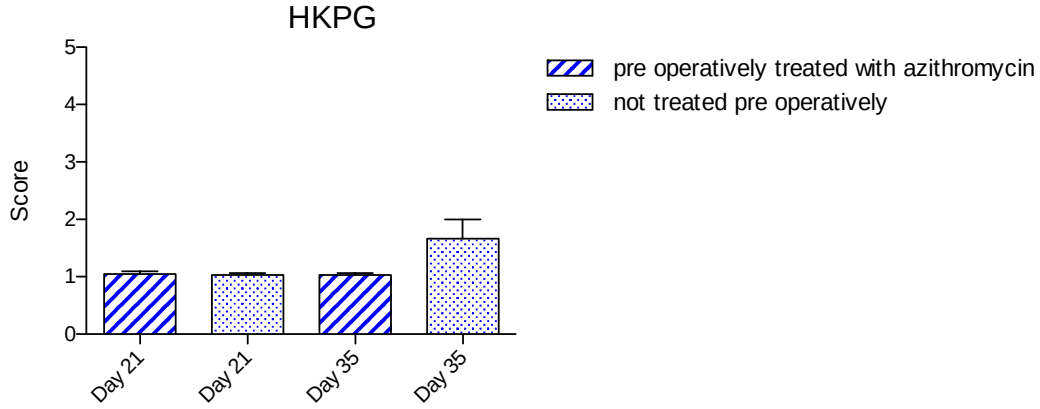



**Figure 13c.**CD 163 positive cell scores of rats with HKPG sponge implants sacrificed at days 21 and 35 after sponge implantation for animals treated with azithromycin pre-operatively compared to animals that did not receive the drug.

Representative images demonstrating histological sections with various assigned scores can be seen in Figure 14.

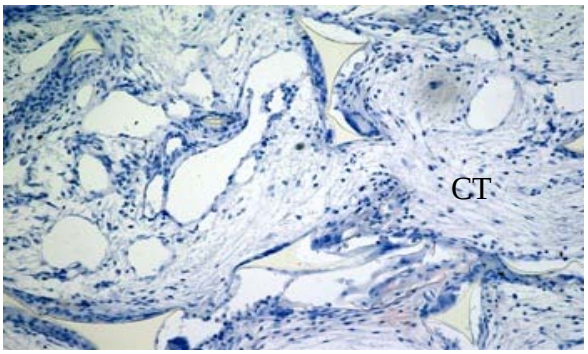



**Figure 14a.** CD 68 staining of PBS sponge retrieved at day 35 after pre-operative azithromycin administration (PBS Pre-operatively Treated Sponge Day 35 (Score=0))

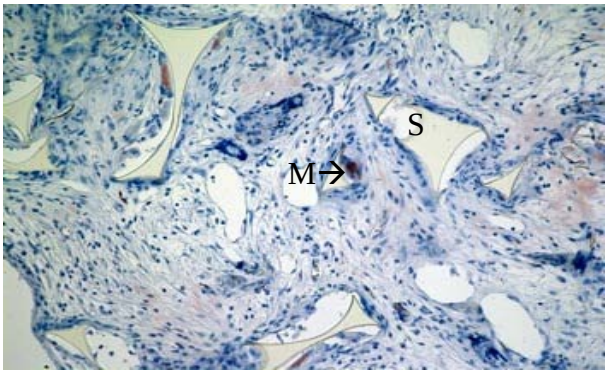



**Figure 14b.** CD 68 staining of PBS sponge retrieved at day 35 after pre-operative azithromycin administration (PBS Pre-operatively Treated Sponge Day 35 (Score=1))

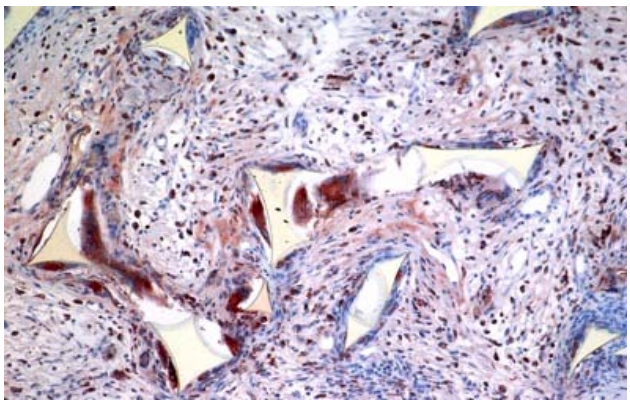


**Figure 14c.** CD 68 staining of HKPG sponge retrieved at day 21 after no pre-operative azithromycin administration (HKPG Untreated Day 21 (Score= 2))

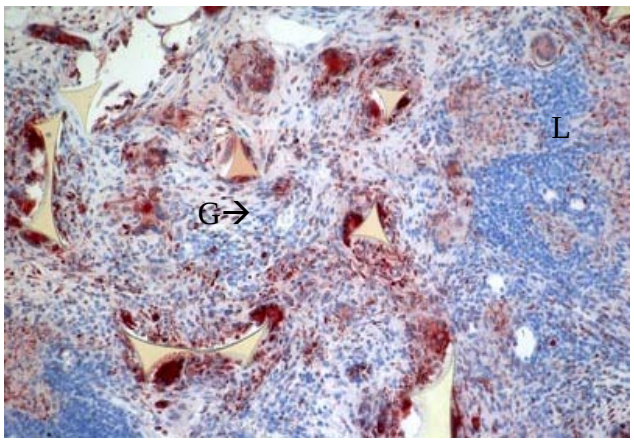



**Figure 14d.** CD 68 staining of HKTB sponge retrieved at day 35 after pre-operative azithromycin administration (HKTB Pre-operatively Treated Day 35 (Score =3))

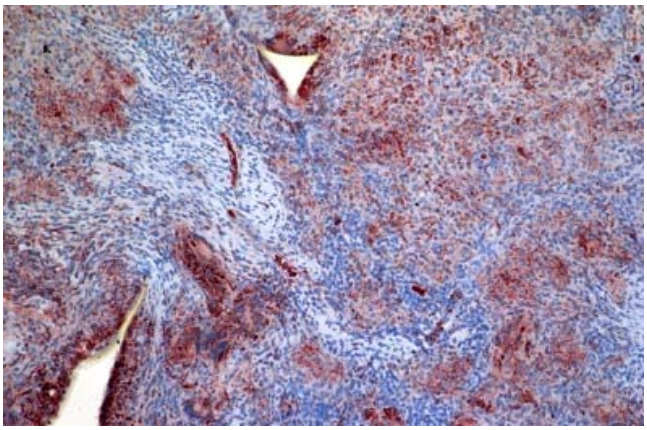


**Figure 14e.** CD 68 staining of HKTB sponge retrieved at day 35 after pre-operative azithromycin administration (HKTB Pre-operatively Treated Day 35 (Score=4))

CT= connective tissue
S= Sponge Matrix
L=Lymphocyte accumulation
M= CD68 positive cells
G= CD68 positive Giant cell

### 2.4.4 The effect of azithromycin administered at day 25 post operatively on chronic inflammation

The following results section relates to those animals into which PBS, HKTB or HKPG sponges were implanted with no pre-operative administration of azithromycin. Chronic inflammation was allowed to develop and animals in each of the respective groups received either no azithromycin or the drug was administered on three consecutive days to test animals commencing on day 25 after which the animals were sacrificed at day 35 and 49 post surgery.

#### *2.4.4.1 Animal weight monitoring*

The weights of the respective rats have been included and reported in section 2.3.2.

#### *2.4.4.2 Sponge wet and dry weights*

As was found in the acute model, there was no clear pattern or difference between the wet and dry weights of sponges obtained from either animals that were treated with the threeday course of azithromycin compared to untreated animals in the PBS, HKTB and HKPG groups. The respective mean wet and dry weights of the sponges can be seen in Table 5.

**Table 5.** Mean wet and dry weights of sponges retrieved from animals in the PBS, HKTB or HKPG groups that did or did not receive azithromycin at day 25.

Dry Weights of Sponges

| **Day of retrieval** | **PBS** | **HKTB** | **HKPG** |
|---|---|---|---|
| Day 35- Not treated with azithromycin | 0.04g±0.008g | 0.062g±0.01g | 0.034g±0.005g |
| Day 35- Treated at day 25 with azithromycin | 0.031g±0.004g | 0.057g±0.006g | 0.029g±0.005g |
| **Day of retrieval** | **PBS** | **HKTB** | **HKPG** |
| Day 49- Not treated with azithromycin | 0.036g±0.011g | 0.065g±0.011g | 0.03g±0.008g |
| Day 49 -Treated at day 25 with azithromycin | 0.041g±0.008g | 0.049g±0.006g | 0.026g±0.006 |

Wet Weights

| **Day of retrieval** | **PBS** | **HKTB** | **HKPG** |
|---|---|---|---|
| Day 35- Not treated with azithromycin | 0.186g±0.034g | 0.27g±0.035g | 0.18g±0.039g |
| Day 35- Treated at day 25 with azithromycin | 0.165g±0.024g | 0.23g±0.016g | 0.145g±0.019g |
| **Day of retrieval** | **PBS** | **HKTB** | **HKPG** |
| Day 49- Not treated with azithromycin | 0.141g±0.009g | 0.225g±0.038g | 0.16g±0.032g |
| Day 49- Treated at day 25 with azithromycin | 0.15g±0.021g | 0.2g±0.021g | 0.12g±0.012g |

### 2.4.5 Biochemical analysis

*2.4.5.1 C-reactive protein levels*

When examining CRP levels, there was no significant difference between animals that received azithromycin at day 25 compared to untreated animals for any of the groups at day 35 or 49 (p=0.443). Figures 15a, b and c illustrate CRP levels for animals treated with azithromycin 25 days after sponge implantation compared to animals that did not receive azithromycin for each of the respective groups.

