The Anti-inflammatory Effect of Macrolide Antibiotics in Chronic Rhinosinusitis

by

Ben Wallwork MB.BS (Hons)

School of Biomolecular and Biomedical Science
Faculty of Science
Griffith University
Queensland

A Thesis Submitted in Fulfillment for the Requirements for the
Degree of Doctor of Philosophy

December 2005
ABSTRACT

Chronic rhinosinusitis is a common disorder of chronic inflammation of the upper respiratory tract. It is associated with significant symptoms and impairment of the quality of life of sufferers. Despite recent advances in the medical and surgical management of chronic rhinosinusitis, there remains a population of patients who fail to obtain relief from their symptoms.

Chronic inflammation of the mucosa of the nasal cavity and paranasal sinuses is one of the hallmarks of chronic rhinosinusitis. This inflammation is demonstrated by an increased number of chronic inflammatory cells, elevated levels of pro-inflammatory cytokines, increased expression of adhesion molecules and metaplastic changes in the epithelium. The current medical treatments for chronic sinusitis aim to reduce this inflammation and consequently improve symptoms.

In recent years, evidence has emerged that macrolide antibiotics have an anti-inflammatory effect that is separate from their anti-bacterial effect. This effect was first described in the treatment of diffuse panbronchiolitis, a disorder of chronic inflammation of the lower respiratory tract. Following the success of macrolides in treating this condition it was trialed in chronic rhinosinusitis. Several open-label trials have subsequently demonstrated a beneficial effect.

Laboratory studies have investigated the mechanism of the anti-inflammatory effect of macrolides. These have shown that macrolides effect cytokine production, inflammatory cell apoptosis, expression of adhesion molecules, neutrophil oxidative burst, bacterial virulence and mucociliary function.

In this thesis we report a series of experiments designed to further investigate the mechanism of action and clinical effect of macrolides. In vitro studies using whole sections of chronic rhinosinusitis mucosa cultured for 24 hours in macrolide, prednisolone or control showed that macrolide and prednisolone produced significant
reductions in the production of interleukin-5, interleukin-8 and granulocyte-macrophage colony stimulating factor. The same cultured specimens also showed a reduction in expression of transforming growth factor-β. No reduction was seen in the expression of the key pro-inflammatory nuclear transcription factor Nuclear factor-κB.

In our in vivo experiments, biopsies were taken from chronic rhinosinusitis patients who had received a 3-month course of macrolide. These biopsies showed a reduction in the number of neutrophils present following treatment. There was no reduction in the number of other inflammatory cells or in the expression of TGF-β and NK-κB.

We have performed the first ever double-blinded, randomized, placebo-controlled trial of macrolide in the treatment of chronic rhinosinusitis. Patients receiving macrolide showed significant improvements in saccharine transit time, nasal endoscopic scoring and symptom scores following a 12 week course. Patients with low levels of serum immunoglobulin E showed significantly improved outcomes compared to those with high levels. Interleukin-8 levels in nasal lavage fluid were significantly reduced in the patients with low levels of IgE following macrolide treatment. No improvements in any of the objective or subjective outcome measures were seen in the placebo-treated patients.

We have performed a series of experiments investigating the anti-inflammatory effect of macrolide antibiotics from ‘the bench to the bedside’. These experiments have provided insight into the mechanism of action of macrolides in the laboratory setting and evidence of a beneficial effect in the treatment of chronic rhinosinusitis patients.
STATEMENT OF ORIGINALITY

The work described in this thesis was carried out in the School of Biomolecular and Biomedical Science, in the Faculty of Science at Griffith University. It was performed under the supervision of Professor Alan Mackay-Sim, Professor William Coman and Associate Professor Anders Cervin. This work has not been submitted previously for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Ben Wallwork
PUBLICATIONS ARISING FROM THIS THESIS


ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisors for the encouragement, advice and support that they have provided me with throughout this research project. Professor Alan Mackay-Sim, my principal supervisor, has been an invaluable source of assistance. In particular he has provided me with much needed instruction in scientific technique and has demonstrated a tremendous ability to refine and clarify my ideas and writing. Associate Professor Anders Cervin provided the original concept and inspiration for this research and I thank him for his assistance and support from Sweden. Professor William Coman initially encouraged me to become involved in research and I thank him for his endless enthusiasm and encouragement.

I also would like to thank my friends and colleagues from the School of Biomedical and Biomolecular Science at Griffith University. In particular, Associate Professor Lennart Greiff and Dr Francois Feron always made themselves available to provide me with advice and support.

To my family and friends, I would like to say thank you for your encouragement and assistance and to Katrina, no thank you can repay you for all of your patience, company and kindness.
TABLE OF CONTENTS

1 INTRODUCTION........................................................................................................1

1.1 CLINICAL RATIONALE..........................................................2

1.2 NASAL CAVITY AND PARANASAL SINUS ANATOMY ............3
  1.2.1 Gross Anatomy.................................................................3
  1.2.2 Histology..............................................................................4
  1.2.3 Nasal Mucous and Mucociliary Transport.........................5

1.3 CHRONIC SINUSITIS.................................................................6
  1.3.1 Epidemiology.................................................................7
  1.3.2 Clinical Features...........................................................7
  1.3.3 Pathophysiology.............................................................8
    Ostiomeatal Complex.........................................................9
    Microbiological Factors.....................................................10
    Immunodeficiency............................................................12
    Allergy...............................................................................12
  1.3.4 Inflammation and Sinusitis..............................................13
    Inflammatory Cells............................................................13
    Cytokines............................................................................15
    Adhesion Molecules........................................................17
    Nuclear factor-kappa B......................................................18
  1.3.5 Treatment of Chronic Sinusitis.........................................19
    Medical Management.......................................................19
    Antibiotics...........................................................................20
    Corticosteroids...............................................................20
    Non-pharmacologic Treatments.......................................22
    Surgical Treatment..........................................................22

1.4 MACROLIDE ANTIBIOTICS......................................................23
  1.4.1 Antimicrobial Activity....................................................24
  1.4.2 Anti-inflammatory Activity..........................................25
    Clinical Studies...............................................................25
    Mechanisms of Action.......................................................27
  1.4.3 Effects on Mucociliary Clearance.................................30

1.5 HYPOTHESIS and AIMS.........................................................32
2 THE ANTI-INFLAMMATORY EFFECT OF MACROLIDE ANTIBIOTICS IN VITRO

2.1 INTRODUCTION

2.2 METHODS

2.2.1 Subjects

2.2.2 Specimen collection

2.2.3 Drug preparation

2.2.4 Specimen preparation and culture

2.2.5 Bradford protein estimation

2.2.6 Cytokine ELISA

2.2.7 Immunohistochemistry

2.3 RESULTS

2.3.1 Cytokine ELISA

2.3.2 In vitro specimen immunohistochemistry

2.4 DISCUSSION

3 THE ANTI-INFLAMMATORY EFFECT OF MACROIDE ANTIBIOTICS IN VIVO

3.1 INTRODUCTION

3.2 MATERIALS
3.3 METHODS ........................................................................................................60
  3.3.1 In vivo subjects .............................................................................................60
  3.3.2 Inflammatory cell immunohistochemical staining .....................................61
  3.3.3 TGF-β staining ...........................................................................................63
  3.3.4 NF-κB staining ...........................................................................................64
  3.3.5 Neutrophil staining .....................................................................................65
  3.3.6 Negative controls .........................................................................................66
  3.3.7 Quantification ..............................................................................................66
  3.3.8 Statistical analysis .......................................................................................67
3.4 RESULTS ...........................................................................................................67
  3.4.1 Inflammatory cell populations .....................................................................67
  3.4.2 TGF-β .........................................................................................................71
  3.4.3 NF-κB .........................................................................................................72
3.5 DISCUSSION ......................................................................................................73
4 A DOUBLE-BLINDED, RANDOMISED, PLACEBO-CONTROLLED TRIAL OF LONG-TERM, LOW-DOSE MACROLIDE IN THE TREATMENT OF CHRONIC RHINOSINUSITIS ........................................75
  4.1 INTRODUCTION ...............................................................................................76
  4.2 METHODS .......................................................................................................78
    4.2.1 Subjects ......................................................................................................78
    4.2.2 Study design ..............................................................................................78
    4.2.3 Microbiology and blood testing .................................................................79
    4.2.4 Subjective outcome measures ..................................................................80
    4.2.5 Objective outcome measures ..................................................................81
    4.2.6 Statistical methods ..................................................................................83
  4.3 RESULTS ..........................................................................................................84
    4.3.1 Subjects ......................................................................................................84
    4.3.2 Roxithromycin vs placebo .........................................................................85
      Saccharine transit time .....................................................................................86
      Peak nasal inspiratory flow ............................................................................87
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a2-mac</td>
<td>α2-macroglobulin</td>
</tr>
<tr>
<td>CRS</td>
<td>Chronic rhinosinusitis</td>
</tr>
<tr>
<td>DPB</td>
<td>Diffuse panbronchiolitis</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>FESS</td>
<td>Functional endoscopic sinus surgery</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitory protein-kappa B</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PNIF</td>
<td>Peak nasal inspiratory flow</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNOT-20</td>
<td>Sinonasal outcome test-20</td>
</tr>
<tr>
<td>STT</td>
<td>Saccharine transit time</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
</tbody>
</table>

**INDEX OF FIGURES**
Figure 1.1 Schematic diagram of the lateral nasal wall

Figure 1.2 The sinusitis cycle

Figure 1.3 The structure of erythromycin

Figure 2.1 Layout of tissue culture wells

Figure 2.2 IL-5 production with clarithromycin and prednisolone compared to control

Figure 2.3 IL-8 production with clarithromycin and prednisolone compared to control

Figure 2.4 GM-CSF production with clarithromycin and prednisolone compared to control

Figure 2.5 Expression of NF-κB in mucosal specimens cultured with clarithromycin or prednisolone compared to control

Figure 2.6 Expression of TGF-β in mucosal specimens cultured with clarithromycin or prednisolone compared to control

Figure 3.1 Section of nasal mucosa stained for CD3 and CD68

Figure 3.2 Section of nasal mucosa stained for CD8

Figure 3.3 Section of nasal mucosa stained as a negative control

Figure 3.4 Section of nasal mucosa stained with TGF-β

Figure 3.5 Section of nasal mucosa stained as a negative control

Figure 3.6 Section of nasal mucosa stained with NF-κB

Figure 3.7 Section of nasal mucosa stained as a negative control

Figure 3.8 Expression of CD3+ve T-lymphocytes in pre- and post-treatment biopsies

Figure 3.9 Expression of CD8+ve T-lymphocytes in pre- and post-treatment biopsies

Figure 3.10 Expression of CD68+ve macrophages in pre- and post-treatment biopsies

Figure 3.11 Number of neutrophils in pre- and post-treatment biopsies

Figure 3.12 Expression of TGF-β in pre- and post-treatment biopsies
Figure 3.13 Expression of NF-κB in pre- and post-treatment biopsies 72
Figure 4.1 Levels of evidence 76
Figure 4.2 Design of the clinical trial 80
Figure 4.3 Timing of outcome measures during clinical trial 83
Figure 4.4 Saccharine transit time pre- and post-treatment with roxithromycin 86
Figure 4.5 Saccharine transit time pre- and post-treatment with placebo 87
Figure 4.6 PNIF pre- and post-treatment with roxithromycin 87
Figure 4.7 PNIF pre- and post-treatment with placebo 88
Figure 4.8 Nasal endoscopic scoring pre- and post-treatment with roxithromycin 88
Figure 4.9 Nasal endoscopic scoring pre- and post-treatment with placebo 89
Figure 4.10 Olfactory function score pre- and post-treatment with roxithromycin 89
Figure 4.11 Olfactory function score pre- and post-treatment with placebo 90
Figure 4.12 SNOT-20 scoring pre- and post-treatment with roxithromycin 90
Figure 4.13 SNOT-20 scoring pre- and post-treatment with placebo 91
Figure 4.14 SNOT-20 scoring pre- and 3 months post-treatment with roxithromycin 91
Figure 4.15 Final response scale in the placebo and roxithromycin groups 92
Figure 4.16 IL-8 levels in nasal lavage pre- and post-treatment with roxithromycin 92
Figure 4.17 IL-8 levels in nasal lavage pre- and post-treatment with placebo 93
Figure 4.18 α2-macroglobulin pre- and post-treatment with roxithromycin 93
Figure 4.19 α2-macroglobulin pre- and post-treatment with placebo 94
Figure 4.20 Fucose levels in nasal lavage pre- and post-treatment with roxithromycin 94
Figure 4.21 Fucose levels in nasal lavage pre- and post-treatment with placebo 95
Figure 4.22 Saccharine transit time pre- and post-treatment in low-IgE patients 99
Figure 4.23 Saccharine transit time pre- and post-treatment in high IgE patients 99
Figure 4.24 PNIF pre- and post-treatment in low IgE patients 100
Figure 4.25 PNIF pre- and post-treatment in high IgE patients 100
Figure 4.26 Nasal endoscopic scoring in low IgE patients 101
Figure 4.27 Nasal endoscopic scoring in high IgE patients 101
Figure 4.28 Olfactory function scores in patients with low levels of IgE 102
Figure 4.29 Olfactory function scores in patients with high levels of IgE 102
Figure 4.30 SNOT-20 scores in patients with low levels of IgE 103
Figure 4.31 SNOT-20 scores in patients with high levels of IgE 103
Figure 4.32 SNOT-20 scores in low IgE patients 3 months following treatment 104
Figure 4.33 SNOT-20 scores in high IgE patients 3 months following treatment 104
Figure 4.34 Nasal lavage levels of IL-8 in low IgE patients 105
Figure 4.35 Nasal lavage levels of IL-8 in high IgE patients 105
Figure 4.36 Final response scale in patients with low compared to high IgE 106
INDEX OF TABLES

Table 1.1 Composition of nasal mucus 5

Table 1.2 Targets of macrolide anti-inflammatory activity 31

Table 2.1 Individual data for the in vitro subjects 38

Table 2.2 Antibodies used during immunohistochemical staining 44

Table 2.3 Effect of clarithromycin on IL-5 production 47

Table 2.4 Effect of prednisolone on IL-5 production 48

Table 2.5 Effect of clarithromycin on IL-8 production 49

Table 2.6 Effect of prednisolone on IL-8 production 50

Table 2.7 Effect of clarithromycin on GM-CSF production 51

Table 2.8 Effect of prednisolone on GM-CSF production 52

Table 2.9 Effect of prednisolone and clarithromycin on NF-κB expression 54

Table 2.10 Effect of prednisolone and clarithromycin on TGF-β expression 56

Table 3.1 Individual data for in the vivo subjects 61

Table 3.2 Antibody dilutions used for immunohistochemical staining 66

Table 3.3 Expression of CD3+ve cells pre- and post-treatment 68

Table 3.4 Expression of CD8+ve cells pre- and post-treatment 68

Table 3.5 Expression of CD68+ve cells pre- and post-treatment 69

Table 3.6 Number of neutrophils in pre- and post-treatment biopsies 70

Table 3.7 Expression of TGF-β in pre- and post-treatment biopsies 71

Table 3.8 Expression of NF-κB in pre- and post-treatment biopsies 72

Table 4.1 Baseline data for subjects enrolled in the clinical trial 85

Table 4.2 Pre- vs post-treatment outcome measures in roxithromycin-treated patients 96
Table 4.3 Pre- vs post-treatment outcome measures in low vs high IgE patients
Chapter 1

INTRODUCTION

1.1 Clinical rationale
Chronic rhinosinusitis remains one of the most frequently reported diseases, despite ongoing advances in medical and surgical management. The National Health Survey in the United States estimated that 14% of the population suffered from chronic rhinosinusitis and that total health care expenditure attributable to sinusitis in 1996 was $5.8 billion U.S. dollars.

Chronic rhinosinusitis is a disease of chronic airway inflammation with a multifactorial aetiology. These causative factors include allergy, infection, anatomical abnormality, immunodeficiency and ciliary dysfunction. The complex interaction between these factors is yet to be delineated and at present there is no single hypothesis to explain the pathogenesis of the disease.

Chronic inflammation in the paranasal sinus mucosa is one of the hallmarks of chronic rhinosinusitis. This inflammation is demonstrated by an increased number of chronic inflammatory cells, elevated levels of pro-inflammatory cytokines, increased expression of adhesion molecules and metaplastic changes in the epithelium. The current medical treatments for chronic sinusitis aim to reduce this inflammation and consequently improve symptoms.

In recent years, evidence has been accumulating that macrolide antibiotics exert an anti-inflammatory effect that is quite separate from their antimicrobial effect. This has been demonstrated in a number of clinical trials of chronic airway inflammatory disorders. Amongst these trials, are preliminary studies that suggest macrolides may be useful in treating chronic sinusitis. Similarly, the mechanisms by which macrolides may exert this anti-inflammatory effect have been under investigation.

Macrolide antibiotics may offer an additional treatment option for a common and often
frustrating condition to treat. Their role in the treatment of chronic sinusitis and the precise mechanisms by which they work are yet to be fully understood.

1.2 Nasal cavity and paranasal sinus anatomy

To fully understand the physiology and pathology of the paranasal sinuses and associated diseases, an understanding of the normal anatomy is essential.

![Figure 1.1](image)

**Figure 1.1** Schematic diagram of the lateral nasal wall. AT, auditory tube; IC, inferior concha/turbinate; MC, middle concha/turbinate; SER, sphenoid recess; SS, sphenoid sinus; V, vestibule.

1.2.1 Gross anatomy

The nasal cavity extends from the face to the nasopharynx (see figure 1.1). It is divided in its midline by a bony and cartilaginous septum. The most anterior part of the cavity, the vestibule, is lined with skin with coarse hairs. The rest of the nasal cavity is divided into the olfactory region and the respiratory region. The olfactory region is in the superior aspect of the cavity and includes part of the roof, septum and lateral wall. The rest of the
cavity, the respiratory region, is lined by columnar or pseudostratified ciliated columnar epithelium.

The lateral wall of the nasal cavity consists of a complex arrangement of portions of seven bones. The conchae or turbinates form three large constant bony projections from the lateral wall in to the nasal cavity. The superior and middle turbinate are part of the ethmoid bone. The inferior turbinate is a bone in its own right.

Underlying each turbinate is a space, the meatus. The meatuses have openings into them from various sources. The most complicated meatus is the middle meatus, underlying the middle turbinate. It contains an opening or groove known as the hiatus semilunaris into which opens the maxillary sinus, anterior ethmoidal air cells and the frontal sinus. In the superior meatus are openings of the posterior ethmoidal cells. The sphenoidal sinus drains into an area above the superior turbinate known as the sphenoethmoidal recess.

The paranasal sinuses include the frontal, ethmoidal, maxillary and sphenoidal sinuses. All the sinuses drain into and are therefore connected to the nasal cavity. They are all lined with mucosa similar to that in the nasal cavity, except that it is not as thick and is less vascular. The anatomy of the sinuses is quite variable and differs from individual to individual and during development.

1.2.2 Histology
The respiratory region of the nasal cavity and the paranasal sinuses are lined by pseudostratified ciliated columnar epithelium. Amongst the epithelium are scattered basal, goblet and inflammatory cells.

The nasal submucosa contains cellular components as well as nerves, blood vessels and nasal glands. Mononuclear cells, including lymphocytes and monocytes are the predominant cell type in the submucosa. Neutrophils and eosinophils are less
Numerous\textsuperscript{58}. Immunohistochemical studies reveal that T-lymphocytes outnumber B-lymphocytes and that the ratio of T-helper to T-suppressor cells is 2.5:1\textsuperscript{109}.

There are three types of nasal glands. Anterior serous glands occupy the nasal vestibule. Seromucous glands form a superficial and deep layer in the submucosa and number approximately 90000. Intraepithelial glands are located in the epithelium and compared to seromucous glands produce only a small amount of mucous and thus play a minor role in the physiology of nasal secretions\textsuperscript{76}.

1.2.3 Nasal mucous and mucociliary transport

Nasal mucous and mucociliary transport perform several important physiological functions. Amongst these are the humidification, warming and removal of particulate matter from the inspired air. In addition the mucous layer provides immune and mechanical protection.

Nasal secretions are produced by the glands mentioned above, along with exudation from blood vessels, goblet cells and tears. These secretions form a thin blanket of mucous that covers the nasal and sinus epithelium. This blanket consists of a low viscosity periciliary layer (sol phase) and a more viscous layer (gel phase) superficially.

Table 1.1 Composition of nasal mucous\textsuperscript{43}

<table>
<thead>
<tr>
<th>Water and ions from transudation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoproteins: sialomucins, fucomucins, sulfomucins</td>
</tr>
<tr>
<td>Enzymes: lysozymes, lactoferrin</td>
</tr>
<tr>
<td>Circulatory proteins: complement, (\beta)-macroglobulin, cytokines</td>
</tr>
<tr>
<td>Immunoglobulins: IgA, IgE, IgG, IgM, IgD</td>
</tr>
<tr>
<td>Cells: surface epithelium, basophils, eosinophils, leukocytes</td>
</tr>
</tbody>
</table>
Table 1.1 shows some of the components of nasal mucous. Glycoproteins constitute approximately 80% of the dry weight of nasal mucous and are responsible for its elasticity and viscosity. IgA and IgE, being the immunoglobulins involved in mucosal defence, are present in greater quantities than in serum and IgA itself constitutes 50% of the total protein content.

In the sinuses, the cilia constantly propel the mucous layer towards the sinus openings in a genetically predetermined pattern. The mucous is then ultimately transported to the nasopharynx and swallowed. To enable proper mucociliary clearance, an intact mucous membrane, normal ciliary function and normal mucous production are essential. Impairment in any of these normal functions can lead to sinus disease.

1.2 Chronic sinusitis

It has proven difficult to provide an exact definition for chronic sinusitis due to the lack of objective criteria that may be used in defining it. The International Conference on Sinus Disease in 1993 proposed that the criteria for chronic sinusitis in adults was persistent symptoms and signs for eight weeks or four episodes per year of recurrent acute sinusitis, each lasting at least 10 days, in association with persistent changes on the CT scan four weeks after medical treatment without intervening acute infection. The American rhinosinusitis task force in 1996 developed a number of major and minor criteria used for making the diagnosis of rhinosinusitis. The major criteria were: facial pain/pressure, nasal obstruction/blockage, nasal discharge/purulence/discoloured postnasal discharge, hyposmia/anosmia, purulence in the nasal cavity on examination, fever (acute rhinosinusitis only). The minor criteria were: headache, fever, halitosis, fatigue, dental pain, cough, ear pain/pressure/fullness. Rhinosinusitis was defined as the presence of 2 or more major criteria or 1 major and 2 or more minor criteria. In 2003 the taskforce recommended the criteria for diagnosis in research projects should be more rigorous. It was recommended that in addition to the clinical criteria described above, patients should have a CT scan or nasendoscopy to confirm the diagnosis.
1.2.1 Epidemiology

Sinusitis is one of the most common chronic diseases. Despite this, there is a relative paucity of epidemiological data that give its precise incidence. This is partly due to difficulty in providing objective criteria for diagnosis and also, under-reporting of cases\(^\text{17}\).

Much of the available data has been obtained from the United States population. The National Health Survey carried out between 1990-1992 suggested that 14% of the population suffered from the disease\(^\text{13}\). Approximately 12% of Americans below the age of 45 report symptoms of chronic sinusitis\(^\text{83}\). In one study, the occurrence of signs of sinusitis on an MRI scan in a normal population was 39% in adults and 45% in children\(^\text{23}\).

The economic impact of chronic sinusitis in terms of medical consultations, medication prescriptions, surgery and lost workdays is considerable. In 1996 in the U.S.A., the overall health-care expenditure attributable to sinusitis was $5.8 billion\(^\text{86}\). An estimated 200,000 sinus operations were performed in the United States in 1994\(^\text{40}\).

There is evidence that the incidence of chronic sinusitis is rising. In the U.S.A. in 1992, there were 24 million visits to doctors attributable to the disease, compared to 16 million in 1989\(^\text{10}\). Between 1990 and 1992, sinusitis patients reported approximately 73 million days of restricted activity. This represented a 23 million day increase when compared with the period from 1986 to 1988\(^\text{40}\).

1.2.2 Clinical features

In spite of the fact that chronic sinusitis is a common condition, it can be a challenge to diagnose as it mimics several other conditions such as dental, ophthalmological and neurological complaints\(^\text{19}\). Patients generally present with one or a number of a spectrum
of complaints. These include facial pain, headache, fatigue, increased volume and viscosity of nasal secretions, anosmia, nasal congestion, chronic cough, post-nasal drip and auditory-tube dysfunction.

The clinical signs of chronic sinusitis vary depending on the site and nature of the inflammation and whether there is any superimposed acute inflammation. The area over the involved sinus may be tender. The nasal mucous membrane tends to be red and turgescent and yellow or green purulent rhinorrhea may be present.  

A 1995 study by Gliklich and Metson examined the general health and well-being of these patients in attempt to further understand the overall health impact of chronic sinusitis. Using the Medical Outcome Short-form 36-item Health Survey (SF-36) the authors compared chronic sinusitis patients with patient populations suffering from different chronic diseases. The study concluded that individuals with chronic sinusitis showed significant decrements, compared to population norms, in the domains of bodily pain, social functioning, general health, vitality and physical functioning. In addition, comparisons with other chronic diseases such as angina pectoris, congestive heart failure, chronic obstructive pulmonary disease and chronic back pain revealed significantly lower scores in measures of bodily pain and social functioning in sufferers of chronic sinusitis. This study suggested that patients with chronic sinusitis suffer in a more general manner than in terms of sinus-specific symptoms alone and that the health impact of chronic sinusitis is greatly under-appreciated.

1.2.3 Pathophysiology
Recent advances in allergy and immunology, imaging techniques and endoscopy have resulted in a greater understanding of the pathophysiology of chronic sinusitis. It is now understood that there is a complex interaction of several factors that give rise to the disease. This interaction has become known as the ‘sinusitis cycle’ (see figure 1.2). The following section contains a discussion of the factors associated with the development of chronic sinusitis with an emphasis on those that are examined in this thesis.
The Sinusitis Cycle

- Ostium closes
- Mucosal thickening creating further blockage
- Blockage of air flow and drainage
  - Secretions stagnate
  - Secretions thicken, pH changes
  - Bacterial growth enhanced
  - Increased tissue inflammation
  - Cilia and epithelium damaged
  - Mucosal gas metabolism changes

Figure 1.2 The sinusitis cycle (Adapted from Fernandes 2000)

Ostiomeatal complex

The traditional belief, held until approximately 30 years ago, was that the primary site of mucosal inflammation leading to chronic sinusitis was the maxillary sinus. Consequently the majority of surgical treatments were focused towards this area. More recently, Messerklinger emphasised that the apposition of mucociliary surfaces in the paranasal sinuses has the potential to block sinus ostia, leading to obstruction of drainage and in turn inflammation and infection. The middle meatus, as the site of opening of the majority of the ostia therefore became the site of interest and the associated anatomical area became known as the ostiomeatal complex.

The ostiomeatal complex is defined as the area bounded by the medial orbital wall and the middle turbinate. It contains the ostia of the anterior ethmoidal, frontal and maxillary sinuses where they open into the middle meatus. A critical bony structure in this region is the uncinate process. It is a thin sagitally-oriented, crescent-shaped fragment of bone. The free posterior margin of the uncinate process is concave and lies parallel to the
convex surface of the bulla ethmoidalis. The two therefore create a 2-dimensional cleft known as the hiatus semilunaris which opens into the 3-dimensional ethmoidal infundibulum. The ethmoidal infundibulum lies within the ethmoid bone and contains the opening of the maxillary sinus ostium. It is evident therefore that disease in the anterior ethmoid and the ethmoidal infundibulum may therefore result in maxillary sinus ostial obstruction and disease. Similarly, the frontal recess, which drains the frontal sinus, may drain directly into the ethmoidal infundibulum if the uncinate process attaches superiorly to the roof of the ethmoid or the middle turbinate. This complex and intricate anatomic arrangement explains why even minor inflammatory swelling in this region can result in obstruction of sinus drainage and hence the ‘sinusitis cycle.’

**Microbiological factors**

Certain microbial organisms are found normally in the nasal cavity and nasopharynx. *Staphylococcus aureus* occurs primarily in the nasal vestibule; *Moraxella catarrhalis*; streptococcal species including *S. Pneumoniae* and *S. pyogenes* and *Haemophilus influenzae* are found in the posterior nasal passages and nasopharynx. In contrast to the bacteria, the viruses of importance in acute sinusitis are not part of the normal nasal microbial flora but appear during acute infections.

Until recently it was generally held that the paranasal sinuses are sterile under normal conditions. The current belief however is that all sinuses contain some aerobic organisms and some contain a mixed environment of aerobic and anaerobic organisms. These normal bacteria flora may proliferate as a result of ostial occlusion without the introduction of external pathogenic organisms.

The role of microbial organisms in the pathogenesis of acute community-acquired sinusitis is relatively well understood. Viral infection of the nasal passage and sinuses promotes secondary bacterial infection of the sinus cavities by promoting inflammatory changes in the ostiomeatal complex and by damaging the epithelial lining of the sinuses, hence harming the normal protective mechanisms of the upper airway. The
predominant bacteria in acute sinusitis are *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*\(^3^0\).

In contrast to the situation in acute sinusitis, the precise role of microbial organisms in the aetiology of chronic sinusitis is poorly understood. This uncertainty arises because the organisms recovered from the sinus cavity of patients with chronic sinus disease are those that would be expected to secondarily colonize a functionally impaired sinus. For example, the human upper airway is normally populated with anaerobic bacteria in concentrations of up to \(10^{11}\) organisms per gram of tissue and it is therefore not surprising that anaerobic organisms are frequently isolated in a sinus cavity with impaired drainage\(^1^7\). Other factors which confound attempts to assign a microbial cause to chronic sinusitis are previous antibiotic therapy, previous sinus surgery, the method of obtaining the sinus culture and culturing techniques.

Infection in chronic sinusitis tends to be polymicrobial. Frequently cultured organisms include those mentioned above in reference to acute sinusitis, along with anaerobic streptococci, veillonella, corynebacterium, *Pseudomonas aeruginosa* and fungal species\(^1^9\).

In recent years, new theories regarding the pathogenesis of chronic rhinosinusitis have been proposed in which microbial infection is thought to be the principle underlying abnormality. Ponikau has theorised that chronic rhinosinusitis may arise due to a non-immunoglobulin-E mediated reaction to ubiquitous airborne fungi such as *Alternaria*\(^8^4\). Bachert has suggested that enterotoxin release by intraepithelial *S. aureus* may result in multiclonal IgE production and subsequent eosinophilic inflammation\(^1^1^2\). These new theories, if proven correct, may lead to advances in the treatment of chronic rhinosinusitis.

**Mucociliary clearance**
As mentioned previously, normal mucociliary clearance is necessary for adequate sinus drainage. Impaired mucociliary function may be secondary to stasis of secretions, hypoxia or infection and in turn lead to an exacerbation of the chronic inflammation and a prolongation of the ‘sinusitis cycle.’ A decrease in mucociliary clearance has been consistently demonstrated in chronic sinusitis.

In contrast to the secondary impairment of clearance noted above, conditions that primarily affect ciliary function or mucous production may also cause chronic sinusitis. These conditions include cystic fibrosis, in which tenacious mucous blocks the sinus ostia and immotile cilia syndrome, which predisposes to mucous retention, closure of ostia and sinusitis.

**Immunodeficiency**

Conditions that affect immune function are associated with chronic sinusitis. Amongst these are diabetes mellitus, leukaemia, lymphoma, iatrogenic immunosuppression and AIDS.

Patients with deficiencies in normal antibody production are also prone to sinus infection. Secretory IgA deficiency is associated with chronic sinusitis presumably because there is a decreased level of this protective antibody in the sinus secretions. Similarly, abnormalities in production of IgG or one of its subclasses can predispose to sinusitis.

**Allergy**

The role of allergy in the causation of chronic sinusitis is not clear. There is however an association between allergic diseases and chronic sinusitis. 15-25% of cases of chronic sinusitis occur in patients who suffer from an allergic disease. Patients with chronic sinusitis frequently demonstrate positive skin test reactions to a variety of allergens.
Some features of the inflammatory response in chronic sinusitis such as eosinophilia and elevated serum IgE suggest an allergic component. Vasodilation, plasma exudation and glandular secretion occurring secondarily to allergic inflammation may lead to recruitment of parasympathetic reflexes, oedema of nasal mucosa and ostial obstruction.

1.2.4 Inflammation and sinusitis

Chronic inflammation plays a key role in the pathogenesis of sinusitis. The ensuing tissue damage and mucosal swelling prolongs the ‘sinusitis cycle’. Many of the therapeutic options for chronic sinusitis are aimed at suppressing this inflammation and breaking the vicious cycle. Despite the importance of this inflammatory process, it is in many ways still poorly understood, partly because once established, it is difficult to discern the causative factors in its development.

Most of the information available concerning chronic inflammation in the nasal cavity comes from studies investigating nasal polyps. Nasal polyposis is a condition in which exuberant mucosal thickening leads to the production of polypoid protuberances in the nasal cavity. These polyps provide a rich source of material for analysis and hence are frequently used for experimental work. There are some studies that examine chronic sinusitis mucosa exclusively and others that demonstrate that polyps and sinusitis mucosa display similar immunopathologic findings.

The following review discusses a large number of inflammatory mediators that have been implicated in the pathogenesis of chronic sinusitis. Studies vary regarding the involvement of some of these mediators and in some cases there is conflicting evidence regarding their roles. These discrepancies probably arise due to differences in methods of determining the expression of these factors and differences in patient selection.

Inflammatory cells
Lymphocytes are significantly the most abundant cell type in nasal polyps. Stoop et al. stated that lymphocytes are present in greater numbers in nasal polyps than in inferior turbinate biopsies and suggested that a T–lymphocyte dependent disturbance is associated with chronic inflammation in the paranasal mucosa. Other studies have reported that the relative proportion and spatial distribution of T- and B-lymphocytes were similar in nasal polyps and disease-free controls. Hamilos suggests that the number of CD4+ T-lymphocytes is increased in patients with positive allergy tests but not in those with negative tests. CD8+ T-lymphocytes (suppressor/cytotoxic T-cells) outnumber CD4+ T-lymphocytes (helper/inducer T-lymphocytes).

Nasal polyps have been shown to have significantly increased numbers of eosinophils compared to normal nasal mucosa. The levels of tissue eosinophilia are equal in allergic and non-allergic cases of nasal polyposis. Eosinophils produce a number of substances that contribute to the inflammatory state in chronic sinusitis. These include leukotriene C4 (a potent mucous secretagogue) and platelet-activating factor, which promotes vasodilatation, increased permeability and further chemotaxis of eosinophils and neutrophils. When activated, eosinophils produce positively charged proteins such as major basic protein and eosinophilic cationic protein that are highly toxic to human respiratory epithelium. Eosinophilic cationic protein (EG2) is used as a marker of activated eosinophils. The majority of eosinophils have been shown to be EG2+ suggesting that active eosinophils play a role in the pathogenesis of nasal polyps and chronic sinusitis.

A high degree of infiltration with plasma cells has been shown in nasal polyps in a number of studies. Plasma cells play an integral role in the humoral immune response through the production of antibodies. These findings of elevated levels of plasma cells suggest that the humoral immune response is associated with chronic inflammation in the upper airway.

Hamilos reports that in chronic hyperplastic sinusitis/nasal polyposis there are mildly increased numbers of mast cells compared to controls but no increase in the number of
macrophages or neutrophils. These findings differ from the results of Morinaka et al.\textsuperscript{74} who found that neutrophil numbers are significantly increased in chronic sinus inflammation.

**Cytokines**

Cytokines are peptides or glycoprotein molecules that act as intercellular signals in various immune and inflammatory responses. They are key regulators of immune cell growth and differentiation and are thought to regulate or determine the activity of a disease process. The term cytokine constitutes a large family of molecules that includes the interleukins, the interferons, the colony stimulating factors and the tumour necrosis factors. They are produced by a wide variety of cell types and each individual cytokine may have multiple functions.\textsuperscript{55} In recent years there has been extensive interest in determining the cytokine patterns involved in all inflammatory conditions, including chronic sinusitis.

**Interleukin-8 (IL-8)** exerts a potent neutrophil chemotactic activity. It is produced by various cells including fibroblasts, epithelial cells, peripheral blood monocytes and neutrophils.\textsuperscript{101} IL-8 expression, as determined by immunohistochemistry, RT-PCR and Southern blot has been shown to be elevated in chronic sinusitis mucosa compared to controls.\textsuperscript{71,81} IL-8 is secreted from exudative cells in the nasal discharge of patients with chronic sinusitis. This secretion in turn induces further neutrophil migration and maintain the vicious cycle of local purulence.\textsuperscript{98} Similarly, maxillary sinus lavage fluid of patients with chronic sinusitis has been shown to have significantly increased numbers of neutrophils and dramatically increased levels of IL-8.\textsuperscript{30}

**Interleukin-6 (IL-6)** is a pro-inflammatory cytokine produced by a variety of cells. It stimulates T-lymphocyte activation and proliferation, along with B-lymphocyte differentiation and immunoglobulin secretion. It causes fibroblast proliferation and collagen deposition and is thought to be partly responsible for the thickening of the basement membrane and subepithelial fibrosis seen in chronic sinusitis mucosa. IL-6
mRNA and immunoreactivity have been shown to be elevated in both allergic and non-allergic sinusitis\textsuperscript{21,56}.