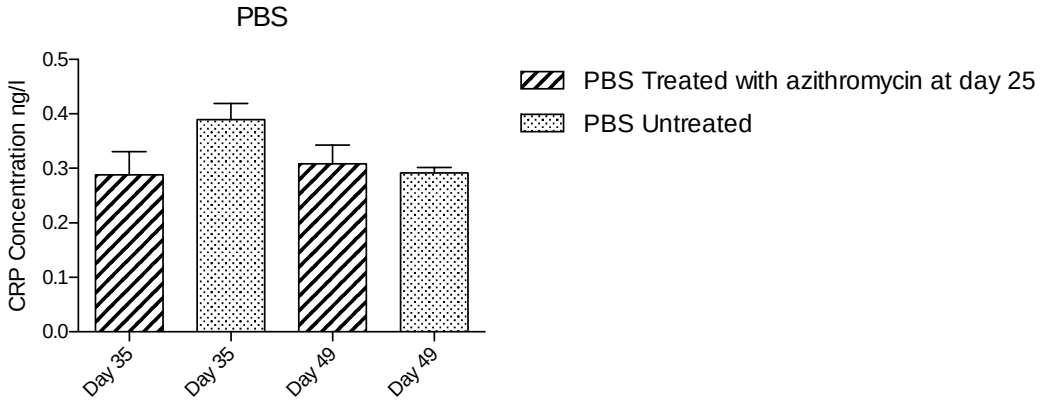



**Figure 15a.** CRP levels obtained from rats with PBS sponge implants sacrificed at days 35 and 49 after sponge implantation for animals treated with azithromycin at day 25 compared to animals that did not receive the drug

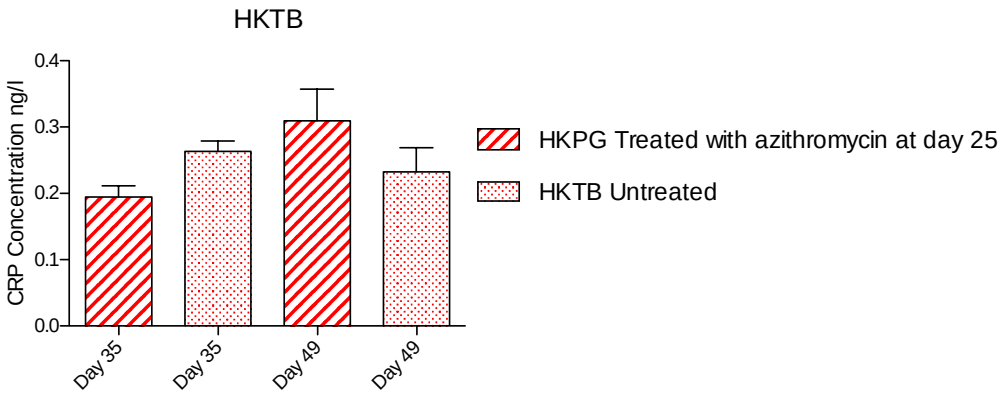



**Figure 15b.** CRP levels obtained from rats with HKTB sponge implants sacrificed at days 35 and 49 after sponge implantation for animals treated with azithromycin at day 25 compared to animals that did not receive the drug

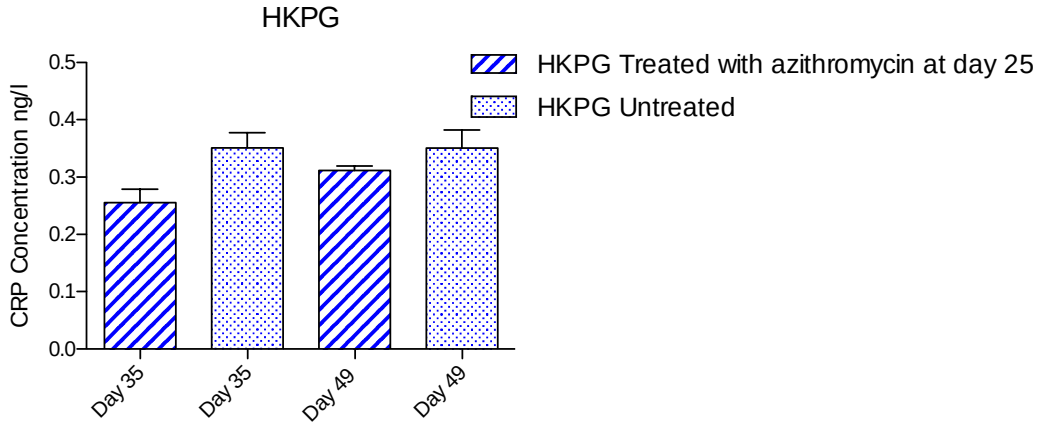



**Figure 15c.** CRP levels obtained from rats with HKPG sponge implants sacrificed at days 35 and 49 after sponge implantation for animals treated with azithromycin at day 25 compared to animals that did not receive the drug

*2.4.5.2 Hydroxyproline*

The level of HP is shown in Figures 16a, b and c for the groups of animals that received azithromycin at 25 days after sponge implantation, compared to those that did not at the day 35 and day 49 time points. HP values were significantly lower for animals in the HKPG ($p=0.025$) (Figure 16c) and HKTB ($p=0.012$) (Figure 16b) groups when they had received azithromycin at day 25 compared to the animals in their respective groups that did not. Examining sponges from animals with PBS implants, it was found that there was no significant difference in HP levels from the animals that had received the drug compared to those that had not (Figure 16a). Looking at the HP levels at day 49, this significant difference between treated and untreated animals for the HKPG ($p=0.315$) and HKTB ($p=0.22$) groups were lost.

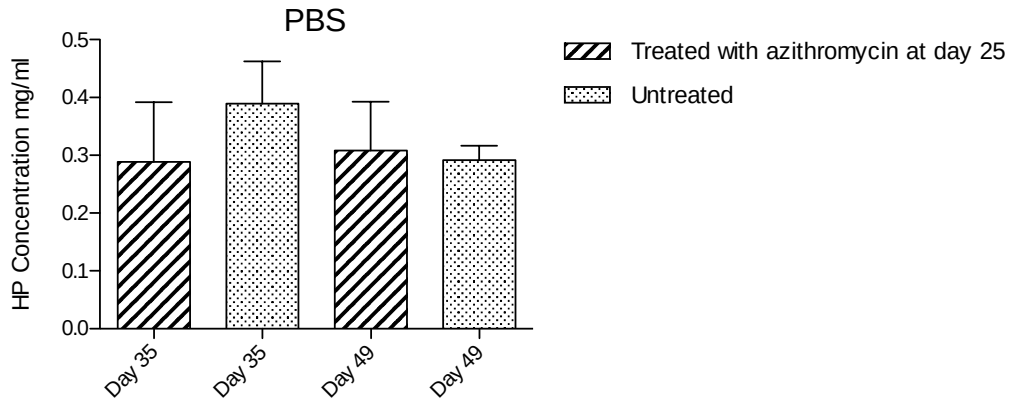



**Figure 16a.** Showing HP levels between animals with PBS sponge implants treated with azithromycin at day 25 compared to their respective untreated controls, retrieved at the day 35 and 49 time points

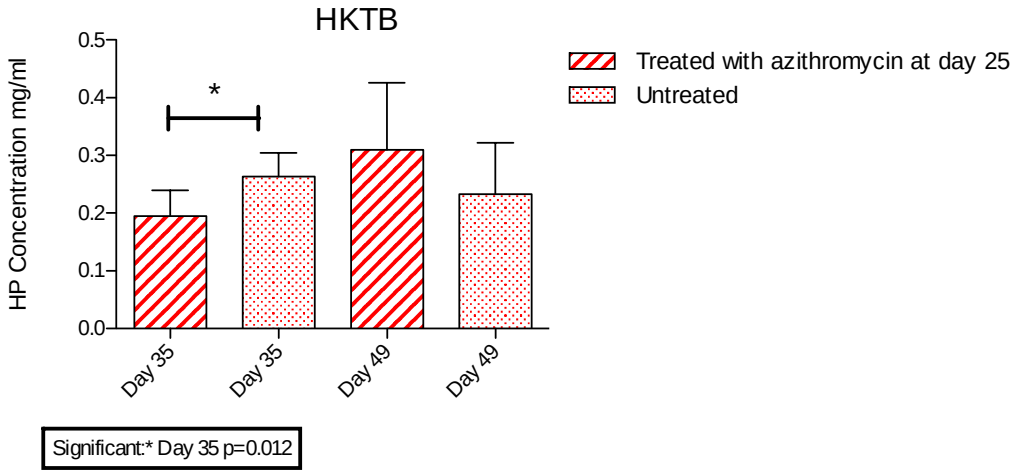



**Figure 16b.** Showing HP levels between animals with HKTB sponge implants treated with azithromycin at day 25 compared to their respective untreated controls, retrieved at the day 35 and 49 time points