**Interleukin-5** (IL-5) is a pro-inflammatory cytokine associated with eosinophilic inflammation and its level of expression directly correlates with the degree of eosinophilia in allergic chronic sinusitis. IL-5 is a haematopoietic growth factor that is an important regulator of eosinophil growth and survival and is primarily responsible for the terminal differentiation and activation of eosinophils. It is expressed in significantly greater levels in the nasal mucosa of chronic sinusitis patients\textsuperscript{55}. In addition, the expression of IL-5 receptor messenger-RNA is increased in patients with allergic chronic sinusitis compared to those with non-allergic disease and controls. This suggests that these patients may have increased IL-5 receptors and thus an amplified response\textsuperscript{110}.

**Interleukin-4** (IL-4) is another cytokine associated with allergic and eosinophilic inflammation. It is essential for isotype switching in favour of IgE production and up-regulates expression of vascular cell adhesion molecule-1, which promotes eosinophil transendothelial migration\textsuperscript{110}. IL-4 mRNA levels are increased in patients with allergic chronic sinusitis but not in non-allergic patients\textsuperscript{30}.

**Interleukin-3** (IL-3) is thought to play a dominant role in the pathogenesis of chronic sinusitis. This cytokine is produced by a variety of cells including activated T-lymphocytes, mast cells and eosinophils. It displays multiple colony-stimulating effects and stimulates the differentiation and activation of macrophages, neutrophils, mast cells and eosinophils. These actions may contribute to fibrosis and lead to constant thickening of sinus mucosa\textsuperscript{72}. IL-3 has been shown to be present in abundance in nasal polyps and the expression of IL-3 mRNA correlates with the degree of eosinophilia in allergic and non-allergic chronic sinusitis\textsuperscript{28}.

**Granulocyte-macrophage colony-stimulating factor** (GM-CSF) has actions similar to those of IL-5 and is also associated with allergic and eosinophilic inflammation. In addition it may be important in the activation of macrophages at peripheral tissue sites\textsuperscript{110}. 
GM-CSF levels are increased greatly in nasal polyps and the degree of eosinophilia seen correlates with the expression of GM-CSF mRNA\textsuperscript{26}. Interestingly, unlike IL-5, which is predominantly elevated in allergic chronic sinusitis, GM-CSF receptor expression is greatest in non-allergic disease, suggesting that it has an additional role in the recruitment of neutrophils and/or macrophages\textsuperscript{51}.

**Transforming growth factor-β** (TGF-β) is a protein that exists in 5 isoforms, of which 3 have been identified in human tissues. It is involved in the process of tissue repair and can be produced by a number of cells including macrophages and epithelial cells. TGF-β is a potent chemoattractant for neutrophils, mononuclear cells and fibroblasts. It greatly enhances extracellular matrix production and down-regulates the expression of matrix-degrading enzymes. In addition it influences airway epithelial cell growth and differentiation. TGF-β has been shown to be present in nasal polyps and to a lesser extent chronic inflammatory mucosa but not in the nasal mucosa from disease-free controls\textsuperscript{14}.

Several other cytokines have been implicated in the pathogenesis of chronic sinusitis. Amongst these are IL-1β, IL-13 and IFN-gamma. RANTES and eotaxin, both of which facilitate the transendothelial migration of eosinophils, are strongly expressed in nasal polyps\textsuperscript{30}. Interleukin-12, thought to play a suppressive role in the development of allergic sinonasal responses, is down-regulated in chronic sinusitis mucosa compared to controls\textsuperscript{72}.

**Adhesion molecules**

Leukocyte-endothelial adhesion molecules are expressed on endothelial surfaces in blood vessels. They act as ligands for leukocyte cell surface receptors and enable adhesion of leukocytes and subsequent migration into inflamed tissue. Their expression is regulated locally by cytokines\textsuperscript{15}.
**Interacellular adhesion molecule-1 (ICAM-1)** and **Vascular cell adhesion molecule-1 (VCAM-1)** are both members of the immunoglobulin supergene family. ICAM-1 mediates the adhesion of most leukocyte types. VCAM-1 is considered selective for lymphocytes, monocytes and eosinophils. Immunoreactivity for ICAM-1 has been shown to be significantly elevated in maxillary sinus mucosa of patients with chronic sinusitis. VCAM-1 in the same study was only minimally expressed or absent\(^{81}\). An alternative study revealed significantly increased expression of VCAM-1 in nasal polyps compared to controls. This increased expression was most notable in non-allergic subjects\(^{27}\).

**Nuclear factor-κB**

Nuclear factor-κB (NF-κB) is a nuclear transcription factor involved in the regulation of expression of a large number of pro-inflammatory genes. As a consequence, it has been the subject of considerable interest in order to determine its precise associations and its role in various disease processes. It exists as a heterodimer comprising the subunits p50 and p65. Each subunit consists of an N-terminal DNA binding site and a C-terminal nuclear localisation signal that is necessary for its translocation across the nuclear membrane. In the cytoplasm, NF-κB exists in its inactive form due to binding with an inhibitory protein (IκB). This protein conceals the nuclear localisation signal and thus prevents passage into the nucleus. When IκB is phosphorylated, it dissociates from the complex and is degraded, allowing the activation of NF-κB\(^4\).

NF-κB mediates the transcription of genes for the adhesion molecules ICAM-1, VCAM-1 and E-selectin. In addition it regulates GM-CSF, TNFα, inducible nitric oxide synthase, IL-2, IL-6 and IL-8 production amongst others\(^{4,24,65}\).

NF-κB activation has been demonstrated in a number of key cell types including mast cells, eosinophils, lymphocytes, endothelial cells and epithelial cells. In nasal polyp tissue activated NF-κB occurs in the endothelium, epithelium and a variety of cells in the submucosa. This suggests that NF-κB plays an important role in the regulation of inflammation in nasal polyps\(^{108}\).
1.2.5 Treatment of chronic rhinosinusitis

Despite recent advances in medical and surgical management, chronic rhinosinusitis frequently remains a frustrating condition to treat. The condition attracts interest from otorhinolaryngologists, allergists and infectious diseases specialists amongst others. This interest underscores the multifactorial aetiology of the disease and the fact that treatment is occasionally unsuccessful. Considering its prevalence, there is a relative paucity of controlled clinical trials investigating the efficacy of management strategies.

The guiding principle of management should be the reversal of the ‘sinusitis cycle’ and the subsequent re-ventilation, improved drainage and recovery of the sinonasal tract. To enable this, the chronic sinusitis sufferer needs to be completely evaluated with a medical history, physical examination and a review of previous medical history. Contributing factors to sinusitis, such as allergen exposure and hypogammaglobulinaemia should be sought and addressed appropriately.

Computed tomography (CT) scanning has replaced plain radiography as the imaging investigation of choice in assessing the chronic sinusitis patient. It allows non-invasive evaluation of the paranasal sinuses and nasal cavity, including the ostiomeatal complex. It also provides good anatomical definition to guide surgery and may demonstrate the underlying cause of a patient’s disease\textsuperscript{17,19,30}.

Medical Management

Studies that have investigated the role of medical management in chronic sinusitis suggest that it is efficacious. In one study, 20 patients were treated with topical dexamethasone and decongestant sprays and 10 with matched placebo sprays. Significantly more patients receiving active treatment demonstrated improvement in symptoms\textsuperscript{102}. A study by McNally et al\textsuperscript{67} examined 200 patients with sinusitis. Treatment
of this group consisted of 4 weeks of oral antibiotics, nasal lavage, nasal corticosteroids and topical decongestants. After 1 month of treatment, all patients were reported to have some improvement in symptoms and/or signs. In a retrospective study of 19 patients with chronic sinusitis, subjects were given a 10-day course of oral prednisone and a 4 to 6 week course of antibiotic. 17 of the 19 patients had improvement in both symptoms and CT scan scores⁹⁶.

### Antibiotics

As mentioned previously, infection does play a role in the pathogenesis of chronic sinusitis, however this role is often secondary to other factors.

The goals of antimicrobial therapy are:

- to relieve symptoms of acute illness
- to prevent subacute morbidity
- to forestall the development of chronic sinusitis
- to prevent intracranial complications

The choice of antibiotic should be guided by the most likely organism, patterns of antibiotic resistance and penicillin allergy history. It should be kept in mind that whilst anaerobic bacteria are found in 10% of acute sinus infections, the incidence in chronic sinusitis is approximately 88%¹⁹. First-line antibiotics should be broad-spectrum agents such as amoxicillin-clavulanic acid or sulfamethoxazole-trimethoprim in penicillin-allergic cases. Other antibiotics with potential benefit include the cephalosporins, macrolides and ciprofloxacin⁴³.

7 to 10-day courses of antibiotics may lead to a partial and temporary improvement in symptoms, however, longer courses may be more efficacious. Persistence of obvious signs of infection such as pus in the nasal cavity or air-fluid levels in the sinuses should prompt bacterial and fungal cultures¹⁷.

### Corticosteroids
Corticosteroids are used in the treatment of chronic sinusitis on the premise that through their anti-inflammatory effect they are able to suppress inflammation of the nasal mucosa and thus improve sinus ventilation and drainage. They can be administered topically or systemically. Corticosteroids have well-established side effects, including insomnia, mood swings, peptic ulcer disease, osteopenia, glaucoma, hypertension, immunosuppression and disorders of glucose metabolism. As such, systemic corticosteroids are not used in prolonged courses and tend to be given for short periods in a tapering dose pre-operatively or to alleviate an exacerbation of severe symptoms. Topical corticosteroids have proven long-term safety and provided that the patient does not develop overt symptoms attributable to the drug, such as epistaxis, prolonged courses are frequently used.

Numerous trials have investigated the mechanism of the anti-inflammatory effect of corticosteroids on nasal polyp tissue. Tinsgaard et al showed that the intensity of endothelial ICAM-1 expression in polyps was significantly reduced during topical glucocorticoid treatment. A study by Kenai et al demonstrated that the proportion of activated eosinophils (EG2+ve versus total eosinophil count) was significantly lower in polyps from patients treated with topical nasal steroid than in polyps from untreated patients. Polyps from treated patients contained significantly lower tissue density of CD3, CD4 and CD8 lymphocytes. ICAM-1 expression was shown to be reduced but not to a significant degree. An in-vitro study has been carried out to investigate the effect of prednisolone on cultured nasal polyp tissue. The study revealed that GM-CSF and IL-5 levels, as detected by ELISA on supernatants and homogenates were significantly reduced after treatment with prednisolone. Hamilos et al using the intranasal steroid fluticasone, reported a reduction in the number of MBP and EG2+ve eosinophils and CD4 lymphocytes in nasal polyps of the treated group compared to controls. A statistically significant reduction in IL-4 and IL-13 mRNA-positive cells was shown. In contrast, no significant reduction in endothelial expression of VCAM-1 or mRNA for TNF-α or IL-1β was shown.
Fewer studies have been performed examining the mechanisms of action of corticosteroids on chronic sinusitis mucosa. Wright et al.\textsuperscript{110} demonstrated a down-regulation of mRNA expression for IL-4 and IL-5 receptors in chronic sinusitis patients treated with intranasal corticosteroid. Another study examined pre- and post-treatment biopsies of patients with chronic sinusitis who were each given a 10-day course of oral prednisone. Immunohistochemistry revealed a significant decrease in IL-6 levels in the post-treatment biopsies and a strong trend towards decreased TNF-\(\alpha\) levels. No significant decrease was shown in the levels of IL-5, IL-8 and IL-1\(\beta\) levels.

The effect of corticosteroids on NF-\(\kappa\)B activation is uncertain. At least two studies, performed on airway epithelial cells, have shown that they do not inhibit activation of NF-\(\kappa\)B\textsuperscript{33,78}.

Despite their widespread use, the evidence supporting topical corticosteroids in the treatment of chronic rhinosinusitis is somewhat scarce and unconvincing. Studies investigating the use of topical corticosteroids for the treatment of chronic sinusitis are much fewer in number but have shown a benefit when compared to placebo\textsuperscript{68}.

**Non-pharmacologic treatments**

A number of non-pharmacological measures are widely used in the treatment of chronic sinusitis to provide symptomatic relief. These include:

- Steam inhalations to provide symptom relief
- Saline sprays to help liquefy secretions and moisturise the nasal mucosa
- Mucoevacuants to reduce mucous viscosity

**Surgical treatment**

Advances in endoscopy and imaging techniques have led to the development of a new surgical technique named functional endoscopic sinus surgery (FESS). FESS aims to remove diseased tissue, preserve normal nasal structure and restore normal nasal
function. The surgery is directed towards the ostiomeatal complex, which as discussed earlier, is the crucial area of disease involvement in chronic sinusitis. This new surgical technique represents a departure from traditional techniques in which radical removal of sinus mucosa was performed. The emphasis instead is on re-establishing ventilation and drainage and allowing the secondarily involved mucosa to recover over time\cite{19}.

In general terms, the indications for FESS are as follows:\cite{17,43}

- For patients with chronic sinusitis that is symptomatic and persists despite adequate medical therapy.
- For patients with recurrent sinusitis in whom it is determined that bony or mucosal abnormalities of the ostiomeatal complex are a pre-disposing factor.
- For sino-nasal polyposis when medical management fails.
- In cases of acute sinusitis when medical therapy fails to adequately treat the disease or if complications arise.

### 1.3 Macrolide antibiotics

The macrolides are a group of antibiotics that have in common a macrocyclic lactone ring (see figure 1.3). The first macrolide to be discovered was erythromycin. It was recovered from a soil sample in the Philippines and was shown to be a metabolic product of a strain of *Streptomyces erythreus*\cite{11}. Erythromycin continues to be widely used as an antibiotic agent as an alternative to beta-lactam antibiotics for the prevention and treatment of infections caused by gram-positive cocci and for the treatment of respiratory infections. It is one of the safest antibiotics available but is poorly tolerated by many patients due to gastrointestinal side effects\cite{94}. These side effects led to the development of new improved macrolides that have increased acid-stability and thus reduced conversion of the molecule to inactive forms in the stomach. These modifications resulted in improved bioavailability following oral dosing, improved gastrointestinal tolerance and increased
antibacterial activity when compared to erythromycin\textsuperscript{32}. The new 14-membered ring macrolides are clarithromycin, azithromycin and roxithromycin.

An interesting aspect of the pharmacokinetics of macrolide antibiotics is their extensive tissue uptake and intracellular accumulation. Macrolides accumulate in inflammatory cells at concentrations up to several hundred-fold higher than concentrations in extracellular fluid. For example, azithromycin concentrations in most tissues types are 10 to 100 times serum levels and it has been shown to accumulate to a high degree in a number of cell types, including neutrophils, macrophages and fibroblasts\textsuperscript{94}. Bermudez et al.\textsuperscript{7} showed that cytokines stimulate the accumulation of macrolide antibiotics into macrophages in vitro. This suggests that at sites of inflammation, cells may accumulate even more macrolide than under normal physiological conditions. This intracellular accumulation has led to interest in the capability of macrolide antibiotics to treat intracellular pathogens, as well as their capacity to alter host cell intrinsic functions.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{erythromycin_structure.png}
\caption{The structure of erythromycin}
\end{figure}

\subsection{1.3.1 Antimicrobial activity}
Macrolides have a well-established antimicrobial activity and have been used in the treatment of a variety of bacterial infections for several decades. They are primarily bacteriostatic and bind to the 50S subunit of the ribosome, thus inhibiting bacterial protein synthesis. Macrolides are active against gram-positive cocci (including anaerobes) with the exception of enterococci and have limited gram-negative activity. They were initially predominantly used for the treatment of staphylococcal and streptococcal pathogens, however, resistant strains subsequently emerged and the macrolides were replaced by the penicillins and cephalosporins. They are currently used as an alternative to the beta-lactam antibiotics for the treatment of gram-positive cocci in beta-lactam allergic patients. In addition macrolides are the drug of choice for the treatment of respiratory infections caused by Corynebacterium diptheriae, Bordetella pertussis, Legionella pneumophila, Mycoplasma pneumoniae and Chlamydia pneumoniae.

Apart from their direct bacteriostatic and bactericidal effects, macrolides have been shown to reduce the virulence of certain organisms. Macrolides do not have a direct antibacterial effect against Pseudomonas aeruginosa. However, Hirakata et al showed that erythromycin inhibits the release of elastase, protease, phospholipase C and eotaxin A by P. aeruginosa. Another study showed that erythromycin was able to suppress the production of toxic lectins, protease and hemolysin. These findings suggest that macrolides may be able to reduce tissue damage caused by certain bacteria, without having a direct antibacterial effect.