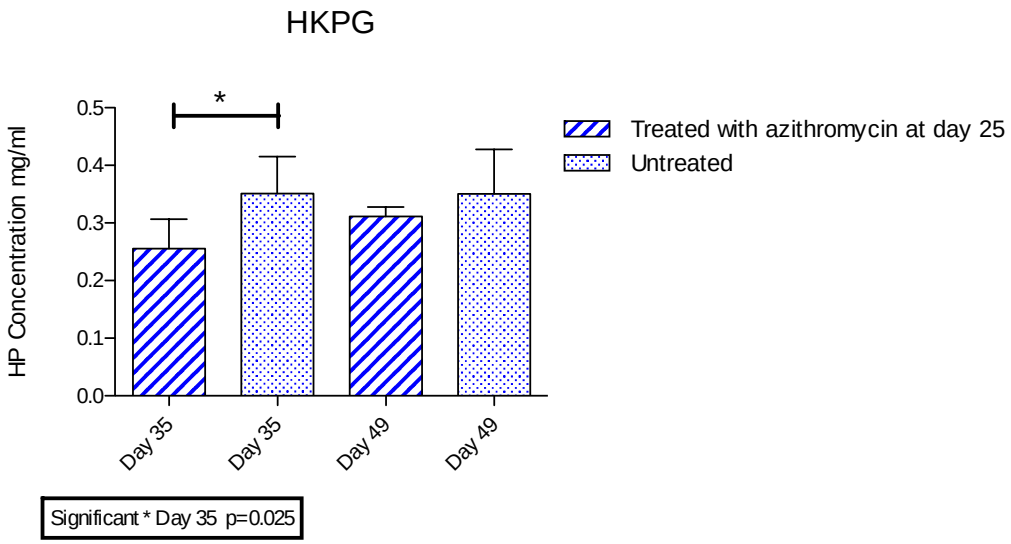



**Figure 16c.** Showing HP levels between animals with HKPG sponge implants treated with azithromycin at day 25 compared to their respective untreated controls, retrieved at the day 35 and 49 time points

### 2.4.6 Histological analysis

*2.4.6.1 Qualitative assessment*

The qualitative analysis of the respective sponges, PBS, HKTB and HKPG, for animals either treated with azithromycin at day 25 or untreated animals showed little difference. Images for sections stained with H&E can be seen in Figure 17. By day 35 approximately 90 to 100% of the sponges were infiltrated with fibrous tissue and inflammatory infiltrate, consisting mainly of lymphocytes. It was noted that sponges with HKTB were substantially more infiltrated with lymphocytes when compared to the PBS and HKPG groups.

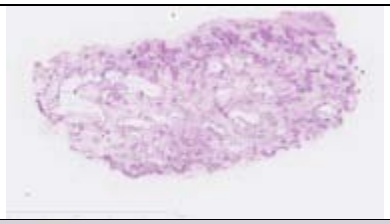
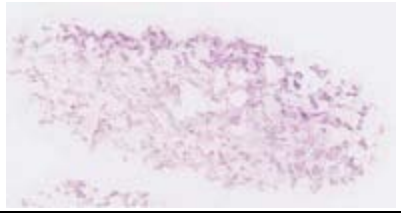
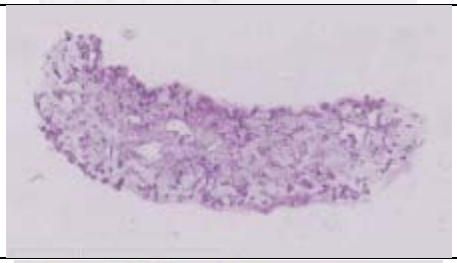
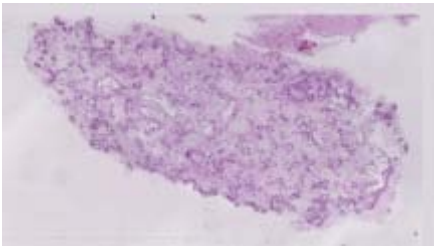
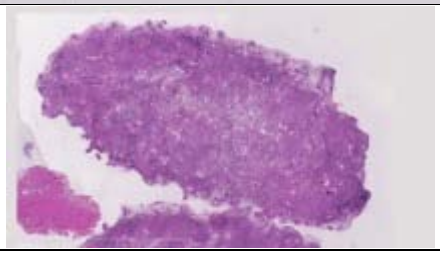
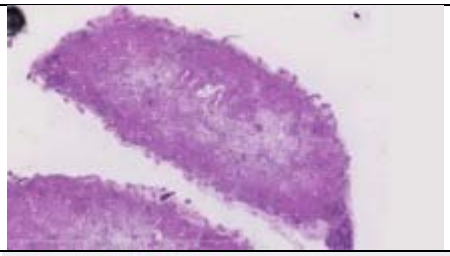
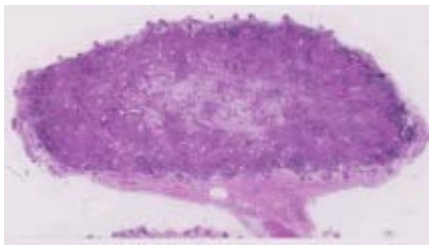
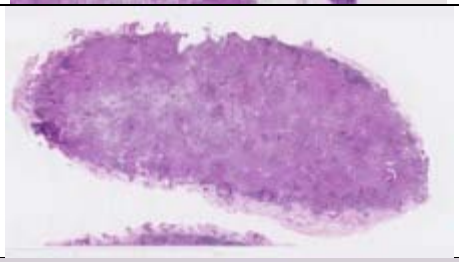
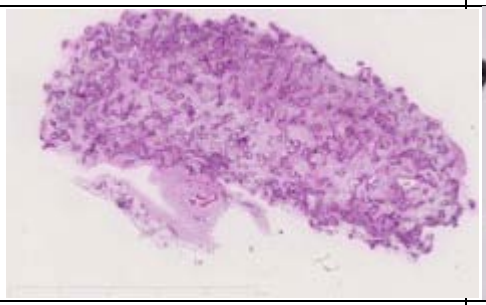
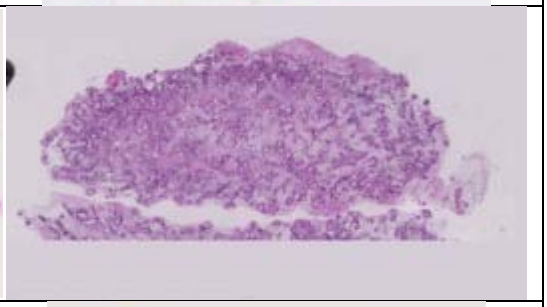
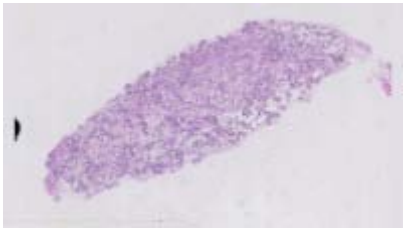
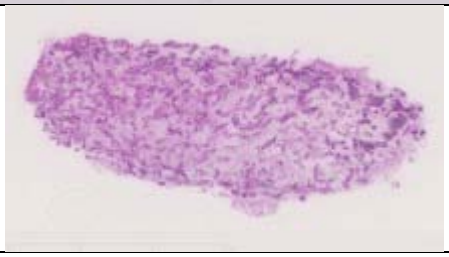
| Group and day of retrieval | Treated at day 25 post op | Untreated |
|---|---|---|
| PBS Day 35 |  |  |
| PBS Day 49 |  |  |
| HKTB Day 35 |  |  |
| HKTB Day 49 |  |  |
| HKPG Day 35 |  |  |
| HKPG Day 49 |  |  |

**Figure 17.** Representative histological sections for each time point in the PBS, HKTB and HKPG groups comparing animals that received azithromycin at 25 days post sponge implantation compared to untreated animals

*2.4.6.2 Combined inflammation and infiltration score*

Similarly to the findings in the sponges retrieved from animals in the acute groups, it was found that at day 35, there was no significant difference between sponges from animals that were treated with azithromycin at day 25 compared to untreated animals in the PBS (p=0.88), the HKTB (p=1.00) and the HKPG (p=0.55) groups. The same pattern was observed for sponges retrieved at day 49 for PBS (p=0.82), the HKTB (p=0.54) and the HKPG (p=0.71) groups (Figures 18a, b and c)