1.3.2 Anti-inflammatory activity

Clinical studies
The initial evidence that macrolides may attenuate the inflammatory response emerged several decades ago in studies investigating the treatment of asthma. A number of studies demonstrated that concomitant administration of macrolide antibiotics with corticosteroids resulted in a reduction in the required dose of steroid. Initially it was
thought that this effect was due to an alteration in the metabolism of corticosteroids and an inhibition of theophylline clearance, which is also used in the treatment of asthma. However, it became apparent that these effects alone were not sufficient to explain the effect and therefore that other factors must be at play.\textsuperscript{53}

The most convincing evidence that macrolides demonstrate an anti-inflammatory activity separate from their antibiotic activity has come from Japan in studies concerning the condition diffuse pan-bronchiolitis (DPB). DPB is a disease of adults characterised by chronic inflammation of the respiratory bronchioles and the infiltration of chronic inflammatory cells. Infection with \textit{P. aeruginosa} is frequently present. In 1984, the 5-year survival for patients with DPB infected with \textit{P. aeruginosa} was 26\% and for those without infection the rate was 55\%, despite aggressive treatment with antibiotics and corticosteroids. Since the introduction of erythromycin as the standard therapy, the 10-year survival rate for all cases has increased to 94\%\textsuperscript{47}. In addition, it was noted that the chronic sinusitis that these patients also frequently suffered from, was symptomatically relieved during the erythromycin therapy.\textsuperscript{52}

Following the spectacular results achieved in the treatment of DPB, studies were performed to investigate the effect of macrolide antibiotics in the treatment of related conditions such as bronchiectasis, cystic fibrosis, asthma and chronic sinusitis. Hashiba et al\textsuperscript{34} carried out a study involving 45 patients with chronic sinusitis, 20 of whom had had previous surgery. Patients received an 8 to 12-week course of 400mg of clarithromycin per day. Outcome measures were assessed during the period of treatment and consisted of subjective symptom scores and objective examination findings. Improvement in symptoms and rhinoscopic findings occurred in 71.1\% of patients. The efficacy in terms of duration of administration was noted to increase from 48\% at 4 weeks to 71\% at 12 weeks. The results obtained in this study were similar to those achieved in earlier Japanese studies. Cervin et al\textsuperscript{12} studied 17 patients with persistent rhinosinusitis after sinus surgery. The patients received 3 months of erythromycin at a dose of 250mg daily. The 12 patients who reported a subjective response to treatment continued erythromycin for a further 9 months. At 12 months, there were statistically significant improvements in
saccharine transit time, endoscopic nasal scoring and symptom-specific visual analog scoring. Yamada et al\textsuperscript{111} reported that administration of macrolides to patients with nasal polyps resulted in a decrease in the size of polyps in some patients and that there was a correlation between the reduction in the size of the polyps and the reduction of IL-8 levels in the nasal lavage. Ragab et al\textsuperscript{85} randomised 90 patients with chronic rhinosinusitis to receive either surgical or medical treatment. Those patients receiving medical treatment were given erythromycin 500mg daily for 2 weeks followed by 250mg daily for a further 10 weeks. Patients in the surgical and medical groups used a topical nasal steroid and nasal douches with a saline and sodium bicarbonate solution. At 6 and 12 months follow-up, patients in both groups showed significant improvement in their total visual analogue scores, their individual symptom scores and in their quality of life scores. There was no significant difference between the 2 groups in terms of these outcome measures.

These open-label clinical trials suggest that long-term, low-dose macrolides may be beneficial in the treatment of chronic rhinosinusitis. However, there has never been a published clinical trial which provides level I evidence to support or discourage their use in this setting. In chapter 4 we describe our findings from the first ever double-blinded, randomised, placebo-controlled trial.

Some studies suggest that macrolide antibiotics may also have a role in the treatment of non-infectious asthma. In one such study, patients with asthma were treated with roxithromycin (150 mg) for 8 weeks. Significant reductions were noted in symptoms, eosinophil count, serum eosinophilic cationic protein (ECP), sputum eosinophils and sputum ECP\textsuperscript{91}. It should be noted however that these findings and improvement in airway function may in part be due to the treatment of superimposed infection.

**Mechanisms of action**

In recent years there has been considerable interest in determining the mechanism by which macrolides exert their anti-inflammatory activity. This research has been performed on a number of different tissue types and in a number of different disease
states. The difficulties in performing this analysis reside in our incomplete understanding of the immune system and in the limitations of the in vitro models used for analysis.

**Cytokines**

Suzuki et al\textsuperscript{101} examined the effect of macrolides on IL-8 secretion from cultured nasal epithelial cells obtained from nasal polyps. IL-8 levels as detected by ELISA on culture supernatants were significantly reduced by four different macrolides and the corticosteroid dexamethasone. In that study, IL-8 secretion was not inhibited unless the cells had been pre-treated with macrolides and it was suggested that this may be because the drug needs to be accumulated intracellularly before it can exert its biological action. The 16-membered ring macrolide, josamycin, was not as effective at reducing IL-8 secretion at lower concentrations, which may in part explain why this group of macrolides are not as clinically effective as their 14-membered ring counterparts. Erythromycin has also been shown to reduce IL-8 production by peripheral blood neutrophils\textsuperscript{82}. Neutrophils in the nasal discharge of patients with chronic sinusitis secrete approximately twice as much IL-8 as those in peripheral blood, indicating that they are activated and may induce further neutrophil migration. Erythromycin at concentrations of \(10^{-5}\) and \(10^{-6}\) has been shown to significantly inhibit IL-8 secretion by exudative neutrophils by 54% and 34% respectively. These drug concentrations are approximately the same as levels found in sinus mucosa and nasal discharge during macrolide therapy\textsuperscript{98}. Macrolides by decreasing IL-8 synthesis may therefore reduce neutrophil recruitment and block the vicious cycle of IL-8 production and neutrophil exudation.

A study conducted by Nonaka et al\textsuperscript{80} revealed that roxithromycin suppressed the proliferation of nasal polyp fibroblasts in a dose-dependent manner but had no effect on IL-8 synthesis by those fibroblasts. The release of IL-6, IL-8 and soluble ICAM-1 by cultured human bronchial epithelial cells stimulated with *Haemophilus influenzae* endotoxin or IL-1B, has also been shown to be inhibited by erythromycin\textsuperscript{44}.
TGF-B is upregulated in nasal polyps and inflammatory nasal mucosa. The macrolide FK506 (Tacrolimus) blocks the TGF-B receptor in yeast cells and this may represent a mechanism by which macrolides exert their anti-inflammatory activity.

In clinical studies, macrolides have also been shown to reduce cytokine production. Patients with chronic sinusitis were treated for 14 days with 500 mg of clarithromycin daily. Statistically significant reductions in IL-6, IL-8, TNF-α, elastase and oedema score were observed. A second study, involving chronic bronchitis patients treated for 7 days with clarithromycin, demonstrated reductions in neutrophil activity, IL-8 levels and mucous viscosity.

**Apoptosis**

Apoptosis or programmed cell death of inflammatory cells is accompanied by an attenuation of the activity of these cells. Therapeutic induction of apoptosis therefore provides an opportunity to modulate the inflammatory response. Erythromycin and roxithromycin have been shown to accelerate apoptosis in isolated human neutrophils. Aoshiba et al reported similar findings with erythromycin, roxithromycin and midecamycin.

**Oxidative burst**

Phagocytic cells are capable of producing toxic, reactive oxygen species that are used to destroy phagocytosed microorganisms. These oxygen species are damaging to bacteria and also potentially to host tissues if generated in excess. Macrolides have been reported to produce a dose-dependent reduction in superoxide production by neutrophils. An interesting study by Braga et al showed that rokitamycin was also capable of inhibiting the oxidative burst of neutrophils and that after washing the cells to remove the macrolide, the oxidative burst ability was restored.

**Adhesion**

Recruitment of inflammatory cells to a site of inflammation involves the cells adhering to the vascular endothelium prior to transmigration. Erythromycin has been shown to be
capable of down-regulating the expression of cell-surface adhesion molecules on neutrophils. Matsuoka et al. reported that clarithromycin markedly inhibited the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 by synovial (fibroblast-like) cells. Inhibition of adhesion molecule expression therefore appears to be another possible mechanism by which macrolides exert their anti-inflammatory activity.

**Molecular mechanism of activity**

As discussed above, the clinical effect of macrolides and their effect on inflammatory parameters are being increasingly defined. However, the molecular mechanism by which they produce these effects remains unclear. As discussed earlier the nuclear transcription factor NF-κB plays a key role in the inflammatory process and hence there has recently been interest in determining if macrolides are able to alter its activity.

Miyanohara et al. examined the activity of clarithromycin on cultured human nasal epithelial cells and fibroblasts obtained from nasal polyps. They demonstrated that IL-1β mRNA production was significantly decreased in the presence of clarithromycin. The same cells showed a decrease in the DNA-binding activity of NF-κB following clarithromycin treatment. The authors suggested that clarithromycin may decrease the expression of IL-1β mRNA through suppression of activation of the pro-inflammatory nuclear transcription factor NF-κB. Erythromycin has been shown to inhibit activation of both the NF-κB and AP-1 transcription factors in human bronchial epithelial cells. Ichiyama et al. reported that clarithromycin inhibits NF-κB activation in pulmonary epithelial and blood mononuclear cells. This inhibition was not associated with preservation of the inhibitory IκB protein.

### 1.3.3 Effects on mucociliary clearance

Macrolides may be beneficial in the treatment of chronic sinusitis not only because of their anti-inflammatory and antibiotic effects but also due to effects on mucous
production and mucociliary clearance. The findings of a number of animal and human studies support this theory. Nakano et al\textsuperscript{77} have shown that roxithromycin treatment in rabbits increases the rate of tracheal mucociliary transport. Clarithromycin decreases lipopolysaccharide-induced goblet cell hypersecretion in the guinea-pig trachea\textsuperscript{103}. The abnormal visco-elastic properties of nasal mucous in patients with chronic sinusitis have been improved and thus made more suitable for effective mucociliary clearance after clarithromycin treatment\textsuperscript{87}. These in-vitro findings support the observations of clinical studies in which mucous secretion was reduced and mucociliary clearance was increased\textsuperscript{79,88}.

\begin{table}[h]
\centering
\caption{Targets of macrolide anti-inflammatory activity}
\begin{tabular}{|l|l|l|}
\hline
Cell type & Macrolide effect & Result \\ \hline
Fibroblasts & \downarrow proliferation & \downarrow fibrosis \\ \hline
Fibroblasts & \downarrow ICAM-1 mRNA & \downarrow inflammatory cell recruitment \\ \hline
Fibroblasts & \downarrow NF-kB DNA-binding & \downarrow production inflammatory mediators \\ \hline
Nasal epithelial cells & \downarrow IL-8 production & \downarrow neutrophil recruitment \\ \hline
Peripheral neutrophils & As above & As above \\ \hline
Exudative neutrophils & As above & As above \\ \hline
Nasal epithelial cells & \downarrow IL-1B mRNA & Reduced inflammation \\ \hline
Bronchial epithelial cells & \downarrow IL-6, IL-8, ICAM-1 & \downarrow Inflammatory cell recruitment \\ \hline
Yeast cells & Block TGF-B receptors & \downarrow Inflammatory cell recruitment \\
& & Extracellular matrix production \\
\hline
\end{tabular}
\end{table}
Neutrophils  
Membrane stabilizing  
release of cytotoxic substances

Neutrophils  
↓ oxidant production  
As above

1.5 Hypothesis and aims

This literature review illustrates the fact that in recent years there has been considerable interest in the anti-inflammatory activity of macrolide antibiotics and significant advances in the understanding of how they exert this activity. However, there remains significant areas of interest that have not yet been satisfactorily addressed and which need to be studied in order to enable the clinical role of macrolides to be fully ascertained.

The deficiencies as I see them are:

- An incomplete understanding of the molecular and cellular basis of chronic airway inflammation
- An incomplete determination of the mechanisms by which macrolides exert their anti-inflammatory effect
- A lack of comparisons between existing anti-inflammatory agents (in particular corticosteroids) and macrolides, in terms of their mechanisms of action and the relative efficacy of each
- A lack of studies examining the effect of macrolides in-vivo
- A lack of in-vitro studies that examine the effect of macrolides on whole tissue specimens (i.e. the tissue as it occurs in the body), as opposed to those which examine the artificial situation of individual cell lines
- No level I evidence investigating the use of long-term, low-dose macrolides in the treatment of chronic rhinosinusitis

The hypothesis and specific objectives of this thesis are designed to address some of these deficiencies.

The hypothesis

Macrolide antibiotics exert an anti-inflammatory effect on chronically inflamed upper airway mucosa and this effect is beneficial in the treatment of chronic rhinosinusitis
Aims:

1. To further understanding of the molecular and cellular basis of inflammation in chronic sinusitis
2. To compare the effect of macrolide antibiotics and corticosteroids on inflammatory mediators
3. To investigate the effect of macrolide antibiotics in-vivo and in-vitro
4. To perform in-vitro studies that attempt to mimic the interaction of tissue and drug as it would occur in the natural living state
5. To perform the first double-blinded, randomized, placebo-controlled trial of long-term, low-dose macrolide in the treatment of chronic rhinosinusitis
6. To identify patients who are more or less likely to respond to macrolide treatment
Chapter 2

The anti-inflammatory effect of macrolide antibiotics in vitro
2.1 Introduction

As discussed in the introductory chapter, a number of studies have been performed to investigate the mechanism of the anti-inflammatory effect of macrolide antibiotics. The majority of these studies have been performed on individual cell lines, such as respiratory epithelial cells or inflammatory cells. This represents a somewhat artificial situation in which the effect of macrolide is examined on a particular cell type, rather than on the entire tissue with all the cell types present and with the usual interactions between those cells preserved. For that reason, in the in vitro experiments performed as part of this thesis, whole sections of upper airway mucosa have been used for analysis. In addition, the mucosal specimens were obtained from patients with a confirmed diagnosis of chronic rhinosinusitis. It is hoped that by using such tissue for the in vitro experiments, a more reliable model of chronic upper airway inflammation is obtained and therefore the effect of macrolide in vivo may be more reliably predicted.

**Aims of the in vitro experiments**

1. To examine the effect of macrolide antibiotic on whole sections of chronic rhinosinusitis mucosa in vitro
2. To compare the effect of different concentrations of macrolide with a negative control
3. To compare the in vitro anti-inflammatory effect of macrolide antibiotics with corticosteroids
4. To specifically examine the effect of macrolide and corticosteroid on inflammatory cytokines known to play a role in the pathogenesis of chronic rhinosinusitis (Interleukin-5, Interleukin-8, Granulocyte-macrophage colony stimulating factor, Transforming growth factor-β)
5. To examine and compare the effect of macrolide and corticosteroid on the key pro-inflammatory nuclear transcription factor NF-κB
2.2 Methods

The in vitro experiments described in this chapter were performed on whole sections of chronic rhinosinusitis mucosa obtained from patients undergoing endoscopic sinus surgery. These mucosal biopsies were then cultured for 24 hours in varying concentrations of clarithromycin, prednisolone and control solution. Following culture, specimens were either formalin-fixed for immunohistochemical analysis or homogenized for the purposes of performing cytokine ELISA. A list of materials used in these experiments is provided in Appendix A and a detailed description of experimental methods is given below.

2.2.1 Subjects

As discussed in the introduction to this chapter, an important aim of our in vitro experiments was to use a model of chronic rhinosinusitis that resembled the in vivo situation as closely as possible. To enable this, sinus mucosal specimens were obtained from 11 patients undergoing functional endoscopic sinus surgery for treatment of chronic rhinosinusitis at the Princess Alexandra and Holy Spirit Hospitals, Brisbane, Australia. The diagnosis was made according to the American Task Force on Rhinosinusitis criteria. All patients had experienced symptoms for a minimum of three months. Mucosal biopsies were taken from the lateral nasal wall in the region of the middle meatus.

Patients with a known history of any of the following were excluded:

- Fungal sinusitis
- Immuno-deficiency
- Cystic fibrosis
- Ciliary disorders
- A course of corticosteroids or antibiotics in the 2 weeks preceding surgery

Informed consent was obtained from each of the subjects. The Griffith University, Holy Spirit Hospital and Princess Alexandra Hospital ethics committees approved all
procedures. All procedures were carried out according to the guidelines of the National Health and Medical Research Council of Australia.

### Table 2.1 Individual data for in vitro subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Sex</th>
<th>Age</th>
<th>Asthma History</th>
<th>Skin Prick Test Result</th>
<th>ASA Intolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>62</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>48</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>47</td>
<td>No</td>
<td>Positive</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>60</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>56</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>51</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>27</td>
<td>No</td>
<td>Positive</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>48</td>
<td>Yes</td>
<td>Positive</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>31</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>48</td>
<td>Yes</td>
<td>Positive</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>35</td>
<td>No</td>
<td>Positive</td>
<td>No</td>
</tr>
</tbody>
</table>

#### 2.2.2 Specimen collection

Mucosal specimens were obtained from the 11 subjects discussed above. These specimens were collected by the operating surgeon during endoscopic sinus surgery. Care was taken to obtain mucosa from the nasal cavity and paranasal sinuses that was not polypoid. These specimens were immediately immersed in RPMI Medium 1640 and placed on ice for transport.

#### 2.2.3 Drug preparation

One of the aims of this study was to examine the effect of clarithromycin on the mucosal specimens and to compare this effect with that of the corticosteroid prednisolone and control solutions.
Specimens were treated with prednisolone at concentrations of $10^{-4}$M, $10^{-5}$M, $10^{-6}$M and $10^{-7}$M. To enable this, a prednisolone stock solution at a concentration of $10^{-2}$M was prepared by dissolving prednisolone in 100% ethanol. This stock solution was later added to the appropriate culture wells. The maximum concentration of ethanol in the culture wells was 1% and hence 1% ethanol was used as the treatment control for the prednisolone treatment group.

Specimens were treated with clarithromycin at concentrations of $10^{-5}$M, $10^{-6}$M and $10^{-7}$M. Clarithromycin was not readily soluble in ethanol and hence a $10^{-3}$M stock solution was prepared by dissolving it in 100% methanol with the aid of a 37°C water bath. This stock solution was later added to the appropriate wells. The maximum methanol concentration in the wells was 1% and hence 1% methanol was used as the treatment control for the clarithromycin treatment group.

### 2.2.4 Specimen preparation and culture

On receipt in the laboratory, mucosal specimens were divided into pieces each of approximately 50mg weight. Each of these pieces was then placed into a tissue culture well containing 1 ml of RPMI Medium 1640. Care was taken to ensure that adjacent pieces of the mucosal specimens were placed into wells from which direct comparisons were later to be made. For example, 4 adjacent pieces of mucosa were placed in the four wells that would later be used for immunohistochemical analysis (Fig ****).

To each of the wells was added the appropriate concentration of drug or control. The specimens were then incubated for 24 h at 37°C and 5% CO$_2$.

Following incubation, the mucosal pieces used for immunohistochemical analysis were removed from the wells and immersed in 10% formalin for 4 hours. Each piece was then rinsed 3 times in P.B.S. for 5 minutes and then stored in P.B.S. until paraffin blocks were made.
The pieces planned for frozen section were treated in the same fashion as above except that they were stored in P.B.S. with 30% sucrose overnight. These pieces were then mounted in Cryoform™ embedding tissue medium and frozen at –80°C.

Mucosal pieces scheduled for ELISA analysis were homogenized. To enable this, they were immersed in 500ul of 0.32M sucrose containing Complete™ protease inhibitor cocktail. The procedure was performed on ice to prevent the samples overheating. Following homogenization, the samples were centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was separated and aliquots were made to minimize the number of freeze-thaw cycles necessary. Aliquots were frozen at –80°C.