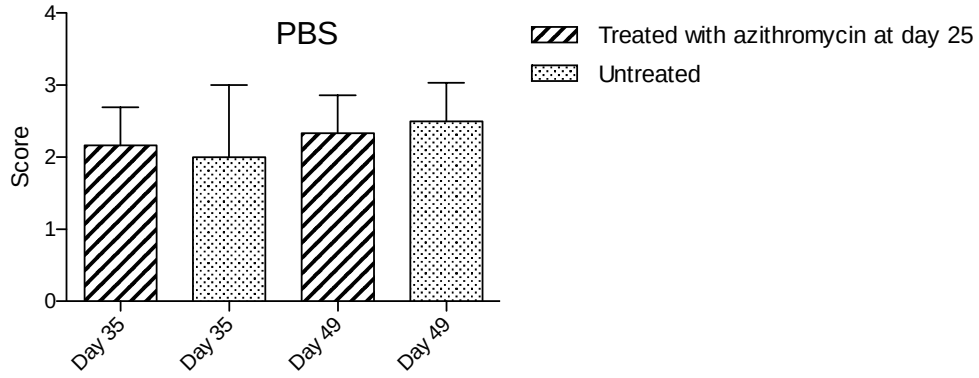



**Figure 18a.** Combined Inflammation and infiltration score for animals with PBS sponge implants treated at day 25 with azithromycin compared to untreated animals

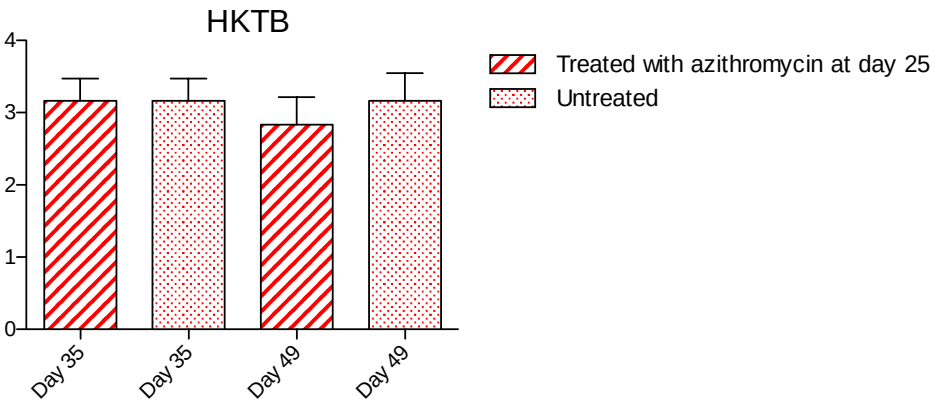



**Figure 18b.** Combined Inflammation and infiltration score for animals with HKTB sponge implants treated at day 25 with azithromycin compared to untreated animals

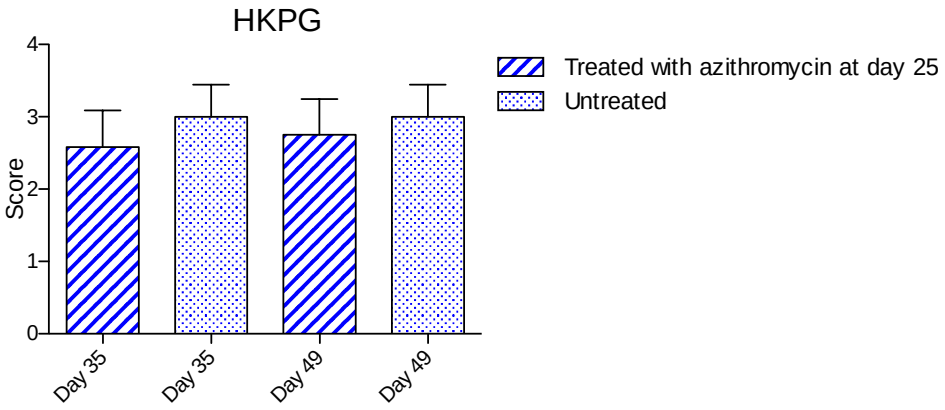



**Figure 18c.** Combined Inflammation and infiltration scores for animals with HKPG sponge implants treated at day 25 with azithromycin compared to untreated animals

*2.4.6.3 Lymphocyte counts*

When looking at lymphocyte counts for sponges retrieved from the animals, it was found that at day 35, there was no significant difference between sponges from animals that were treated with azithromycin at day 25 compared to untreated animals in the PBS (p=0.48), the HKTB (p=0.48) and the HKPG (p=0.24) groups. The same pattern was observed for sponges retrieved at day 49 for PBS (p=0.66), the HKTB (p=0.81) and the HKPG (p=0.35) groups (Figures 19a, b and c).

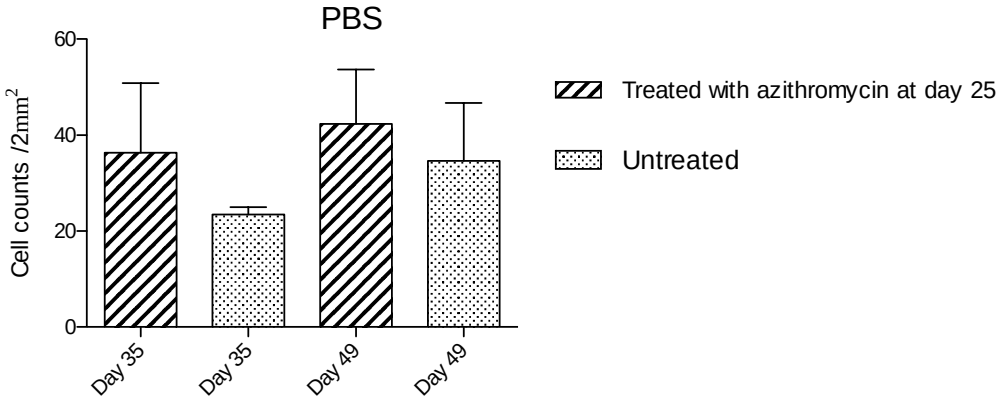



**Figure 19a.** Lymphocyte counts for animals with PBS sponge implants treated at day 25 with azithromycin compared to untreated animals

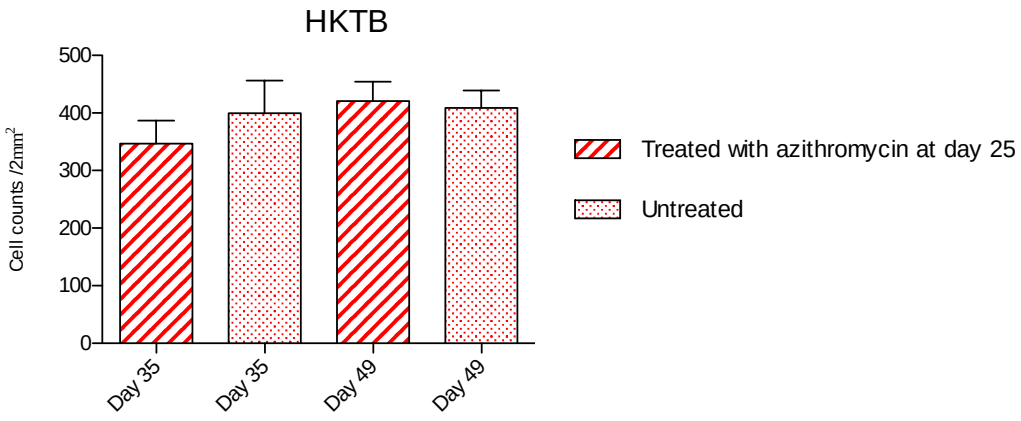



**Figure 19b.** Lymphocyte counts for animals with HKTB sponge implants treated at day 25 with azithromycin compared to untreated animals

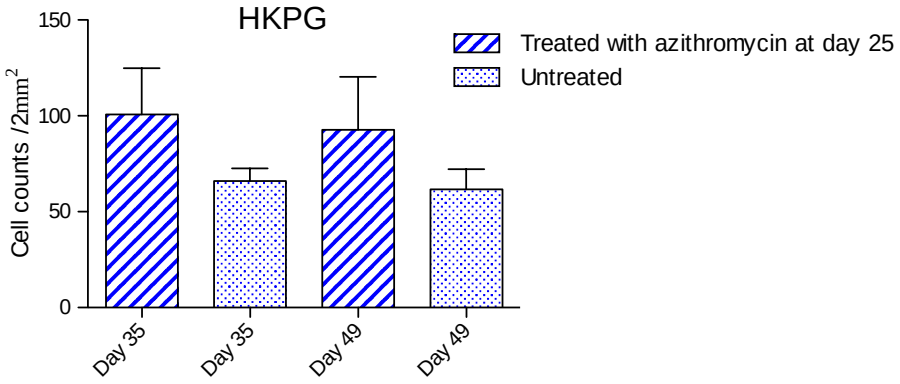



**Figure 19c.** Lymphocyte counts for animals with HKPG sponge implants treated at day 25 with azithromycin compared to untreated animals

## 2.5 Discussion

The results of this study confirmed the hypothesis that azithromycin has the ability to modulate acute inflammation in a subcutaneous rat model, although this could not be confirmed for chronic inflammation.