**Figure 2.1 Layout of tissue culture wells** (A mucosal piece was placed in each of the marked wells and exposed to treatment or control as indicated)

2.2.5 **Bradford protein estimation**
As stated earlier, one of the objectives of this study was to examine the effect of macrolide antibiotics on an in vitro model that resembled as closely as possible the natural state. This is in contrast to the majority of previous similar studies that have focused on cultures of individual cell lines. The advantage of the models used in these earlier studies, is that it is relatively easy to standardize the levels of cytokine production between different specimens by dividing the levels of production by the number of cells in the culture. This technique was not possible in our experiment because it used heterogenous whole tissue sections and hence an alternative was needed.

Two different methods were initially used unsuccessfully as they produced highly erratic results:

- Accurate weighing of the specimens prior to culture and then expression of cytokine production per mg of tissue.
- Culturing the specimens for 24 hours before adding the treatment or control and then culturing for a further 24 hours. ELISA was performed on the culture media after 24 and 48 hours to see if there was a change in cytokine production following the addition of treatment compared to controls.

Following these unsuccessful attempts, it was decided to use the Bradford protein estimation technique to quantify the total protein per ml present in the homogenate. The results of the ELISA could then be expressed as pg of cytokine production per mg of total protein.

A 96-well plate was used for the protein estimation. Six standards of bovine serum albumin were added in triplicate to the wells and three were left blank. The homogenate samples were added in triplicate to the remainder of the wells. 200ul of Bradford solution was added to each. This solution consisted of 2ml Bradford stock solution, 1ml 3M NaCl and 7ml of distilled water.
The plate was read at 595nm using a plate reader and the Softmax Pro software program. A standard curve and results for the individual samples were produced.

### 2.2.6 Cytokine ELISA

Enzyme linked-immunosorbent assay (ELISA) was performed on the homogenates to detect the levels of cytokine production by the cultured mucosal specimens. The cytokines investigated were IL-5, IL-8 and GM-CSF. Commercially available ELISA kits obtained from Biosource International (Camarillo, California) were used for the assays.

Initial testing was carried out to determine the optimum dilution of the homogenates for the assay. These dilutions were:

- **IL-5**: No dilution, 100 ul of homogenate added to each microtiter well
- **IL-8**: 1:5 dilution, 50 ul of homogenate added to each microtiter well
- **GM-CSF**: No dilution, 50 ul of homogenate added to each microtiter well

Dilutions were made with the standard diluent buffer present in the assay kit. A range of human cytokine standards were prepared as per the kit instructions. The appropriate volume of standard or homogenate sample was then added to the microtiter wells, onto which was coated an antibody specific for the cytokine. During the first 90-minute incubation, the cytokine antigen bound to the immobilized antibody in the wells. After washing with the provided wash buffer, 100ul of a biotinylated antibody specific for the human cytokine was added. During this second 30-minute incubation, this antibody bound to the human cytokine captured during the first incubation. After another wash cycle, 100ul of streptavidin-peroxidase was added. During the third incubation of 30-minutes, the enzyme bound to the biotinylated antibody to complete the four-member sandwich. 100ul of a substrate solution was then added for a further 30-minutes. This substrate was acted upon by the bound enzyme to produce a colour change. 100ul of a stop solution was added to terminate the reaction.

Within 2 hours of adding the stop solution, the absorbance of each well was read at 450 nm. The individual results for the samples and a standard curve were printed.
2.2.7 Immunohistochemistry

Immunohistochemistry was performed on sections from the tissue culture specimens. All specimens had been fixed in formalin as previously described. 5μm sections were cut from the paraffin blocks and placed on Superfrost Plus™ slides (Menzel Glaser, Germany).

De-waxing

Slides were dried overnight in an oven at 37°C. They were then transferred to an oven at 60-62°C for 1 hour. After removal the slides were immersed in Xylene for 2 periods of 5 minutes. The sections were then rehydrated through baths containing descending grades of ethanol as follows:

- Ethanol 100% - 5 minutes
- Ethanol 100% - 5 minutes
- Ethanol 100% - 5 minutes
- Ethanol 90% - 5 minutes
- Ethanol 70% - 5 minutes

The sections were at no stage allowed to dry out from this point on during the staining process.

Antigen retrieval

Antigen retrieval was performed prior to immunohistochemical staining in all cases. Slides were transferred to 10mM EDTA pH 7.5 for 2 minutes. The buffer was tipped off and the slide carrier was refilled with EDTA. The slides were autoclaved for 10 minutes at 121°C. The autoclave was then allowed to cool to room temperature before the slides were removed.

A number of alternative methods of antigen retrieval were trialed during this study however, none of these were as effective as the high temperature protocol described above. The methods attempted included:

- Immersion in 0.1 or 1M NaOH for 20 minutes
- Boiling in citrate buffer for 10 minutes
- Immersion in Xylene for 10 minutes

**Antibodies**
Details of the antibodies used in the project are given in appendix A. For each of these antibodies, a series of experiments were initially performed to determine the optimal staining procedure and the appropriate dilution. Table 2.2 shows the dilutions and concentrations at which these antibodies were used. Antibodies were diluted with T.B.S.

**Table 2.2  Antibodies used during immunohistochemical staining**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human NFkB</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-human TGF-β₁₋₃</td>
<td>1:100</td>
</tr>
<tr>
<td>Biotinylated anti-mouse</td>
<td>1:200</td>
</tr>
<tr>
<td>Biotinylated anti-rabbit</td>
<td>1:200</td>
</tr>
<tr>
<td>Mouse immunoglobulin</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit immunoglobulin</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

**TGF-β Immunohistochemistry technique**
After antigen retrieval, slides were transferred into T.B.S. twice for 5 minutes and then dried and painted with a Dako pen. The slides were blocked for 60 minutes in 3% skim milk. Following a 10 second wash in T.B.S., the primary antibody (TGF-β or NF-κB) was applied to the sections for 60 minutes. The slides were washed twice for 5 minutes in T.B.S. Endogenous peroxidase activity was blocked using 3% H₂O₂ in T.B.S. for 30 minutes. The slides were again washed twice for 5 minutes in T.B.S. Following this a rabbit anti-mouse biotinylated secondary antibody was applied for 30 minutes. The slides were washed twice for 5 minutes in T.B.S and then Streptavidin-Horse radish peroxidase (1:300) was applied for 30 minutes. The slides were washed twice for 5 minutes in T.B.S. and then the streptavidin ABC/Alkaline phosphatase complex was applied for 30 minutes. Another 2 washes with T.B.S. was performed and then DAB was applied for 60-90 seconds. The slides were again washed twice for 5 minutes in T.B.S. Fast Red substrate was applied for 3-5 minutes. The slides were washed twice for 5 minutes in
T.B.S. Nuclei were counterstained with Mayer’s hematoxylin for 45 seconds and slides were then held under running water for 2 minutes. Cover slips were applied using an aqueous mounting medium. All incubations were performed at room temperature.

**NF-κB immunohistochemistry technique**

Nuclear counterstaining was not performed with NFκB staining as it tended to obscure the nuclear localization of the primary antibody. The staining method is described below. After antigen retrieval slides were washed twice for 5 minutes in T.B.S. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ for 30 minutes. The slides were then immersed in 3% skim milk powder for 60 minutes. After a 10 second wash in T.B.S. the mouse anti-human NFκB antibody was applied for 60 minutes. Triton X-100 was added to the primary antibody at a strength of 0.01%. The slides were washed twice for 5 minutes in T.B.S. A rabbit anti-mouse biotinylated secondary antibody was applied for 30 minutes. The slides were again washed twice for 5 minutes in T.B.S. and then solution from the Vectastain ABC kit was applied to the sections for 30 minutes. The slides were washed twice for 5 minutes in T.B.S. DAB was applied for 60-90 seconds. Cover slips were applied using an aqueous mounting medium. All incubations were carried out at room temperature.

**Negative controls**

For all of the cultured biopsy specimens, sections were stained exactly as described above except that the primary antibody was omitted and was replaced with mouse immunoglobulin as the negative control. In addition, on every staining run, sections were allocated to serve as negative controls and were treated with rabbit or mouse immunoglobulin instead of the primary antibody.

**Quantification**

All sections were encoded and counted by the author in a blinded fashion. An Olympus microscope was used with a x 10 eyepiece and an objective with a magnification of x 40. For each specimen at least 2 sections were immunostained and at least 4 fields were counted on each section. The area of interest was the superficial stromal layer and thus one edge of the field was aligned along the epithelial basement membrane. When
aligning the field, areas with a prominent glandular component were avoided in order to give a representative count. All positively staining cells in the field were counted and the way in which the counts were expressed for the different stains is discussed in the relevant results section.

2.2.8 Statistical analysis
Statistical evaluation was performed using the statistical software package SPSS (Chicago, USA). A $p$ value $< 0.05$ was considered to be statistically significant. Results are expressed as mean +/- standard error of the mean (SEM). The statistical tests used for the analysis of the data from each experiment are discussed in the relevant results section.

2.3 Results
2.3.1 Cytokine ELISA
As mentioned previously, it was necessary to derive a method of expressing the amount of cytokine production by the cultured mucosal specimens which took into account the different sizes of the mucosal pieces and the inevitable differences in the population of cells. In order to do this, the results were expressed as pg of cytokine in the tissue homogenate per mg of total protein.

In order to determine a concentration-dependent drug effect on cytokine production, a repeated measures ANOVA was performed on the log-transformed data. Tests of within-subjects contrasts were performed to determine if the mean levels of cytokine production in the treatment and control groups were significantly different. The Wilcoxon signed rank test was used to determine if there were significant differences in cytokine production between the clarithromycin and prednisolone groups. A $p$ value $< 0.05$ was considered to be statistically significant. Results are expressed as mean +/- standard error of the mean (SEM).

IL-5 ELISA
The concentration-dependent reduction in IL-5 production by specimens cultured in the presence of clarithromycin was significant ($F_{1,10}=5.627, p<0.039$). Maximum effect was seen at a concentration of $10^{-6}$ M, at which production compared to controls was reduced by 67 +/- 9%. Individual subject results are shown in Table 2.3 and the means are represented graphically in Fig 2.2.

Prednisolone also demonstrated a significant concentration effect ($F_{1,10}=8.986, p<0.013$). Maximal reduction was seen at a concentration of $10^{-5}$ M and was 59 +/- 18% (Table 2.4 and Figure 2.2).

Table 2.3 Effect of various concentrations of clarithromycin on IL-5 production compared to control (Data are expressed as pg per mg total protein)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>$10^{-8}$M</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.8</td>
<td>26.8</td>
<td>6.2</td>
<td>38.7</td>
</tr>
<tr>
<td>2</td>
<td>8.6</td>
<td>0.4</td>
<td>0.7</td>
<td>39.5</td>
</tr>
<tr>
<td>3</td>
<td>35.8</td>
<td>10.5</td>
<td>4.9</td>
<td>13.5</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>52.8</td>
<td>316.7</td>
<td>350.5</td>
</tr>
<tr>
<td>5</td>
<td>237.9</td>
<td>101.9</td>
<td>202.8</td>
<td>395</td>
</tr>
<tr>
<td>6</td>
<td>333</td>
<td>130.7</td>
<td>320</td>
<td>315</td>
</tr>
<tr>
<td>7</td>
<td>30.1</td>
<td>22.2</td>
<td>35.6</td>
<td>19.3</td>
</tr>
<tr>
<td>8</td>
<td>166.2</td>
<td>217</td>
<td>497.2</td>
<td>990</td>
</tr>
<tr>
<td>9</td>
<td>39.1</td>
<td>84.7</td>
<td>107.9</td>
<td>71.2</td>
</tr>
<tr>
<td>10</td>
<td>39.2</td>
<td>62.3</td>
<td>77.1</td>
<td>58.4</td>
</tr>
<tr>
<td>11</td>
<td>14.5</td>
<td>129.8</td>
<td>83.4</td>
<td>234.7</td>
</tr>
<tr>
<td>Mean</td>
<td>84.6</td>
<td>76.3</td>
<td>150.2</td>
<td>229.6</td>
</tr>
<tr>
<td>Standard error of the mean</td>
<td>33.3</td>
<td>19.7</td>
<td>49.4</td>
<td>87.6</td>
</tr>
</tbody>
</table>

Table 2.4 Effect of various concentrations of prednisolone on IL-5 production compared to control (Data are expressed as pg per mg total protein)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>$10^{-4}$M</th>
<th>$10^{-5}$M</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91.4</td>
<td>133</td>
<td>12.1</td>
<td>37</td>
<td>138</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>51.8</td>
<td>73.7</td>
<td>56.6</td>
<td>29.5</td>
</tr>
<tr>
<td>3</td>
<td>9.4</td>
<td>17.1</td>
<td>16.1</td>
<td>21.4</td>
<td>19.3</td>
</tr>
</tbody>
</table>
Table

<table>
<thead>
<tr>
<th></th>
<th>359</th>
<th>30.8</th>
<th>418.9</th>
<th>588.3</th>
<th>641.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>371.5</td>
<td>149.3</td>
<td>94.5</td>
<td>20.8</td>
<td>187.6</td>
</tr>
<tr>
<td>5</td>
<td>47.5</td>
<td>81.8</td>
<td>98.7</td>
<td>465</td>
<td>113.3</td>
</tr>
<tr>
<td>6</td>
<td>16.7</td>
<td>3</td>
<td>16.2</td>
<td>35.6</td>
<td>41.5</td>
</tr>
<tr>
<td>7</td>
<td>453.8</td>
<td>424.5</td>
<td>650</td>
<td>512</td>
<td>704.5</td>
</tr>
<tr>
<td>8</td>
<td>70.6</td>
<td>24.2</td>
<td>168.7</td>
<td>100.2</td>
<td>145.4</td>
</tr>
<tr>
<td>9</td>
<td>54.5</td>
<td>4</td>
<td>74.8</td>
<td>37.6</td>
<td>118.5</td>
</tr>
<tr>
<td>10</td>
<td>2.1</td>
<td>5</td>
<td>8.9</td>
<td>9.1</td>
<td>101</td>
</tr>
</tbody>
</table>

Mean 134.3 84 151.5 171.2 203.6
Standard error of the mean 51.6 37.4 60.6 68.8 71.8

Figure 2.2 IL-5 production in the presence of clarithromycin and prednisolone compared to controls. A statistically significant, linear concentration-dependent reduction was seen with clarithromycin \( p<0.039 \) and prednisolone \( p<0.013 \). (The y-axis denotes cytokine levels expressed as pg per mg total protein).

**IL-8 ELISA**

Clarithromycin produced a significant concentration-dependent reduction in IL-8 production \( F_{1.10}=10.479, p<0.009 \). Maximal reduction was obtained at a concentration of \( 10^{-5} \)M and was 34 +/- 16% (Table 2.5 and Figure 2.3).
Prednisolone also produced a significant reduction in IL-8 production ($F_{1,10}=11.578$, $p<0.007$). $10^{-5}$M prednisolone reduced IL-8 production compared to control by 45 +/- 13% (Table 2.6 and Figure 2.3).

Table 2.5 Effect of various concentrations of clarithromycin on IL-8 production compared to control (Data are expressed as pg per mg total protein)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>$10^{-5}$M</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>133.4</td>
<td>345.5</td>
<td>146.8</td>
<td>458</td>
</tr>
<tr>
<td>2</td>
<td>237.7</td>
<td>281.1</td>
<td>233</td>
<td>491</td>
</tr>
<tr>
<td>3</td>
<td>1131.8</td>
<td>423.9</td>
<td>730.9</td>
<td>1120</td>
</tr>
<tr>
<td>4</td>
<td>1344.7</td>
<td>876.4</td>
<td>2574</td>
<td>900</td>
</tr>
<tr>
<td>5</td>
<td>1378</td>
<td>805.2</td>
<td>1499</td>
<td>1350</td>
</tr>
<tr>
<td>6</td>
<td>1341.3</td>
<td>1818.7</td>
<td>3600</td>
<td>2260.5</td>
</tr>
<tr>
<td>7</td>
<td>940.2</td>
<td>1473.1</td>
<td>913.8</td>
<td>1810.4</td>
</tr>
<tr>
<td>8</td>
<td>422.1</td>
<td>930.5</td>
<td>1087.3</td>
<td>562.3</td>
</tr>
<tr>
<td>9</td>
<td>181.9</td>
<td>297</td>
<td>1043</td>
<td>783.7</td>
</tr>
<tr>
<td>10</td>
<td>150</td>
<td>622.3</td>
<td>845.4</td>
<td>937.5</td>
</tr>
<tr>
<td>11</td>
<td>204.1</td>
<td>335.8</td>
<td>351.2</td>
<td>573.6</td>
</tr>
<tr>
<td>Mean</td>
<td>678.5</td>
<td>976.1</td>
<td>1183.9</td>
<td>1022.3</td>
</tr>
<tr>
<td>Standard error of the mean</td>
<td>163.9</td>
<td>234.8</td>
<td>315.7</td>
<td>174.8</td>
</tr>
</tbody>
</table>

Table 2.6 Effect of various concentrations of prednisolone on IL-8 production compared to control (Data are expressed as pg per mg total protein)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>$10^{-3}$M</th>
<th>$10^{-5}$M</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>112.3</td>
<td>214.1</td>
<td>203.7</td>
<td>126.8</td>
<td>219.3</td>
</tr>
<tr>
<td>2</td>
<td>52.6</td>
<td>188.6</td>
<td>63</td>
<td>222</td>
<td>340</td>
</tr>
<tr>
<td>3</td>
<td>623.1</td>
<td>623.5</td>
<td>289</td>
<td>1067.5</td>
<td>760.3</td>
</tr>
<tr>
<td>4</td>
<td>706.1</td>
<td>1184.5</td>
<td>633.2</td>
<td>438.1</td>
<td>1400</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>674</td>
<td>1140</td>
<td>1733</td>
<td>2530</td>
<td>1340</td>
</tr>
<tr>
<td></td>
<td>3167.2</td>
<td>1238.1</td>
<td>2044.3</td>
<td>2668.7</td>
<td>2400.2</td>
</tr>
<tr>
<td></td>
<td>726.1</td>
<td>537.1</td>
<td>858.4</td>
<td>908.5</td>
<td>1880</td>
</tr>
<tr>
<td></td>
<td>362.5</td>
<td>174.1</td>
<td>207.6</td>
<td>730</td>
<td>700.6</td>
</tr>
<tr>
<td></td>
<td>56.7</td>
<td>101</td>
<td>114</td>
<td>80.2</td>
<td>620.4</td>
</tr>
<tr>
<td></td>
<td>499.5</td>
<td>404.7</td>
<td>588.7</td>
<td>707</td>
<td>375.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>254.1</td>
<td>40</td>
<td>447.2</td>
<td>901.2</td>
</tr>
</tbody>
</table>

Mean | 637.2 | 550.8 | 615.8 | 902.3 | 994  |
Standard error of the mean | 266.9 | 132.3 | 206.2 | 270.1 | 207.7 |

**IL-8 (pg/mg total protein)**

**Figure 2.3** IL-8 production in the presence of clarithromycin and prednisolone compared to controls. A statistically significant, linear concentration-dependent reduction was seen with clarithromycin ($p<0.009$) and prednisolone ($p<0.007$). (The y-axis denotes cytokine levels expressed as pg per mg total protein)

**GM-CSF ELISA**

Clarithromycin demonstrated a significant concentration effect on GM-CSF production ($F_{1,10}=8.165$, $p<0.017$). Maximal reduction was seen at a concentration of $10^{-5} M$ and was $55 +/- 13\%$ (Table 2.7 and Figure 2.4).
Prednisolone also showed a significant concentration effect ($F_{1,10}=7.021, p<0.024$). Maximal reduction was seen at a concentration of $10^{-4}$M and was 59 +/- 16% (Table 2.8 and Figure 2.4).

Table 2.7 Effect of various concentrations of clarithromycin on GM-CSF production compared to control (Data are expressed as pg per mg total protein)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>$10^{-5}$M</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>7.4</td>
<td>21</td>
<td>66.3</td>
</tr>
<tr>
<td>2</td>
<td>20.1</td>
<td>11.3</td>
<td>137.2</td>
<td>65.3</td>
</tr>
<tr>
<td>3</td>
<td>34.8</td>
<td>91.1</td>
<td>31.8</td>
<td>49.2</td>
</tr>
<tr>
<td>4</td>
<td>49.9</td>
<td>29.7</td>
<td>39.4</td>
<td>19.7</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>59.6</td>
<td>41.3</td>
<td>129</td>
</tr>
<tr>
<td>6</td>
<td>27.6</td>
<td>40.6</td>
<td>10.7</td>
<td>45.8</td>
</tr>
<tr>
<td>7</td>
<td>92.9</td>
<td>47</td>
<td>525.4</td>
<td>143.2</td>
</tr>
<tr>
<td>8</td>
<td>11.8</td>
<td>1.2</td>
<td>56.2</td>
<td>137.5</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3</td>
<td>14.9</td>
<td>19.5</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>7.5</td>
<td>6.4</td>
<td>11.3</td>
</tr>
<tr>
<td>11</td>
<td>15.5</td>
<td>40</td>
<td>4.5</td>
<td>25.8</td>
</tr>
</tbody>
</table>

Mean: 29.2, 30.8, 80.8, 64.8

Standard error of the mean: 8.4, 8.5, 45.9, 14.9

Table 2.8 Effect of various concentrations of prednisolone on GM-CSF production compared to control (Data are expressed as pg per mg total protein)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>$10^{-4}$M</th>
<th>$10^{-5}$M</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.4</td>
<td>80.8</td>
<td>31.2</td>
<td>4.6</td>
<td>22.4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>32.3</td>
<td>23.8</td>
<td>43.1</td>
<td>29.2</td>
</tr>
<tr>
<td>3</td>
<td>8.8</td>
<td>34.1</td>
<td>52.3</td>
<td>70.2</td>
<td>61.5</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>16.4</td>
<td>11.6</td>
<td>11.4</td>
<td>178.5</td>
</tr>
<tr>
<td>5</td>
<td>86.2</td>
<td>66.2</td>
<td>8.3</td>
<td>117.5</td>
<td>59.6</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>28.8</td>
<td>49.3</td>
<td>44.5</td>
<td>33.5</td>
</tr>
<tr>
<td>7</td>
<td>17.4</td>
<td>6.5</td>
<td>25.4</td>
<td>86.3</td>
<td>81.5</td>
</tr>
<tr>
<td>8</td>
<td>22.4</td>
<td>4.7</td>
<td>1.1</td>
<td>6</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>12.7</td>
<td>25.7</td>
<td>21.3</td>
<td>39.4</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1.1</td>
<td>7.7</td>
<td>3.8</td>
<td>59.2</td>
</tr>
<tr>
<td>11</td>
<td>2.1</td>
<td>31</td>
<td>62.5</td>
<td>24.6</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td><strong>Mean</strong></td>
<td>23.6</td>
<td>29.7</td>
<td>26.8</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td><strong>Standard error of the mean</strong></td>
<td>9.2</td>
<td>7.5</td>
<td>6.1</td>
<td>11.2</td>
</tr>
</tbody>
</table>

*Figure 2.4* GM-CSF production in the presence of clarithromycin and prednisolone compared to controls. A statistically significant, linear concentration dependent reduction was seen with clarithromycin \((p<0.017)\) and prednisolone \((p<0.024)\). (The y-axis denotes cytokine levels expressed as pg per mg total protein)

**Comparing clarithromycin and prednisolone**

The Wilcoxon signed rank test was performed to determine if there were differences in cytokine production by the mucosal specimens when cultured in clarithromycin compared to prednisolone. No significant differences were noted between the 2 treatment groups for any of the cytokines tested.
2.3.3 In vitro specimen immunohistochemistry

Mucosal specimens obtained from each of the 10 patients undergoing sinus surgery were cultured for 24 hours in the presence of clarithromycin and prednisolone, each at a concentration of $10^{-5}$M. To serve as controls, specimens from each patient were also cultured in methanol and ethanol. Immunohistochemistry was performed on these cultured specimens as described in the methods section. The Wilcoxon signed-rank test was applied to the data in order to determine if there was a difference between the treatment and control groups. A p-value of <0.05 was considered to be significant.

Nuclear factor-kappaB

Eight of the 10 mucosal specimens cultured in clarithromycin showed a reduction in the expression of NF-κB. However, this reduction failed to reach statistical significance (Table 2.9 and Figure 2.5). Of the 10 specimens cultured in prednisolone, 6 demonstrated reduced expression of NF-κB and once again, a statistically significant reduction was not seen.

Table 2.9 Effect of clarithromycin and prednisolone on NFκB expression compared to control (Results are expressed as the number of positively staining cells per field)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Clarithromycin $10^{-5}$M</th>
<th>Control (Methanol)</th>
<th>Prednisolone $10^{-5}$M</th>
<th>Control (Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>4.5</td>
<td>8.25</td>
<td>9.5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.25</td>
<td>2.25</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>6.5</td>
<td>3</td>
<td>1.25</td>
<td>8.5</td>
</tr>
<tr>
<td>5</td>
<td>1.75</td>
<td>3.25</td>
<td>3.66</td>
<td>4.25</td>
</tr>
<tr>
<td>6</td>
<td>1.25</td>
<td>2.25</td>
<td>4.25</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>3.25</td>
<td>6.66</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>5.5</td>
<td>4</td>
<td>9.25</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>1</td>
<td>2.75</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>0.75</td>
<td>2</td>
<td>1</td>
<td>3.25</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
<td>Control</td>
<td>Prednisolone</td>
<td>Control</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------</td>
<td>----------</td>
<td>--------------</td>
<td>----------</td>
</tr>
<tr>
<td>Mean</td>
<td>2.5</td>
<td>3.49</td>
<td>3.44</td>
<td>4.55</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.61</td>
<td>0.67</td>
<td>0.60</td>
<td>1.11</td>
</tr>
</tbody>
</table>

**Figure 2.5** Expression of NF-κB in mucosal specimens cultured in clarithromycin or prednisolone compared to controls. Statistically significant reductions in NF-κB expression were not seen with clarithromycin or prednisolone.

**Transforming growth factor-β**

TGF-β expression was reduced in 9 of the 10 mucosal specimens cultured in both clarithromycin and prednisolone. Compared to specimens cultured in control solution, clarithromycin and prednisolone produced statistically significantly reductions in TGF-β expression (p=0.009 and 0.04 respectively) (Table 2.10 and Figure 2.6).
Figure 2.6 Effect of clarithromycin and prednisolone on TGF-β expression compared to controls (Results are expressed as the percentage of positively staining cells per field)

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Clarithromycin $10^{-5}$M</th>
<th>Control (Methanol)</th>
<th>Prednisolone $10^{-5}$M</th>
<th>Control (Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>72</td>
<td>69</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>52</td>
<td>44</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>93</td>
<td>58</td>
<td>87</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>71</td>
<td>81</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>78</td>
<td>47</td>
<td>78</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>60</td>
<td>39</td>
<td>55</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>81</td>
<td>73</td>
<td>81</td>
</tr>
<tr>
<td>8</td>
<td>51</td>
<td>54</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>79</td>
<td>70</td>
<td>76</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>73</td>
<td>64</td>
<td>71</td>
</tr>
<tr>
<td>Mean</td>
<td>53</td>
<td>71</td>
<td>58</td>
<td>69</td>
</tr>
<tr>
<td>SEM</td>
<td>4.1</td>
<td>4.0</td>
<td>5.1</td>
<td>4.2</td>
</tr>
</tbody>
</table>
Table 2.10 Expression of TGF-B in mucosal specimens cultured in clarithromycin or prednisolone compared to controls. Statistically significant reductions in TGF-B expression were seen with clarithromycin and prednisolone.

2.4 Discussion

In the experiments described above we have shown that clarithromycin produced a statistically significant, dose-dependent reduction in production of interleukin-8, interleukin-5 and granulocyte-macrophage colony stimulating factor by whole sections of chronic rhinosinusitis mucosa. This reduction was equivalent to that seen with prednisolone. We have also demonstrated a statistically significant reduction in the expression of TGF-β following in vitro treatment with clarithromycin and prednisolone. No significant reduction was seen in the expression of NF-κB.

In the introduction to this chapter the aims of our in vitro experiments were outlined. Our first aim was to determine the mechanism of action of the anti-inflammatory effect of macrolide antibiotics in an in vitro model of chronic rhinosinusitis. ELISA and immunohistochemical staining were performed on whole sections of diseased mucosa after culture with varying concentrations of clarithromycin, prednisolone and control solution. This experimental technique differed from the majority of previously published studies in which isolated cell lines were used. We believe that our model provides a truer representation of the in vivo effect of macrolide antibiotics as the full complement of resident cells with the usual complex interactions between them were present in the whole mucosal sections.

The ELISA experiments showed that clarithromycin is capable of producing statistically significant reductions in the production of the pro-inflammatory cytokines interleukin-8, interleukin-5 and GM-CSF. The inhibition of production of the latter two cytokines has not previously been reported in experiments involving nasal mucosa. Our ELISA experiments also showed a significant inhibition of cytokine production by the
corticosteroid prednisolone. Interestingly, there was no significant difference between the reduction produced by clarithromycin and that produced by prednisolone. This finding helps to demonstrate the magnitude of the in vitro anti-inflammatory effect of macrolides.

Transforming growth factor-β is a potent inflammatory cell chemo-attractant and enhances extracellular matrix production. Increased expression has previously been demonstrated in inflamed nasal mucosa but not in disease-free controls\textsuperscript{14}. Immunohistochemical staining for TGF-β showed that both clarithromycin and prednisolone in vitro were capable of producing a statistically significant reduction in expression. Inhibition of TGF-β expression by macrolide antibiotics in any tissue type has not previously been reported in the literature and represents an additional possible mechanism of the anti-inflammatory effect of macrolides.

Nuclear factor-kappaB is a key pro-inflammatory nuclear transcription factor that is upregulated in many disorders of inflammation, including chronic rhinosinusitis. Previous studies have shown that macrolides are capable of inhibiting the activation of NF-κB and have suggested this action as the underlying molecular mechanism of their anti-inflammatory effect\textsuperscript{73}. In our in vitro experiments we did not show a statistically significant reduction in NF-κB expression with macrolide treatment although reductions were seen in eight of the ten specimens.

Further discussion of our results and how they compare with those of previous authors can be found in the discussion chapter of this thesis.
Chapter 3
The anti-inflammatory effect of macrolide antibiotics in vivo

3.1 Introduction

In our second series of experiments we aimed to examine the potential anti-inflammatory effect of macrolide antibiotics in vivo. Specifically, we wanted to determine if the in vitro effect of macrolide that we had noted in the experiments described in the previous chapter, were present in patients with chronic rhinosinusitis who were treated with long-term, low-dose macrolide. We hypothesized that the anti-inflammatory effect seen in vitro would also be seen in patients receiving a course of macrolide antibiotic and that this effect would explain the apparent clinical benefit of macrolide treatment in patients with chronic rhinosinusitis.

Ten patients with chronic rhinosinusitis who were enrolled in a clinical trial of long-term, low-dose macrolide treatment, had nasal mucosa biopsies taken before and after their treatment. These biopsies were examined using immunohistochemical techniques to determine the expression of markers for inflammatory cell populations, TGF-β and NF-κB.
Aims of the in vivo experiments

1. To examine the potential mechanism of the anti-inflammatory effect of macrolide antibiotics in patients with chronic rhinosinusitis receiving a course of clarithromycin
2. To compare any possible in vivo effect with that seen in vitro
3. To determine whether macrolide treatment alters inflammatory cell populations in patients with chronic rhinosinusitis
4. To determine whether macrolide treatment alters the expression of TGF-β
5. To determine whether macrolide treatment alters the expression of the key pro-inflammatory nuclear transcription factor NF-κB

3.2 Materials

The materials used in the immunohistochemical staining of the in vivo biopsy specimens were the same as those used in the in vitro experiments and are listed along with the antibodies used in appendix A.

3.3 Methods

Ten patients with chronic rhinosinusitis were treated for 12 weeks with 250mg of clarithromycin daily. Nasal mucosal biopsies were taken before and following macrolide treatment. These biopsies were formalin-fixed and stained immunohistochemically for inflammatory cell markers, NF-κB and TGF-β.

3.3.1 In vivo subjects

Nasal mucosal specimens were obtained from 10 subjects for immunohistochemical analysis. These specimens were obtained from subjects who were recruited into a study
being conducted at the Princess Alexandra Hospital and Griffith University by Associate Professor Anders Cervin. In brief, adult patients with a history of chronic sinusitis were recruited by ENT specialists. All subjects had previously had endoscopic sinus surgery and suffered from persistent symptoms. Each was treated with a 3-month course of Clarithromycin (Abbott Australasia, Sydney, Australia) 250mg once daily. Concurrent treatment with corticosteroids was not permitted during the trial period. Subjects were reviewed at the commencement of treatment, at 6 weeks and at the end of their course. During these visits, possible side effects were discussed, questionnaires were completed and several objectives tests were performed to determine clinical progress. Informed consent was obtained from each of the subjects. The Griffith University and Princess Alexandra Hospital ethics committees approved all procedures. All procedures were carried out according to the guidelines of the National Health and Medical Research Council of Australia. Results obtained from this clinical study are awaiting publication.

Ten of the subjects, recruited into this clinical trial, underwent middle turbinate biopsy at the commencement and conclusion of the 3-month trial period. Individual data for these patients are shown in table 3.1. Once obtained the biopsies were formalin-fixed and used for immunohistochemical analysis.

**Table 3.1 Individual data for the subjects involved in the in vivo experiments**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>Asthma History</th>
<th>Skin Prick Test Result</th>
<th>ASA Intolerance</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>62</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>38</td>
<td>Yes</td>
<td>Positive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>61</td>
<td>Yes</td>
<td>Positive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>57</td>
<td>No</td>
<td>Positive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>49</td>
<td>Yes</td>
<td>Negative</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>54</td>
<td>No</td>
<td>Negative</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
3.3.2 Inflammatory cell immunohistochemical staining

Double immunohistochemical staining was performed for staining with CD 3 (T-lymphocytes) and CD 68 (Macrophages) and for CD 8 (Cytotoxic T-lymphocytes). Representative sections of stained slides and a negative control are shown in figures 3.1-3.3. The method used is described below. All incubations were carried out at room temperature.

After antigen retrieval, slides were transferred into T.B.S. twice for 5 minutes. Slides were then dried and painted with a Dako pen. The slides were blocked for 60 minutes in 3% skim milk. Following a 10 second wash in T.B.S., the first primary antibody (CD 3 or CD 8) was applied to the sections for 60 minutes. The slides were washed twice for 5 minutes in T.B.S. Endogenous peroxidase activity was blocked using 3% H$_2$O$_2$ in T.B.S. for 30 minutes. The slides were again washed twice for 5 minutes in T.B.S. The appropriate biotinylated secondary antibody was then applied for 30 minutes. The slides were washed twice for 5 minutes in T.B.S. Streptavidin-Horse radish peroxidase (1:300) was applied for 30 minutes. The slides were washed twice for 5 minutes in T.B.S. The second primary antibody (CD 68 or CD 20) was applied for 60 minutes. The slides were washed twice for 5 minutes in T.B.S. The appropriate biotinylated secondary antibody was then applied for 30 minutes and the slides were washed twice for 5 minutes in T.B.S. The streptavidin ABC/Alkaline phosphatase complex was applied for 30 minutes. The slides were washed twice for 5 minutes in T.B.S. DAB was applied for 60-90 seconds. The slides were again washed twice for 5 minutes in T.B.S. Fast Red substrate was applied for 3-5 minutes and the slides were washed twice for 5 minutes in T.B.S.
Nuclei were counterstained with Mayer’s hematoxylin for 45 seconds. Slides were then held under running water for 2 minutes. Cover slips were applied using an aqueous mounting medium.

Figure 3.1 Representative section of nasal mucosa stained for CD3 +ve T-lymphocytes and CD68 +ve macrophages. Nuclear counterstaining with haematoxylin was performed.

Figure 3.2 Representative section of nasal mucosa stained for CD8 +ve T-lymphocytes. Nuclear counterstaining with haematoxylin was performed.
Figure 3.3 Representative section of nasal mucosa prepared as negative control. Nuclear counterstaining with haematoxylin was performed.

3.3.3 Transforming growth factor-B$_{1-3}$ staining

The procedure for TGF-B$_{1-3}$ staining was the same as that described in the methods section of chapter 2. A representative section of a stained slide and negative control are shown in figures 3.4 and 3.5.

Figure 3.4 Representative section of nasal mucosa stained for TGF-β. Nuclear counterstaining with haematoxylin was performed.
Figure 3.5 Representative section of negative control of nasal mucosa, Nuclear counterstaining with haematoxylin was performed.

3.3.4 Nuclear factor-kappaB staining

NF-κB staining was performed using the same technique described in the methods section of chapter 2. A representative section of a stained slide and negative control are shown in figures 3.6 and 3.7.

Figure 3.6 Representative section of nasal mucosa stained for NF-κB.

Figure 3.7 Representative section of negative control of nasal mucosa.

3.3.5 Neutrophil staining
Formalin-fixed mucosal biopsies were stained with haematoxylin and eosin in the Princess Alexandra Pathology laboratory according to the laboratory’s standard technique. Neutrophils on these slides were identified and quantified by their characteristic cytologic appearance.