### 2.5.1 Inflammatory cell response

The histological data and hydroxyproline levels found in this study for all groups were consistent with previous reports in the literature. Histologically, it was observed that the infiltration pattern of the sponges commenced at the periphery and progressed towards the centre of the sponge. Similarly, the pattern of quantitative cell infiltrate of neutrophils and lymphocytes was consistent with what has already been described in other studies (Anselme et al., 1990). This pattern was initially made up of high neutrophil numbers, which were

significantly reduced by week 3, a feature commonly observed in acute inflammation. Lymphocyte appearance and numbers also increased from days 21 to 49, consistent with findings reported by other researchers (Walter and Chiaramonte, 1965, Bartold et al., 2010).

The ability of azithromycin to significantly reduce neutrophil numbers in acute inflammation, as observed in the HKTB sponges, is consistent with previous literature that has demonstrated similar effects (Culic et al., 2002, Bosnar et al., 2009, Legssyer et al., 2006).

In our study the reduction of acute inflammation by azithromycin as demonstrated by the reduction in neutrophil counts may have been mediated through various mechanisms. Azithromycin has been demonstrated to decrease the production of key inflammatory cytokines such as IL-6 (Ohara et al., 2002, Hanada et al., 2003, Feola et al., 2010, Culic et al., 2002, Bosnar et al., 2009, Beigelman et al., 2009), IL-1 (Tsai et al., 2009, Reato et al., 2004, Ohara et al., 2002, Ho et al., 2010, Bosnar et al., 2009) TNF-α (Tsai et al., 2004, Tsai et al., 2009, Reato et al., 2004, Li et al., 2010, Feola et al., 2010, Cigana et al., 2007) and IL-12 (Tsai et al., 2004).

This effect could also be attributed to the suppression of NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells), which is thought to be a key immune modulatory mechanism of azithromycin (Cigana 2006, Cigana 2007). NFkB is a protein complex involved in the cellular response to a variety of stimuli such as stress, cytokines, free radicals and bacterial and viral antigens; and plays a key role in regulating the immune response to infection by controlling DNA transcription. Several studies have demonstrated that azithromycin has the potential to inhibit NFkB in a variety of cells, ranging from vascular smooth muscle (Miller et al., 2000), to epithelial cells (Cigana et al., 2007, Cigana et al., 2006), macrophages (Yamauchi et al., 2009, Iwamoto et al., 2011) and human corneal epithelial cells (Li et al., 2010); although this has not consistently been reported in the literature (Blau et al., 2007, Morinaga et al., 2009).

More recent work has evaluated the effect of azithromycin on the activity and expression of the toll like receptors (TLR), especially TLR-2 and TLR-4, which are associated with the activation of NFkB and play a key role in innate immunity and neutrophil activation. Consequently, it was found that azithromycin has the ability to reduce both the expression of TLR-2 (Karlstrom et al., 2011) as well as TLR-4 (Maezono et al., 2011), with both being associated with reductions in the recruitment of neutrophils. Other mechanisms which have been identified are the decreased reaction of neutrophils to chemo attractants (Oda et al., 1994), decrease in adhesion molecules such as of L-selectin and integrin α1 (Mac-1) (Enomoto et al., 2002) and ICAM-1 and E-selectin (Akamatsu et al., 2001), as well as increased apoptosis through the inhibition of GM-SF (Koch et al., 2000).

An unexpected finding was that azithromycin was found to induce a more marked reduction (three fold) in neutrophil numbers in the HKTB group compared to the HKPG group. This finding is interesting, as the HKTB sponges were the most noxious stimulus in the current experiment and were included as positive control. In contrast to this, no change in neutrophil numbers was noted in animals in the PBS groups. Although it is unclear why this pattern was observed in the heat killed organism loaded sponges, a possible explanation could be the tendency of azithromycin to accumulate intracellularly, where it has been found at concentrations 2000 times higher than plasma concentrations (Wilms et al., 2006). It would be plausible to suggest that in areas of severe inflammation, higher concentrations of azithromycin may exist due to the active transport of azithromycin in inflammatory cells and local release (Mandell and Coleman, 2001), which may confer an increased local anti-inflammatory effect. Alternatively, especially for the HKPG group, it may be that since a trend for a reduction in neutrophils was detected, it may have been possible to find a significant difference if a higher number of experimental animals was included.

The finding that azithromycin had no effect on lymphocyte counts in any of the groups in both the acute and chronic models is difficult to interpret, as it was expected azithromycin would reduce lymphocyte numbers.

However, differential effects of azithromycin on lymphocytes have previously been demonstrated. A study of a small group of healthy human volunteers demonstrated that azithromycin had a stimulatory effect on the proliferation of activated lymphocytes (Tomazic et al., 1993). However much variation was identified in the study and results should thus be interpreted with caution. On the other hand, it has been reported that azithromycin accelerated lymphocyte apoptosis (Kadota et al., 2005), which was thought to be due to effects on the Fas-Fas (Ishimatsu et al., 2004) and Bcl-cL and BAX pathways (Mizunoe et al., 2004) in *in vitro* studies. However it has been suggested that these results could not be directly translated to the *in vivo* situation. This was demonstrated by a study showing that although azithromycin had the ability to reduce lymphocyte proliferation *in vitro*, this was not always the case *ex vivo,* when the drug was administered to mice. Although some inhibition in lymphocyte proliferation occurred when azithromycin was administered in low dosage (7 mg/kg and 14 mg/kg), this effect was lost when higher dosages of the drug were administered (28 mg/kg and 56 mg/kg), presumably due to compensatory mechanisms (Ortega et al., 2002). It could thus be that the current lack of effect of azithromycin on lymphocyte cell counts may be due to such compensatory mechanisms, although more research is needed in this area.

### 2.5.2 C-reactive protein

No clear effect on the acute phase reactant CRP was noted with the administration of azithromycin in any of the experimental groups. This result is unexpected when compared to other reported studies where the anti-inflammatory effect of azithromycin is demonstrated (Altenburg et al., 2010). There are two possible explanations for this finding in this study. The levels of CRP show only a moderate increase (1-10 fold) with inflammation in rats (Cray et al., 2009) and the Elisa kit used in this experiment for CRP determination states the CRP levels in rats may increase 2 fold. The maximum increase seen in any of the groups was 1.5 fold. Given this small effect and the low number of animals used it would be difficult to detect small changes between the treated and untreated groups, unless the numbers were increased or another more reactive acute phase reactant was chosen, such as α2-macroglobulin (Cray et al., 2009). Alternatively, it could be suggested that the chosen dosage of azithromycin is insufficient to affect CRP levels in the chosen model of acute and chronic inflammation.

### 2.5.3 Hydroxyproline

Hydroxyproline assaying was done as it forms 12% of collagen and is thus a direct measure of the collagen content of the tissue (Woessner Jr, 1961). The results from this study have shown a reduction in hydroxyproline during chronic inflammation 10 days after the administration of azithromycin in the HKTB and HKPG groups. This is the first time that a reduction in fibrosis during chronic inflammation has been demonstrated. A reduction in fibrosis is supported by the findings of others (Wuyts et al., 2010), who have shown that low dosage of azithromycin (3.5 mg/kg) had the ability to reduce bleomycin induced lung fibrosis in a mouse model. That study differed from the current work in that no actual assay was performed to evaluate collagen or hydroxyproline levels, and fibrosis was evaluated in the acute stages of tissue damage. Furthermore the hydroxyproline levels found in the current study follow a similar pattern commonly reported during wound healing and scar tissue formation (Madden and Peacock, 1971). The differences in the ability of azithromycin to reduce hydroxyproline levels between the different groups in the acute phase could be due to differences in the inflammatory processes invoked by the different irritants with which the sponges were loaded. As such, in the case of the PBS sponges, the comparative cell counts demonstrate mild inflammation and mainly fibrous scar tissue formation compared to the HKPG and HKTB sponges, which could account for the consistent finding of a reduced HP level in animals that had received azithromycin pre-operatively. The initially lower levels of

HP that were found in untreated animals in the HKTB group at days 7 and 14 can be explained by the strong acute inflammatory reaction in the untreated animals, with overwhelming neutrophil infiltration caused by the irritant, which could limit scar tissue formation. Although this effect could be seen at day 14 in this group, it was less pronounced than at day 7. Once the initial phases of inflammation had subsided, it appears as though administration of azithromycin once again reduced fibrosis at day 21 with the trend remaining for the rest of the study. These findings are of significance with regards to gingival overgrowth as it endorses several reports in the last two decades of reductions of drug induced gingival enlargement by azithromycin administration (Wahlstrom et al., 1995). Clinical studies showed that most of the reduction in gingival enlargement occurs within the first month (Tokgoz et al., 2004),with the biggest effect during the first week (Tokgoz et al., 2004, Palomar et al., 1998) and the effect lasting up to 6 months post administration (Gomez et al., 1997) with a success rate of 89% (Citterio et al., 2001). A possible mechanism of action for this, it was found *in vitro* that azithromycin inhibits the proliferation of fibroblasts and elevated the reduced levels of especially MMP-2 (and less MMP-1) as well as blocking the accumulation of collagen associated with gingival overgrowth. This was substantiated by the finding from the same study that cyclosporin-A treated fibroblasts had up regulated collagen I and down regulated MMP-2 mRNA expression, which was blocked by azithromycin (Kim et al., 2008). However *in vivo* experiments found that there was no change in the mRNA expression of collagen type I after azithromycin administration to rats with gingival enlargement, although a reduction in overgrowth was noted which was associated with increased phagocytic activity of fibroblasts (Paik et al., 2004). A more recent study (Condé et al., 2009) showed that the release of TGF-β, which has been associated with gingival overgrowth (Vieira Jr et al., 1999), was reduced in rats after roxithromycin administration, in conjunction with a reduction in fibroblast numbers as well as a reduction in inflammatory infiltrate (Condé et al., 2009).

When the wet and dry weights of the retrieved sponges in the different groups were analysed, no differences were noted between the groups. This finding corresponds to the limited effect that was detected for both collagen content and inflammatory infiltrate between treated and untreated animals in the treated groups. Earlier work supports this as it was found that reductions in both wet and dry weight of implanted sponges and cotton pellets related to the amount of granulation tissue formation and inflammation (Robinson and Robson, 1964). Interestingly, it has also been reported by others that azithromycin has limited effect on exudate volume in induced inflammation (Ianaro et al., 2000).

### 2.5.4 Immunohistochemistry

To date, very limited data are available on how azithromycin affects macrophage numbers. From the current literature, it appears that the drug has the ability to alter macrophage phenotype, both *in vitro* (Murphy et al., 2008) and *in vivo* (Feola et al., 2010) although the exact mechanisms are not known. However the results from our study did not support these findings. Although azithromycin did have a limited impact on macrophage numbers, it did not change the expression of specific cell surface receptors (CD68 and CD163). As a consequence, no significant increase in the ratio of CD163 (Scavenger Phenotype maker) was noted compared to CD68 (Pan Macrophage marker) (Brown et al., 2009). A possible explanation for this could be due to differences between *in vitro* and *in vivo* experiments. Furthermore, in the study conducted by Feola et al. (2010), azithromycin was administered to mice at a concentration of 160 mg/kg for four days, as opposed to the 20 mg/kg daily for three days (recommended clinical dosage for the drug) used in this experiment. The reason this dosage was chosen in the current experiment, as opposed to the dosage used in the experiment by Feola et al. (2010) is that it represents the suggested clinical dosage for the drug.

## 2.6 Limitations of study

It should be mentioned that there are several limitations in the current study. The inclusion of several different groups (PBS, HKPG and HKTB) for both acute and chronic models resulted in a small number of rats (n=3) in each group. It could very well be that with a higher number of animals more significant differences could have been detected. Furthermore, HPLC serum or tissue levels of azithromycin would have added to the validity of the results and would possibly have enabled us to correlate inflammatory changes to drug tissue concentrations, but due to budget and time constraints this was not possible.

## 2.7 Significance of study

The findings from the current study are valuable in regards to periodontitis, as it was clearly demonstrated that azithromycin decreased neutrophil numbers, which seems to be especially true for situations with more pronounced inflammation. Neutrophils in general have been associated with periodontitis and it is thought that they are responsible for a large part of tissue destruction that occurs in periodontitis. Aggressive periodontitis in particular has been linked to a “hyper responsive” phenotype (Kantarci et al., 2003), resulting in increased tissue destruction. It may be that azithromycin is a valuable drug for these patients.

It should also be remembered that the current study only evaluated the effect of azithromycin on inflammation induced by heat killed bacteria. However, it should not be forgotten that azithromycin is primarily an antibiotic and as such the combination of its antimicrobial and immune modulatory properties may especially applicable to periodontitis, as both bacteria and the host response play an important part in its pathogenesis.

## 2.8 Conclusion

From the results obtained in this study, it can be concluded that azithromycin has the potential to suppress neutrophil infiltration and thus modulate acute inflammation in a subcutaneous rat model for inflammation. Furthermore, the drug has limited effect on macrophage infiltration but no significant impact on CRP levels. As assessed by hydroxyproline levels, azithromycin reduced the collagen content in sponges during chronic inflammation, a novel finding that has not been previously demonstrated. Although the effects on acute inflammation were clear, once chronic inflammation had ensued, the anti-inflammatory effect of azithromycin was less conclusive with a tendency for diminished inflammation observed.

## 2.9 References


Akamatsu, H., Yamawaki, M. & Horio, T. (2001) Effects of roxithromycin on adhesion molecules expressed on endothelial cells of the dermal microvasculature. *Journal of Internal Medicine Research* **29,** 523-527.

Altenburg, J., de Graaff, C. S., van der Werf, T. S. & Boersma, W. G. (2010) Immunomodulatory effects of macrolide antibiotics - Part 1: Biological mechanisms. *Respiration*.

Anselme, K., Bacques, C., Charriere, G., Hartmann, D. J., Herbage, D. & Garrone, R. (1990) Tissue reaction to subcutaneous implantation of a collagen sponge. A histological, ultrastructural, and immunological study. *Journal of Biomedical Materials Research* **24,** 689-703.

Bartold, P., Marino, V., Cantley, M. & Haynes, D. R. (2010a) Effect of Porphyromonas gingivalis-induced inflammation on the development of rheumatoid arthritis. *Journal of Clinical Periodontology* **37,** 405- 411.

Bartold, P. M., Cantley, M. D. & Haynes, D. R. (2010b) Mechanisms and control of pathologic bone loss in periodontitis. *Periodontology 2000* **53,** 55-69.

Beigelman, A., Gunsten, S., Mikols, C. L., Vidavsky, I., Cannon, C. L., Brody, S. L. & Walter, M. J. (2009) Azithromycin attenuates airway inflammation in a noninfectious mouse model of allergic asthma. *Chest* **136,** 498-506.

Birkedal-Hansen, H. (1993) Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research* **28,** 500-510.

Borrell, L. N., Burt, B. & Taylor, G. W. (2005) Prevalence and trends in periodontitis in the USA: from NHANES III to the NHANES, 1988 to 2000. *Journal of Dental Research* **84,** 924-930.

Bosnar, M., Bosnjak, B., Cuzic, S., Hrvacic, B., Marjanovic, N., Glojnaric, I., Culic, O., Parnham, M. J. & Erakovic Haber, V. (2009) Azithromycin and clarithromycin inhibit lipopolysaccharide-induced murine pulmonary neutrophilia mainly through effects on macrophage-derived granulocyte-macrophage colony-stimulating factor and interleukin-1beta. *Journal of Pharmacological Experimental Therapy* **331,** 104-113.

Brown, B. N., Valentin, J. E., Stewart-Akers, A. M., McCabe, G. P. & Badylak, S. F. (2009) Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials* **30,** 1482-1491.

Buckley, C. D., Pilling, D., Lord, J. M., Akbar, A. N., Scheel-Toellner, D. & Salmon, M. (2001) Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends in Immunology* **22,** 199-204.

Burt, B. (2005) Position paper: epidemiology of periodontal diseases. *Journal of Periodontology* **76,** 1406-1419.

Cheung, P. S., Si, E. C. & Hosseini, K. (2010) Anti-inflammatory activity of azithromycin as measured by its NF-KB inhibitory activity. *Ocular Immunology and Inflammation* **18,** 32-37.

Cigana, C., Assael, B. M. & Melotti, P. (2007) Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells. *Antimicrobial Agents and Chemotherapy* **51,** 975-981.

Cigana, C., Nicolis, E., Pasetto, M., Assael, B. M. & Melotti, P. (2006) Anti-inflammatory effects of azithromycin in cystic fibrosis airway epithelial cells. *Biochemical Biophysiology Research Communications* **350,** 977-982.

Citterio, F., Di Pinto, A., Borzi, M. T., Scata, M. C., Foco, M., Pozzetto, U. & Castagneto, M. (2001) Azithromycin treatment of gingival hyperplasia in kidney transplant recipients is effective and safe. *Transplantations Proceedings* **33,** 2134-2135.

Condé, S. A. P., Bastos, M. G., Vieira, B. J. & Aarestrup, F. M. (2009) Down-regulation of transforming growth factor beta-2 expression is associated with the reduction of

cyclosporin induced gingival overgrowth in rats treated with roxithromycin: An experimental study. *BMC Oral Health* **9**.

Cray, C., Zaias, J. & Altman, N. H. (2009) Acute phase response in animals: a review. *Compendium of Medicine* **59,** 517-526.

Culic, O., Erakovic, V., Cepelak, I., Barisic, K., Brajsa, K., Ferencic, Z., Galovic, R., Glojnaric, I., Manojlovic, Z., Munic, V., Novak-Mircetic, R., Pavicic-Beljak, V., Sucic, M., Veljaca, M., Zanic-Grubisic, T. & Parnham, M. J. (2002) Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects. *European Journal of Pharmacology* **450,** 277-289.

Culic, O., Erakovic, V. & Parnham, M. J. (2001) Anti-inflammatory effects of macrolide antibiotics. *European Journal of Pharmacology* **429,** 209-229.

Cutress, T., Powell, R. & Ball, M. (1982) Differing profiles of periodontal disease in two similar South Pacific island populations. *Community Dentistry and Oral Epidemiology* **10,** 193-203.

Dongari-Bagtzoglou, A. (2004) Drug-associated gingival enlargement. *Journal of Periodontology* **75,** 1424-1431.