Table 3.2 Antibody dilutions used in immunohistochemical staining of the in vivo biopsy specimens

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CD 3</td>
<td>1:150</td>
</tr>
<tr>
<td>Anti-human CD 8</td>
<td>1:50</td>
</tr>
<tr>
<td>Anti-human CD 68</td>
<td>1:50</td>
</tr>
<tr>
<td>Biotinylated anti-mouse</td>
<td>1:200</td>
</tr>
<tr>
<td>Biotinylated anti-rabbit</td>
<td>1:200</td>
</tr>
<tr>
<td>Mouse immunoglobulin</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit immunoglobulin</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

3.3.6 Negative controls

For all of the in vivo biopsy specimens, sections were stained exactly as described above except that the primary antibody was omitted and was replaced with either rabbit or mouse immunoglobulin. Rabbit immunoglobulin was used as the negative control for the CD 3 staining as this antibody was raised in rabbits. Mouse immunoglobulin was used as the negative control for all the other antibodies. In addition, on every staining run, sections were allocated to serve as negative controls and were treated with rabbit or mouse immunoglobulin instead of the primary antibody.

3.3.7 Quantification

All sections were encoded and counted by the author in a blinded fashion. An Olympus microscope was used with a x 10 eyepiece and an objective with a magnification of x 40.
For each specimen at least 2 sections were immunostained and at least 4 fields were counted on each section. The area of interest was the superficial stromal layer and thus one edge of the field was aligned along the epithelial basement membrane. When aligning the field, areas with a prominent glandular component were avoided in order to give a representative count. All positively staining cells in the field were counted and the way in which the counts were expressed for the different stains is discussed in the relevant results sections.

3.3.8 Statistical analysis
Statistical evaluation was performed using the statistical software package SPSS (Chicago, USA). A \( p \) value < 0.05 was considered to be statistically significant. Results are expressed as mean +/- standard error of the mean (SEM). The Wilcoxon signed-rank test was applied to the data in order to determine if there was a difference between the pre- and post-treatment groups. A \( p \)-value of <0.05 was considered to be significant.

3.4 Results
3.4.1 Inflammatory cell populations
The figures contained in this section present the data obtained by plotting the pre- and post-treatment values for each individual patient. Data are expressed as number of positively staining cells per high power field (mean, range and standard deviation).

CD 3 +ve T-lymphocytes
Figure 3.8 Expression of CD3+ve cells (T-lymphocytes) in pre- and post-treatment biopsies. No significant difference between the two groups was identified.

Table 3.3 Expression of CD3+ve cells (T-lymphocytes) pre- and post-treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Range)</td>
<td>59.3 (16-163)</td>
<td>58.9 (4-130)</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>42.4</td>
<td>41.3</td>
</tr>
</tbody>
</table>
CD 8 +ve T-lymphocytes

Figure 3.9 Expression of CD8 +ve cells (cytotoxic T-cells) in pre- and post-treatment biopsies. No significant difference between the two groups was identified.

Table 3.4 Expression of CD8 +ve cells (Cytotoxic T-cells) pre- and post-treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Range)</td>
<td>30 (10-56)</td>
<td>25.3 (7-59)</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>16.2</td>
<td>15.1</td>
</tr>
</tbody>
</table>
**CD 68 +ve Macrophages**

**Figure 3.10** Expression of CD68+ve cells (macrophages) in pre- and post-treatment biopsies. No significant difference between the two groups was identified.

**Table 3.5** Expression of CD68+ve cells (macrophages) pre- and post-treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (Range)</strong></td>
<td>4 (1-8)</td>
<td>3 (0-14)</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>2.4</td>
<td>4.3</td>
</tr>
</tbody>
</table>
Neutrophils (H&E staining)

**Figure 3.11** Numbers of neutrophils in pre- and post-treatment biopsies. A significant reduction in the number of neutrophils in the post-treatment biopsies was identified \( (p=0.04) \)

**Table 3.6** Numbers of neutrophils in biopsy specimens pre- and post-treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (Range)</strong></td>
<td>6 (2-18)</td>
<td>2.1 (0-4)</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>4.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>
3.4.2 Transforming growth factor-β

Figure 3.12 Expression of TGF-β in pre- and post-treatment biopsies. No significant difference between the two groups was identified.

Table 3.7 Expression of TGF-β+ve cells pre- and post-treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Range)</td>
<td>32.3 (16-46)</td>
<td>33.6 (25-44)</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>9.4</td>
<td>6</td>
</tr>
</tbody>
</table>

3.4.3 Nuclear factor-kappaB
Figure 3.13 Expression of NF-κB in pre- and post-treatment biopsies. No significant difference between the two groups was identified.

Table 3.8 Expression of NF-κB pre- and post-treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Range)</td>
<td>1.5 (0.7-2.3)</td>
<td>1.4 (0-3.3)</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.6</td>
<td>1.1</td>
</tr>
</tbody>
</table>

3.5 Discussion
In this chapter we have shown that macrolide treatment in vivo results in a statistically significant reduction in neutrophil counts in post-treatment biopsy specimens. There was no such reduction seen in the counts of macrophages, T-lymphocytes and B-lymphocytes. No reduction was seen in the expression of TGF-β and NF-κB following macrolide treatment.

The experiments described in this chapter aimed to examine the possible anti-inflammatory effect of macrolide antibiotics in chronic rhinosinusitis in vivo. These experiments represented a logical progression from our earlier studies which examined this effect in an in vitro model and is in keeping with our principle aim, to determine a possible role for macrolides in clinical practice.

In the 10 subjects who were treated with a 3 month course of macrolide antibiotic, statistically significant reductions were noted in the neutrophil count in the post-treatment biopsies. Neutrophilic inflammation is present in chronic sinus disease and the reduction in neutrophil count that we have described is consistent with the anti-inflammatory effect of macrolides. In particular, in our in vitro experiments in chapter 2 we demonstrated that clarithromycin is capable of significantly inhibiting the production of the important neutrophilic cytokine interleukin-8. Inhibition of the production and action of interleukin-8 by macrolide may be responsible for the decrease in the neutrophil count observed in our group of macrolide-treated patients.

In contrast to neutrophil counts, post-treatment biopsies of the 10 treated patients failed to show a reduction in CD3+ve T-lymphocytes, CD8+ve cytotoxic T-cells, CD68+ve macrophages and nuclear factor-kappaB expression.

Another of our aims in this chapter was to compare the effect of macrolide in vitro with that seen in vivo. In chapter 2 we reported a significant reduction of TGF-β expression by macrolide treatment in vitro. In our in vivo experiments however, a similar reduction was not seen. There are several possible explanations for this finding. Firstly it may be that
macrolide treatment in vivo, in contrast to the in vitro environment, does not have an
effect on TGF-β expression. Alternatively, our negative finding may be due to the small
the numbers of subjects examined. However, there was not even a trend towards a
reduction in expression seen and it is therefore not possible to suggest that with a larger
group of patients a reduction would have been seen. In addition, it is inevitable that in an
in vivo study there are variables and confounding factors which are not as easily able to
be controlled as in the laboratory environment. For example, differences in the
underlying etiology of the patient’s chronic rhinosinusitis or whether or not they
demonstrated a clinical response to treatment was not taken into account in the selection
of patients for biopsy. The small number of patients who had biopsies examined in our in
vivo experiments is an obvious flaw. Ideally we would have been able to examine a
larger number of patients and perform a subgroup analysis to see if there are different
findings noted in those subgroups. Overcoming this flaw in our in vivo study was one of
our principle aims in the clinical trial discussed in the following chapter.
Chapter 4

A double-blinded, randomised, placebo-controlled trial of long-term, low-dose macrolide in the treatment of chronic rhinosinusitis

4.1 Introduction
As we have shown in our experiments in the previous two chapters and as is discussed in the introductory chapter, considerable laboratory evidence now exists to suggest that macrolides may have a beneficial effect in the treatment of chronic rhinosinusitis. There is however, a paucity of clinical evidence to support this statement. The few studies that have been performed have lacked a placebo-control group, have been unblinded and have had limited outcome measures. Such laboratory evidence and limited clinical studies do not provide enough information to allow the recommendation of the use of macrolides in chronic rhinosinusitis.

The levels of evidence as shown in figure 4.1 have gained widespread acceptance in the medical community as a means of describing the scientific validity of clinical evidence. In order for a potential new treatment to gain acceptance it is important that the highest possible level of evidence is available to support its use.

**Levels of Evidence**

- 1a-Meta-analysis of RCT's
- 1b-Individual RCT
- 2a- Meta-analysis of cohort studies
- 2b-Individual cohort studies
- 2c-Ecological Studies
- 3a- Meta-analysis of case-control studies
- 3b-Individual Case-control studies
- 4 – Case-series
- 5- Expert opinion without critical appraisal

*Figure 4.1 Levels of evidence in clinical research (Oxford centre for evidence-based medicine)*

The final component of our research therefore was to conduct a clinical trial of long-term, low-dose macrolide in the treatment of chronic rhinosinusitis and to provide the first piece of level 1 evidence in the world literature. In order to do this, a double-blinded,
randomised, placebo-controlled trial was performed. A wide variety of subjective and objective outcome measures were used to assess response to treatment. Patients were assessed during, at the completion and following treatment in order to determine the onset and duration of any possible treatment effect. In addition, a subgroup analysis was performed in which allergic and non-allergic patients were compared in terms of their response to treatment. Our hope was that by performing such a study, we would be able to provide level 1 evidence to support or refute the findings of previous studies and to therefore cast some light on the potential use of macrolides in clinical practice.

4.2 Methods
4.2.1 Subjects

64 patients were recruited from the ENT departments of the Royal Brisbane Hospital and the Gold Coast Hospital over an 18-month period. Patients were included if they were aged greater than 18 years with a history consistent with the diagnosis of chronic rhinosinusitis as outlined by the Rhinosinusitis task force. A CT scan was performed to confirm the diagnosis and was scored using the Lund-Mackay CT scoring system. Patients were excluded if they had a history of cystic fibrosis, primary ciliary dyskinesia, immune deficiency, allergic fungal sinusitis, nasal polyposis and impairment of liver or renal function. Pregnant and breast feeding women were excluded as were those taking medications with a known adverse interaction with macrolides or with a history of macrolide hypersensitivity. Patients were ineligible for inclusion if they had used topical or systemic corticosteroids within 4 weeks of entering the study.

The study protocol was approved by the ethics committees of the Royal Brisbane and Gold Coast Hospitals and Griffith University.

4.2.2 Study design

After recruitment into the study, subjects were randomised by the pharmacy department, using a random number table, to receive roxithromycin 150mg daily for 3 months or placebo. Patients and investigators were kept blinded to the randomisation until the completion of the study. At the commencement and conclusion of treatment, patients completed the sinonasal outcome test-20 (SNOT-20) questionnaire and had measures of peak nasal inspiratory flow, saccharine transit time, nasal endoscopic scoring, olfactory function and nasal lavage for IL-8, α2-macroglobulin and fucose. In addition the SNOT-20 questionnaire was completed at the midpoint of the treatment period and 3 months following the completion of treatment.

The placebo treatment used for the purposes of the trial was pyridoxine hydrochloride (Vitamin B6). The option of an exact placebo tablet was investigated however the cost of manufacturing a small quantity was prohibitive. Vitamin B6 was suggested by the
pharmacy as an alternative to a true placebo tablet because of their previous experience using it as a ‘placebo’. The dose of 50mg daily is approximately one quarter of the dose frequently taken as a vitamin replacement for treatment of the premenstrual syndrome and is substantially below the dose at which rare side effects have been noted to occur. The tablet itself was virtually identical in appearance to the roxithromycin 150mg tablet. Patients recruited into the trial were made aware of the fact that the ‘placebo’ tablet was in fact vitamin B6.

Roxithromycin was chosen as the macrolide to be given to the patients receiving active treatment because it is cheaper and more readily available than clarithromycin, the agent used in the experiments described in chapters 2 and 3. The clarithromycin used in those experiments was kindly provided by Abbott Australasia. No funding assistance was sought or received for the clinical trial discussed in this chapter.

For the purposes of describing the experimental procedure and results, the following terms will be used in this chapter: \textit{pre-treatment} - at the commencement of treatment; \textit{6 weeks treatment} - at the midpoint of treatment; \textit{post-treatment} - at the conclusion of treatment; \textit{12 weeks post-treatment} - 12 weeks following the conclusion of treatment. Figure 4.2 provides an illustrative version of the design of the study.

\subsection*{4.2.3 Microbiology and blood testing}

Microbiological swabs were obtained from the middle meatus of patients pre- and post-treatment. These swabs were analysed by the microbiology departments of the Royal Brisbane and Gold Coast Hospitals. Analysis involved culture for bacterial microorganisms and testing for antibiotic resistance.

Blood testing at enrolment in the study included serum eosinophil count and serum immunoglobulin E (IgE). Testing was performed by the pathology laboratories of the 2 hospitals mentioned previously.
Study Design

64 adult patients with CRS

- **Roxithromycin group**
  - n=29
  - Roxithromycin 150mg daily for 12 weeks
  - 2 patients withdrew:
    - 1-lost to follow-up
    - 1-Nausea

- **Placebo group**
  - n=35
  - Placebo daily for 12 weeks
  - 3 patients withdrew:
    - 1-lost to follow-up
    - 1-Rash
    - 1-Abdo pain

Randomised
27 patients completed treatment
3 patients withdrew

Figure 4.2 Design of the clinical trial

4.2.4 Subjective outcome measures

**Sinonasal outcome test-20 (SNOT-20).** Patients completed the SNOT-20 questionnaire at their enrolment into the study, after 6 weeks of treatment, post-treatment and 12 weeks post-treatment. The SNOT-20 is a validated, rhinosinusitis-specific quality of life instrument in which patients are asked to answer 20 questions regarding their nasal symptoms and quality of life\(^{57}\). Patients are asked to rate their quality of life and symptoms as they have been over the previous 2 weeks. The patient records a score from 0 (‘no problem’) to 5 (‘problem as bad as it can be’). The score from the 20 items is added and then divided by 20 to give a final score out of 5. The final scores obtained pre-treatment, after 6 weeks of treatment, post-treatment and 12 weeks post-treatment were then able to be compared.

**Final response scale.** At the conclusion of the 3 month treatment period, patients were asked to give an overall indication of their response to treatment on a linear rating scale.
(1. Completely improved, 2. Much improved, 3. Slightly improved, 4. Not improved, 5. Slightly worse, 6. Much worse) The score obtained on this scale was used to provide an overall subjective measure of the patient’s general well-being.

4.2.5 Objective outcome measures

**Peak nasal inspiratory flow (PNIF).** Peak nasal inspiratory flow was measured at the pre- and post-treatment visits (In-Check™, Clement Clark Limited, Harlow, Essex, England). Patients performed 3 inhalations and the mean value was recorded. This test was performed in order to give an indication of nasal patency.

**Saccharine transit time.** A particle of saccharine was placed unilaterally on the lateral nasal wall, 1 cm posterior to the anterior end of the inferior turbinate. The time was recorded from the placement of the particle to the first perception of the saccharine taste. The aim of this test was to provide a measure of mucociliary clearance.

**Nasal endoscopy.** Patient’s nasal cavities were examined with a nasal endoscope at the pre- and post-treatment visits. All examinations were performed by the primary author who was blinded to the treatment the patients received. Endoscopic scoring was carried out according to a template which graded swelling (0-no swelling, 1-mild swelling, 2-severe swelling), mucosal colour (0-pale, 1-red), polyps in the middle meatus (0-absent, 1-present) and nasal secretions (0-normal, 1-watery, 2-mucoid, 3-purulent). The score obtained from this endoscopic scoring therefore ranged from 0-7.

**Olfactory function.** Olfactory testing was performed using the Sniffin’ sticks olfactory function test (Burghardt, Wedel, Germany). Scores were obtained for odour detection threshold, odour discrimination and odour identification and were added to give a possible maximum score of $46^{,62}$. 
Nasal lavage. Nasal lavages were performed for 30 seconds using a compressible plastic nasal pool device filled with 10ml of isotonic saline. The lavage fluid samples were processed by ultrasonication for 15 minutes and then assays were performed for interleukin-8 (IL-8), a2-macroglobulin and fucose. These assays were performed in the Department of Otorhinolaryngology at Lund University, Sweden.

IL-8 was measured using a commercially available ELISA (R&D Systems, United Kingdom).

a2-macroglobulin was measured using a radio-immunoassay sensitive to 7.8 ng/ml. Rabbit anti-human a2-macroglobulin (Dakopatts, Copenhagen, Denmark) was used as anti-serum and human serum (Behringwerke Diagnostica, Marburg, Germany) as standard. Human a2-macroglobulin (Cappel-Organon Teknika, Turnhout, Belgium) was iodinated using the lactoperoxidase method. Tracer and standard (or sample) were mixed with anti-serum before adding goat anti-rabbit anti-serum (AstraZeneca, Lund, Sweden). The bound fraction was measured using a gamma counter (Pharmacia, Uppsala, Sweden).

Nasal lavage fluid levels of fucose were measured using a parallel ligand-exchange chromatography in combination with post-column derivatisation and fluorescence detection. The sample was centrifuged to remove viscoelastic plugs. Fucose was released by hydrolysis with 0.5M sulphuric acid at 100°C for 4 hours. After pH adjustment, the remaining proteins were removed by on-line dialysis. The chromatographic system comprised 2 Bio-Rad Aminex HPX-87H 300 x 7.8 mm columns (Bio-Rad Laboratories, Munchen, Germany) that were separated in a boxcar configuration. The analytical columns were operated at 50°C. As a mobile phase, 2.5mM sulphuric acid was run at a flow-rate of 0.6 ml/min. Post-column derivatisation was carried out in a 314μl-reaction coil, which was thermostated at 100°C, by introducing a 60mM benzamidine and a 2M potassium hydroxide solution at flow-rates of 0.1 ml/min. Fluorescence was monitored at an excitation wavelength of 360 nm, using an optical cut off filter of 420 nm.
Study design

<table>
<thead>
<tr>
<th>At 0 weeks</th>
<th>At 6 weeks</th>
<th>At 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum IgE</td>
<td>SNOT-20</td>
<td>Middle meatal swab</td>
</tr>
<tr>
<td>Middle meatal swab</td>
<td></td>
<td>Patient response scale</td>
</tr>
<tr>
<td>SNOT-20</td>
<td></td>
<td>SNOT-20</td>
</tr>
<tr>
<td>Saccharine transit time</td>
<td></td>
<td>Saccharine transit time</td>
</tr>
<tr>
<td>Peak nasal inspiratory flow</td>
<td></td>
<td>Peak nasal inspiratory flow</td>
</tr>
<tr>
<td>Nasal endoscopy</td>
<td></td>
<td>Nasal endoscopy</td>
</tr>
<tr>
<td>Olfactory function</td>
<td></td>
<td>Olfactory function</td>
</tr>
<tr>
<td>Nasal lavage</td>
<td></td>
<td>Nasal lavage</td>
</tr>
<tr>
<td>•Fucose</td>
<td></td>
<td>•Fucose</td>
</tr>
<tr>
<td>•Interleukin-8</td>
<td></td>
<td>•Interleukin-8</td>
</tr>
<tr>
<td>•a2-macroglobulin</td>
<td></td>
<td>•a2-macroglobulin</td>
</tr>
</tbody>
</table>

**Figure 4.3** Timing of outcome measures during clinical trial

### 4.2.6 Statistical methods

Statistical analysis was performed using the GraphPad Prism 4 software package (GraphPad, SanDiego, CA). A power analysis predicted sample sizes of 25 in each group would be required to achieve power of 80% at the 1% level of significance. The primary endpoint was the patient response scale. Data are expressed as mean +/- standard error of the mean. An intention to treat analysis was used. The Mann-Whitney U-test was applied to the patient response scale data. The Wilcoxon signed rank test was applied to the remaining data. P values <0.05 were considered significant.