Enomoto, F., Andou, I., Nagaoka, I. & Ichikawa, G. (2002) Effect of new macrolides on the expression of adhesion molecules on neutrophils in chronic sinusitis. *Auris Nasus Larynx* **29,** 267-269.

Feola, D. J., Garvy, B. A., Cory, T. J., Birket, S. E., Hoy, H., Hayes, D., Jr. & Murphy, B. S. (2010) Azithromycin alters macrophage phenotype and pulmonary compartmentalization during lung infection with Pseudomonas. *Antimicrobial Agents and Chemother* **54,** 2437-2447.

Fujii, T., Kadota, J., Kawakami, K., Iida, K., Shirai, R., Kaseda, M., Kawamoto, S. & Kohno, S. (1995) Long term effect of erythromycin therapy in patients with chronic Pseudomonas aeruginosa infection. *Thorax* **50,** 1246-1252.

Gomez, E., Sanchez-Nunez, M., Sanchez, J. E., Corte, C., Aguado, S., Portal, C., Baltar, J. & Alvarez-Grande, J. (1997) Treatment of cyclosporin-induced gingival hyperplasia with azithromycin. *Nephrology Dialysis and Transplantation* **12,** 2694-2697.

Haffajee, A. D., Torresyap, G. & Socransky, S. S. (2007) Clinical changes following four different periodontal therapies for the treatment of chronic periodontitis: 1-Year results. *Journal of Clinical Periodontology* **34,** 243-253.

Hanada, H., Ikeda-Dantsuji, Y., Naito, M. & Nagayama, A. (2003) Infection of human fibroblast-like synovial cells with Chlamydia trachomatis results in persistent infection and interleukin-6 production. *Microbiology and Pathology* **34,** 57-63. Ho, W., Eubank, T., Leblebicioglu, B., Marsh, C. & Walters, J. (2010) Azithromycin decreases crevicular fluid volume and mediator content. *Journal of Dental Research* **89,** 831-835.

Ianaro, A., Ialenti, A., Maffia, P., Sautebin, L., Rombola, L., Carnuccio, R., Iuvone, T., D'Acquisto, F. & Di Rosa, M. (2000) Anti-inflammatory activity of macrolide antibiotics. *Journal of Pharmacology and Experimental Therapy* **292,** 156-163.

Ishimatsu, Y., Kadota, J., Iwashita, T., Nagata, T., Ishii, H., Shikuwa, C., Kaida, H., Mukae, H. & Kohno, S. (2004) Macrolide antibiotics induce apoptosis of human peripheral lymphocytes *in vitro*. *International Journal of Antimicrobial Agents* **24,** 247-253.

Kadota, J., Mizunoe, S., Kishi, K., Tokimatsu, I., Nagai, H. & Nasu, M. (2005) Antibiotic-induced apoptosis in human activated peripheral lymphocytes. *International Journal of Antimicrobial Agents* **25,** 216-220.

Karlstrom, A., Heston, S. M., Boyd, K. L., Tuomanen, E. I. & McCullers, J. A. (2011) Toll-like receptor 2 mediates fatal immunopathology in mice during treatment of secondary pneumococcal pneumonia following influenza. *Journal of Infectious Diseases* **204,** 1358-1366.

Kim, J. Y., Park, S. H., Cho, K. S., Kim, H. J., Lee, C. K., Park, K. K., Choi, S. H. & Chung, W. Y. (2008) Mechanism of azithromycin treatment on gingival overgrowth. *Journal of Dental Research* **87,** 1075-1079.

Kinane, D. F. & Bartold, P. M. (2007) Clinical relevance of the host responses of periodontitis. *Periodontology 2000* **43,** 278-293.

Koch, C. C., Esteban, D. J., Chin, A. C., Olson, M. E., Read, R. R., Ceri, H., Morck, D. W. & Buret, A. G. (2000) Apoptosis, oxidative metabolism and interleukin-8 production in human neutrophils exposed to azithromycin: effects of Streptococcus pneumoniae. *Journal of Antimicrobial Chemotherapy* **46,** 19-26.

Kornman, K. S. (1999) Host modulation as a therapeutic strategy in the treatment of periodontal disease. *Clin Infect Dis* **28,** 520-526.

Kornman, K. S. (2008) Mapping the pathogenesis of periodontitis: A new look. *Journal of Periodontology* **79,** 1560-1568.

Legssyer, R., Huaux, F., Lebacq, J., Delos, M., Marbaix, E., Lebecque, P., Lison, D., Scholte, B. J., Wallemacq, P. & Leal, T. (2006) Azithromycin reduces spontaneous and induced inflammation in DeltaF508 cystic fibrosis mice. *Respiratory Research* **7,** 134.

Leibbrandt, A. & Penninger, J. M. (2008) RANK/RANKL: regulators of immune responses and bone physiology. *Annals of the New York Academy of Science* **1143,** 123-150.

Li, D. Q., Zhou, N., Zhang, L., Ma, P. & Pflugfelder, S. C. (2010) Suppressive effects of azithromycin on zymosan-induced production of proinflammatory mediators by human corneal epithelial cells. *Investigative Ophthalmology and Visual Science* **51,** 5623-5629.

Löe, H., Anerud, A., Boysen, H. & Morrison, E. (1986) Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *Journal of Clinical Periodontology* **13,** 431-445.

Loe H, T. E., Jensen SB (1965) Experimental Gingivitis in Man. *Journal of Periodontology* **36,** 177-187.

Madden, J. W. & Peacock, E. E., Jr. (1971) Studies on the biology of collagen during wound healing. 3. Dynamic metabolism of scar collagen and remodeling of dermal wounds. *Annals of Surgery* **174,** 511-520.

Maezono, H., Noiri, Y., Asahi, Y., Yamaguchi, M., Yamamoto, R., Izutani, N., Azakami, H. & Ebisu, S. (2011) Antibiofilm effects of azithromycin and erythromycin on Porphyromonas gingivalis. *Antimicrobial Agents and Chemotherapy* **55,** 5887-5892.

Mandell, G. L. & Coleman, E. (2001) Uptake, transport, and delivery of antimicrobial agents by human polymorphonuclear neutrophils. *Antimicrobial Agents and Chemotherapy* **45,** 1794-1798.

Marsh, P. D. (2003) Are dental diseases examples of ecological catastrophes? *Microbiology* **149,** 279-294.

Marsh, P. D., Moter, A. & Devine, D. A. (2011) Dental plaque biofilms: communities, conflict and control. *Periodontology 2000* **55,** 16-35.

Mascarenhas, P., Gapski, R., Al-Shammari, K., Hill, R., Soehren, S., Fenno, J. C., Giannobile, W. V. & Wang, H. L. (2005) Clinical response of azithromycin as an adjunct to non-surgical periodontal therapy in smokers. *Journal of Periodontology* **76,** 426-436.

Matsumura, Y., Mitani, A., Suga, T., Kamiya, Y., Kikuchi, T., Tanaka, S., Aino, M. & Noguchi, T. (2011) Azithromycin may inhibit interleukin-8 through the suppression of Rac1 and a Nuclear Factor KappaB Pathway in KB cells stimulated with lipopolysaccharide. *Journal of Periodontology*.

Miller, S. A., Selzman, C. H., Shames, B. D., Barton, H. A., Johnson, S. M. & Harken, A. H. (2000) Chlamydia pneumoniae activates nuclear factor kappaB and activator protein 1 in human vascular smooth muscle and induces cellular proliferation. *Journal of Surgical Research* **90,** 76-81.

Mizunoe, S., Kadota, J., Tokimatsu, I., Kishi, K., Nagai, H. & Nasu, M. (2004) Clarithromycin and azithromycin induce apoptosis of activated lymphocytes via down-regulation of Bcl-xL. *International Immunopharmacology* **4,** 1201-1207.

Murphy, B. S., Sundareshan, V., Cory, T. J., Hayes, D., Jr., Anstead, M. I. & Feola, D. J. (2008) Azithromycin alters macrophage phenotype. *Journal of Antimicrobial Chemotherapy* **61,** 554-560.

Navarro-Xavier, R. A., Newson, J., Silveira, V. L., Farrow, S. N., Gilroy, D. W. & Bystrom, J. (2010) A new strategy for the identification of novel molecules with targeted proresolution of inflammation properties. *Journal of Immunology* **184,** 1516-1525.

Oda, H., Kadota, J., Kohno, S. & Hara, K. (1994) Erythromycin inhibits neutrophil chemotaxis in bronchoalveoli of diffuse panbronchiolitis. *Chest* **106,** 1116-1123.

Ohara, T., Kojio, S., Taneike, I., Nakagawa, S., Gondaira, F., Tamura, Y., Gejyo, F., Zhang, H. M. & Yamamoto, T. (2002) Effects of azithromycin on shiga toxin production by Escherichia coli and subsequent host inflammatory response. *Antimicrobial Agents and Chemother* **46,** 3478-3483.

Ortega, E., Escobar, M. A. & de Cienfuegos, G. A. (2002) *In vitro* and *ex vivo* effects of erythromycin A, azithromycin and josamycin on the splenic response to specific antigens and mitogens. *Journal of Antimicrobial Chemotherapy* **49,** 1031-1034.

Oteo, A., Herrera, D., Figuero, E., O'Connor, A., Gonzalez, I. & Sanz, M. (2010) Azithromycin as an adjunct to scaling and root planing in the treatment of Porphyromonas gingivalis-associated periodontitis: a pilot study. *Journal of Clinical Periodontology* **37,** 1005-1015.

Page, R. C., Offenbacher, S., Schroeder, H. E., Seymour, G. J. & Kornman, K. S. (1997) Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontology 2000* **14,** 216-248.

Page, R. C. & Schroeder, H. E. (1976) Pathogenesis of inflammatory periodontal disease: a summary of current work. *Laboratory Investigation* **34,** 235-249.

Paik, J. W., Kim, C. S., Cho, K. S., Chai, J. K., Kim, C. K. & Choi, S. H. (2004) Inhibition of cyclosporin A-induced gingival overgrowth by azithromycin through phagocytosis: an *in vivo* and *in vitro* study. *Journal of Periodontology* **75,** 380-387.

Palomar, R., Belart, M., Soy, D., Oppenheimer, F. & Campistol, J. M. (1998) Effectiveness and safety of azithromycin on the treatment of cyclosporine-induced gingival overgrowth. *Nephron* **79,** 101-102.

Reato, G., Cuffini, A. M., Tullio, V., Mandras, N., Roana, J., Banche, G., Foa, R. & Carlone, N. A. (2004) Immunomodulating effect of antimicrobial agents on cytokine production by human polymorphonuclear neutrophils. *International Journal of Antimicrobial Agents* **23,** 150-154.

Reddy, G. K. & Enwemeka, C. S. (1996) A simplified method for the analysis of hydroxyproline in biological tissues. *Clin Biochem* **29,** 225-229.

Reddy J, Africa CW & JR, P. (1986) Darkfield microscopy of subgingival plaque of an urban black population with poor oral hygiene. *Journal of Clinical Periodontology* **13,** 578-582.

Robinson, B. V. & Robson, J. M. (1964) Production of an anti-inflammatory substance at a site of inflammation. *British Journal of Pharmacology and Chemotherapy* **23,** 420-432.

Rodvold, K. A., Gotfried, M. H., Danziger, L. H. & Servi, R. J. (1997) Intrapulmonary steady-state concentrations of clarithromycin and azithromycin in healthy adult volunteers. *Antimicrobial Agents and Chemother* **41,** 1399-1402.

Ryder, M. I. (2010) Comparison of neutrophil functions in aggressive and chronic periodontitis. *Periodontology 2000* **53,** 124-137.

Sampaio, E., Rocha, M., Figueiredo, L. C., Faveri, M., Duarte, P. M., Gomes Lira, E. A. & Feres, M. (2011) Clinical and microbiological effects of azithromycin in the treatment of generalized chronic periodontitis: a randomized placebo-controlled clinical trial. *Journal of Clinical Periodontology*.

Scaglione, F. & Rossoni, G. (1998) Comparative anti-inflammatory effects of roxithromycin, azithromycin and clarithromycin. *Journal of Antimicrobial Chemotherapy* **41 Suppl B,** 47-50.

Smith, S. R., Foyle, D. M., Daniels, J., Joyston-Bechal, S., Smales, F. C., Sefton, A. & Williams, J. (2002) A double-blind placebo-controlled trial of azithromycin as an

adjunct to non-surgical treatment of periodontitis in adults: clinical results. *Journal of Clinical Periodontology* **29,** 54-61.

Tokgoz, B., Sari, H. I., Yildiz, O., Aslan, S., Sipahioglu, M., Okten, T., Oymak, O. & Utas, C. (2004) Effects of azithromycin on cyclosporine-induced gingival hyperplasia in renal transplant patients. *Transplant Proceedings* **36,** 2699-2702.

Tomazic, J., Kotnik, V. & Wraber, B. (1993) *In vivo* administration of azithromycin affects lymphocyte activity *in vitro*. *Antimicrobial Agents and Chemother* **37,** 1786-1789.

Tsai, W. C., Hershenson, M. B., Zhou, Y. & Sajjan, U. (2009) Azithromycin increases survival and reduces lung inflammation in cystic fibrosis mice. *Inflammation Research* **58,** 491-501.

Tsai, W. C., Rodriguez, M. L., Young, K. S., Deng, J. C., Thannickal, V. J., Tateda, K., Hershenson, M. B. & Standiford, T. J. (2004) Azithromycin blocks neutrophil recruitment in Pseudomonas endobronchial infection. *American Journal of Respiration and Critical Care Medicine* **170,** 1331-1339.

Tyteca, D., Schanck, A., Dufrene, Y. F., Deleu, M., Courtoy, P. J., Tulkens, P. M. & Mingeot-Leclercq, M. P. (2003) The macrolide antibiotic azithromycin interacts with lipids and affects membrane organization and fluidity: studies on Langmuir-Blodgett monolayers, liposomes and J774 macrophages. *Journal of Membrane Biology* **192,** 203-215.

Van Dyke, T. E., Lester, M. A. & Shapira, L. (1993) The role of the host response in periodontal disease progression: Implications for future treatment strategies. *Journal of Periodontology* **64,** 792-806.

Vanaudenaerde, B. M., Wuyts, W. A., Geudens, N., Dupont, L. J., Schoofs, K., Smeets, S., Van Raemdonck, D. E. & Verleden, G. M. (2007) Macrolides inhibit IL17-induced IL8 and 8-isoprostane release from human airway smooth muscle cells. *American Journal of Transplantation* **7,** 76-82.

Verma, A. & Dhawan, A. (2005) Re: Trial of metronidazole vs. azithromycin for treatment of cyclosporine-induced gingival overgrowth. *Pediatric Transplantation* **9,** 132.

Vieira Jr, J. M., Noronha, I. L., Malheiros, D. M. A. C. & Burdmann, E. A. (1999) Cyclosporine-induced interstitial fibrosis and arteriolar TGF-β expression with preserved renal blood flow. *Transplantation* **68,** 1746-1753.

Wahlstrom, E., Zamora, J. U. & Teichman, S. (1995) Improvement in cyclosporine-associated gingival hyperplasia with azithromycin therapy. *New England Journal of Medicine* **332,** 753-754.

Walter, J. B. & Chiaramonte, L. G. (1965) The tissue responses of the rat to implanted ivalon, etheron, and polyfoam plastic sponges. *British Journal of Surgery* **52,** 49-54.

Wenisch, C., Parschalk, B., Zedtwitz-Liebenstein, K., Weihs, A., el Menyawi, I. & Graninger, W. (1996) Effect of single oral dose of azithromycin, clarithromycin, and roxithromycin on polymorphonuclear leukocyte function assessed ex vivo by flow cytometry. *Antimicrobial Agents and Chemotherapy* **40,** 2039-2042.

Wilms, E. B., Touw, D. J. & Heijerman, H. G. (2006) Pharmacokinetics of azithromycin in plasma, blood, polymorphonuclear neutrophils and sputum during long-term therapy in patients with cystic fibrosis. *Therapeutic Drug Monitoring* **28,** 219-225.

Wirnsberger, G. H., Pfragner, R., Mauric, A., Zach, R., Bogiatzis, A. & Holzer, H. (1998) Effect of antibiotic treatment with azithromycin on cyclosporine A-induced gingival hyperplasia among renal transplant recipients. *Transplantation Proceedings* **30,** 2117-2119.

Woessner Jr, J. F. (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Archives of Biochemistry and Biophysics* **93,** 440-447.

Wu, L., Lin, J. H., Bao, K., Li, P. F. & Zhang, W. G. (2009) *In vitro* effects of erythromycin on RANKL and nuclear factor-kappa B by human TNF-alpha stimulated Jurkat cells. *International Immunopharmacology* **9,** 1105-1109.

Wuyts, W. A., Willems, S., Vos, R., Vanaudenaerde, B. M., De Vleeschauwer, S. I., Rinaldi, M., Vanhooren, H. M., Geudens, N., Verleden, S. E., Demedts, M. G., Thomeer, M., Verbeken, E. K. & Verleden, G. M. (2010) Azithromycin reduces pulmonary fibrosis in a bleomycin mouse model. *Experiments in Lung Research* **36,** 602-614.

Yamauchi, K., Shibata, Y., Kimura, T., Abe, S., Inoue, S., Osaka, D., Sato, M., Igarashi, A. & Kubota, I. (2009) Azithromycin suppresses interleukin-12p40 expression in lipopolysaccharide and interferon-gamma stimulated macrophages. *International Journal of Biological Science* **5,** 667-678.

Zhu, C., Rao, K., Xiong, H., Gagnidze, K., Li, F., Horvath, C. & Plevy, S. (2003) Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. *Journal of Biology andChemistry* **278,** 39372-39382.

Zuckerman, J. M., Qamar, F. & Bono, B. R. (2009) Macrolides, Ketolides, and Glycylcyclines: Azithromycin, Clarithromycin, Telithromycin, Tigecycline. *Infectious Disease Clinics of North America* **23,** 997-1026.