### 4.3 Results

#### 4.3.1 Subjects

64 subjects were recruited into the study and were randomised to receive roxithromycin (n=29) or placebo (n=35). Three subjects withdrew from the placebo group (1 subject
was lost to follow-up, 1 developed a rash after 3 days of treatment and 1 developed abdominal pain after 3 weeks of placebo treatment). Two subjects withdrew from the roxithromycin group (1 subject developed nausea and vomiting on commencing treatment and 1 was lost to follow-up). 3 subjects did not complete the 12-week post-treatment SNOT-20 survey because they were lost to follow-up (1 subject in the roxithromycin group and 2 in the placebo group).

No significant differences were identified between the placebo and roxithromycin groups in terms of age, sex, IgE, CT scores or baseline values of SNOT-20 scores, peak nasal inspiratory flow, saccharine transit time, nasal endoscopy, olfactory function scores and lavage data. Table 4.1 illustrates the mean and range of the demographic and clinical data.

Nasal swabs from the middle meatus were taken for microbiological examination prior to and following the completion of treatment. In the 29 roxithromycin-treated patients, there were 12 positive cultures at the commencement of treatment (S. aureus n=4, H. influenzae n=1, P. aeruginosa n=3, S. pneumoniae n=4). Following treatment in these patients there were 9 positive cultures (S. aureus n=5, P. aeruginosa n=2, S. pneumoniae n=2). No macrolide-resistant organisms were noted to develop. In the 35 placebo-treated patients there were 9 positive cultures at the commencement of treatment (S. aureus n=3, S. pneumoniae n=3, H. influenza n=2, M. cattarhalis n=1). Following placebo treatment there were 12 positive cultures (S. aureus n=6, S. pneumoniae n=4, P. aeruginosa n=1, H. influenza n=1).

<table>
<thead>
<tr>
<th>Baseline variable</th>
<th>Roxithromycin group</th>
<th>Placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>48.3 years (20-64)</td>
<td>48.7 years (31-64)</td>
</tr>
<tr>
<td>Sex</td>
<td>21 females, 14 males</td>
<td>19 females, 10 males</td>
</tr>
</tbody>
</table>
Table 4.1 Baseline data for subjects enrolled into the roxithromycin and placebo groups expressed as mean (range). No significant difference was identified between the 2 groups at enrolment into the study.

4.3.2 Roxithromycin vs Placebo

The primary aim of our clinical trial was to determine if there was a difference in any of the outcome measures when patients were treated with roxithromycin or control. The following section contains the results obtained in these 2 groups of subjects. For each of the outcome measures, the pre-treatment results are compared to the post-treatment results. This information is shown graphically and a summary of the data is contained in table 4.2. Data are expressed as mean (standard error of the mean). In brief, the placebo-treated patients showed no significant change in any of the outcome measures. The roxithromycin-treated patients showed significant improvements in saccharine transit time (p<0.01), nasal endoscopic scoring (p<0.01) and SNOT-20 scoring (p=0.01) at the completion of treatment. No significant improvements were noted in SNOT-20 scoring after 6 weeks of treatment or 3 months following the completion of treatment. In
addition, no significant improvements were seen in peak nasal inspiratory flow, olfactory function scoring and nasal lavages for IL-8, fucose and α2-macroglobulin.

Saccharine transit time
Subjects receiving roxithromycin showed a statistically significant improvement in saccharine transit time (p<0.01). (see figure 4.4).

Subjects receiving placebo did not show a statistically significant improvement in saccharine transit time (see figure 4.5).

![Graph](image)

**Figure 4.4** Saccharine transit time pre- and post-treatment with roxithromycin. A statistically significant reduction was noted.
Figure 4.5 Saccharine transit time pre- and post-treatment with placebo. No reduction was noted.

**Peak nasal inspiratory flow**

No significant improvement was noted in peak nasal inspiratory flow following treatment with roxithromycin or placebo (see figures 4.6 and 4.7 and table 4.2).

Figure 4.6 Peak nasal inspiratory flow pre- and post-treatment with roxithromycin. No reduction was noted.
Figure 4.7 Peak nasal inspiratory flow pre- and post-treatment with placebo. No significant reduction was noted.

**Nasal endoscopic scoring**

A statistically significant improvement was noted in nasal endoscopic scoring after treatment with roxithromycin (p<0.01). No improvement was seen following treatment with placebo (see figures 4.8 and 4.9 and table 4.2).

Figure 4.8 Nasal endoscopic scoring pre- and post-treatment with roxithromycin. A statistically significant improvement was noted.
Figure 4.9 Nasal endoscopic scoring pre- and post-treatment with placebo. No improvement was noted.

Olfactory function score

No improvement was noted in the olfactory function score following treatment withroxithromycin or placebo (see figures 4.10 and 4.11 and table 4.2).

Figure 4.10 Olfactory function score pre- and post-treatment with roxithromycin. No improvement was noted following treatment.
Figure 4.11 Olfactory function score pre- and post-treatment with placebo. No improvement was noted following treatment.

**Sino-nasal outcome test-20**

At the midpoint of treatment with roxithromycin and placebo, neither group showed any significant improvement in SNOT-20 scoring (see table 4.2).

After 3-months of treatment there was a significant improvement in SNOT-20 scores in the roxithromycin-treated patients (p=0.01) (see figures 4.12 and 4.13 and table 4.2).

Figure 4.12 SNOT-20 scoring improved significantly in the roxithromycin-treated patients after 3 months.
Figure 4.13 SNOT-20 scoring pre- and post-treatment with placebo.

3 months following the completion of treatment with roxithromycin SNOT-20 scores were obtained. The significant improvements that were noted following the completion of treatment were no longer present after an additional 3 months (see figure 4.14 and table 4.2).

Figure 4.14 SNOT-20 scoring pre-treatment and 3 months following the completion of treatment with roxithromycin. No improvement was noted.
**Final response scale**

Patients treated with roxithromycin and placebo completed the linear rating scale at the post-treatment visit. A statistically significant difference was identified between the roxithromycin (3.11 +/- 0.17) and placebo groups (3.84 +/- 0.12) [p<0.01] (see figure 4.15).

![Final response scale graph]

**Figure 4.15** Final response scale in the placebo and roxithromycin groups. A significant improvement was noted in the roxithromycin-treated patients.

**Interleukin-8 lavage**

There was no significant difference in pre- and post-treatment nasal lavage levels of IL-8 in the roxithromycin and placebo-treated patients (see figures 4.16 and 4.17 and table 4.2).

![Interleukin-8 levels graph]

**Figure 4.16** Interleukin-8 levels in nasal lavage pre- and post-treatment with roxithromycin. No significant difference was noted.
Figure 4.17 Interleukin-8 levels in nasal lavage pre- and post-treatment with placebo. No significant difference was noted.

**α2-macroglobulin lavage**

There was no significant difference in pre- and post-treatment nasal lavage levels of α2-macroglobulin in the roxithromycin and placebo-treated patients (see figures 4.18 and 4.19 and table 4.2).

Figure 4.18 α2-macroglobulin levels in nasal lavage specimens pre-and post-treatment with roxithromycin. No significant difference was noted.
Figure 4.19 α2-macroglobulin levels in nasal lavage specimens pre-and post-treatment with placebo. No significant difference was noted.

Fucose lavage

There was no significant difference in pre- and post-treatment nasal lavage levels of fucose in the roxithromycin and placebo-treated patients (see figures 4.20 and 4.21 and table 4.2).

Figure 4.20 Fucose levels in nasal lavage specimens pre- and post-treatment with roxithromycin. No significant difference was noted.
Figure 4.21 Fucose levels in nasal lavage specimens pre- and post-treatment with placebo. No significant difference was noted.
### Table 4.2
Pre-treatment vs post-treatment outcome measures
All patients in placebo and roxithromycin groups

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Roxithromycin group (n=29)</th>
<th>Placebo group (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>P value</td>
</tr>
<tr>
<td>PNIF (l/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>102.7 (6.5)</td>
<td></td>
</tr>
<tr>
<td>Post-</td>
<td>99.9 (7.8)</td>
<td>NS</td>
</tr>
<tr>
<td>STT (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>11.5 (1.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Post-</td>
<td>8.2 (0.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nasal endoscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>3.2 (0.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Post-</td>
<td>2.6 (0.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Olfactory function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>22.5 (1.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Post-</td>
<td>23.6 (1.7)</td>
<td>NS</td>
</tr>
<tr>
<td>SNOT-20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>2.75 (0.13)</td>
<td></td>
</tr>
<tr>
<td>At 6 weeks</td>
<td>2.61 (0.14)</td>
<td>NS</td>
</tr>
<tr>
<td>At 12 weeks</td>
<td>2.34 (0.19)</td>
<td>0.01</td>
</tr>
<tr>
<td>At 24 weeks</td>
<td>2.49 (0.18)</td>
<td>NS</td>
</tr>
<tr>
<td>Interleukin-8 (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>226.6 (48.2)</td>
<td></td>
</tr>
<tr>
<td>Post-</td>
<td>156 (38.7)</td>
<td>NS</td>
</tr>
<tr>
<td>α2-macro (μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>1.87 (0.39)</td>
<td></td>
</tr>
<tr>
<td>Post-</td>
<td>1.86 (0.72)</td>
<td>NS</td>
</tr>
<tr>
<td>Fucose (μmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>18.6 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Post-</td>
<td>21.5 (7.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>
4.3.3 High IgE vs low IgE patients

As discussed in the introduction to this chapter, one of the aims of our clinical trial was to attempt to identify a subgroup of patients who may show a better response to macrolide treatment. A previous study by Suzuki et al\textsuperscript{100} suggested that patients with low-levels of IgE are more likely to respond. In our study therefore, we performed a subgroup analysis of the patients treated with roxithromycin. Those with low levels of serum IgE (below 200μg/l) were compared with those with high levels (above 200μg/l) to see if there was a difference in any of the outcome measures between the 2 groups. Of the 29 patients in total that were treated with roxithromycin, 14 had low levels of IgE and 15 had high levels. There was no significant difference between the 2 groups of patients in terms of demographic or baseline data.

In the following section, the results obtained pre- and post-treatment with roxithromycin are shown for the patients with low levels of IgE compared to those with high levels. A summary of the data is shown in table 4.3. In brief, patients with low levels of IgE showed statistically significant reductions in saccharine transit time (p<0.01) and nasal endoscopic scoring (p<0.01). SNOT-20 scoring showed no significant improvement after 6 weeks of treatment, however, after 12 weeks there were statistically significant improvements (p<0.01). Twelve weeks post-treatment, the SNOT-20 scores were improved compared to the baseline levels but this improvement failed to reach significance (p=0.06). No significant improvements were shown for peak nasal inspiratory flow and olfactory function scoring. Nasal lavages showed a significant reduction in IL-8 levels in the post-treatment lavages (p=0.02). No significant reductions were seen in the fucose and α2-macroglobulin lavages.

Roxithromycin treatment in the patients with high levels of IgE produced a significant reduction in saccharine transit time (p=0.04). No significant improvement was noted in any of the remaining outcome measures or in the nasal lavages.
Table 4.3
Pre-treatment vs post-treatment outcome measures
Roxithromycin-treated patients with low IgE vs high IgE

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Low IgE group (n=14)</th>
<th>High IgE group (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>P value</td>
</tr>
<tr>
<td>PNIF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>101.4 (12.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Post-</td>
<td>102.4 (10.1)</td>
<td></td>
</tr>
<tr>
<td>STT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>11.2 (1.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Post-</td>
<td>7.5 (0.8)</td>
<td></td>
</tr>
<tr>
<td>Nasal endoscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>3.1 (0.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Post-</td>
<td>2.1 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Olfactory function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>26.3 (2.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Post-</td>
<td>28.3 (1.7)</td>
<td>NS</td>
</tr>
<tr>
<td>SNOT-20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>2.73 (0.24)</td>
<td>NS</td>
</tr>
<tr>
<td>At 6 weeks</td>
<td>2.49 (0.17)</td>
<td>NS</td>
</tr>
<tr>
<td>At 12 weeks</td>
<td>2.03 (0.31)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>At 24 weeks</td>
<td>2.24 (0.25)</td>
<td>0.06</td>
</tr>
<tr>
<td>Interleukin-8 (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>244.1 (82.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>Post-</td>
<td>98.2 (20.9)</td>
<td>NS</td>
</tr>
<tr>
<td>α2-macro (μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>1.67 (0.52)</td>
<td></td>
</tr>
<tr>
<td>Post-</td>
<td>0.89 (0.25)</td>
<td>NS</td>
</tr>
<tr>
<td>Fucose (μmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>17.5 (4.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Post-</td>
<td>23.0 (10.7)</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Saccharine transit time**

Patients with low levels of IgE treated with roxithromycin showed a significant reduction in saccharine transit time (p<0.01) (see figure 4.22 and table 4.3).

![Figure 4.22](image1)

**Figure 4.22** Patients with low levels of IgE showed a significant reduction in saccharine transit time.

Patients with high levels of IgE also showed a statistically significant reduction in saccharine transit time after roxithromycin (p=0.04) (see figure 4.23 and table 4.3).

![Figure 4.23](image2)

**Figure 4.23** Patients with high IgE levels showed a significant reduction in saccharine transit time.
Peak nasal inspiratory flow

No significant improvement was noted in peak nasal inspiratory flow in either the low or high IgE patients (see figures 4.24 and 4.25 and table 4.3).

Figure 4.24 Peak nasal inspiratory flow in patients with low levels of IgE.

Figure 4.25 Peak nasal inspiratory flow in patients with high levels of IgE.
Nasal endoscopic scoring

Patients with low levels of IgE showed a statistically significant improvement in their nasal endoscopic scores following treatment with roxithromycin (p<0.01). No such improvement was seen in the patients with high levels of IgE (see figures 4.26 and 4.27 and table 4.3).

**Figure 4.26** Nasal endoscopic scoring in the low IgE patients. Following treatment with roxithromycin there was a significant improvement in scores.

**Figure 4.27** Nasal endoscopic scoring in the high IgE patients. No improvement was seen following roxithromycin treatment.
Olfactory function scoring

Neither the low IgE or high IgE group of patients showed a significant improvement in olfactory function scores following treatment with roxithromycin (see figures 4.28 and 4.29 and table 4.3).

Figure 4.28 Olfactory function scores in patients with low levels of IgE.

Figure 4.29 Olfactory function scores in patients with high levels of IgE.
Sinonasal outcome test-20 scoring

Patients receiving roxithromycin completed the SNOT-20 questionnaire at the mid-point of their 12 week treatment. At that stage, neither patients in the low IgE or high IgE groups showed improvement in their scores (see table 4.3).

At the conclusion of the 12 weeks of treatment patients with low levels of IgE showed a significant improvement in SNOT-20 scores (p<0.01). In contrast, patients with high levels of IgE showed no significant change (see figures 4.30 and 4.31 and table 4.3).

Figure 4.30 SNOT-20 scores in patients with low levels of IgE.

Figure 4.31 SNOT-20 scores in patients with high levels of IgE.
Three months following the completion of treatment an improvement in SNOT-20 scores was noted in the low IgE group but this improvement failed to reach statistical significance (p=0.06) (figure 4.32).

**Figure 4.32** SNOT-20 scores in patients with low levels of IgE three months following the completion of treatment.

**Figure 4.33** SNOT-20 scores in patients with high levels of IgE three months following the completion of treatment.
**Interleukin-8 lavage**

Following treatment with roxithromycin there was a statistically significant reduction in nasal lavage levels of interleukin-8 in patients with low levels of IgE (p=0.02). In contrast, no significant reduction was seen in patients with high levels of IgE (see figures 4.34 and 4.35 and table 4.3).

**Figure 4.34** Nasal lavage levels of interleukin-8 in patients with low levels of IgE following treatment with roxithromycin.

**Figure 4.35** Nasal lavage levels of interleukin-8 in patients with high levels of IgE following treatment with roxithromycin.
α2-macroglobulin lavage
Neither patients with low or high levels of IgE showed a statistically significant change in nasal lavage levels of α2-macroglobulin following treatment with roxithromycin (see table 4.3).

Fucose lavage
Fucose levels in nasal lavage specimens showed no significant change following treatment with roxithromycin in patients with low or high levels of IgE (see table 4.3).

Final response scale
The final response scale of patients with low levels of IgE differed significantly from those with high levels of IgE (p<0.01) (figure 4.36).

![Figure 4.36](image_url) Final response scale in patients with low IgE compared to high IgE.
4.4 Discussion

At the time of writing, this trial represents the first double-blinded, randomised, placebo-controlled trial of long-term, low-dose macrolide in the treatment of chronic rhinosinusitis. We have shown that roxithromycin, when compared to placebo, is capable of producing significant improvements in a range of subjective and objective outcome measures. This effect is particularly evident in patients with low levels of IgE.

Patients receiving roxithromycin reported significant improvements in the linear rating scale and in their SNOT-20 scores following the 12 week course. Interestingly, these improvements were not noted after only 6 weeks of treatment. These findings are consistent with previous studies that have suggested that a prolonged course of macrolide is required before a clinical effect is seen. Hashiba\textsuperscript{34} reported that response rates of chronic rhinosinusitis patients were 5% at 2 weeks at 71% at 12 weeks. Similarly, Cervin\textsuperscript{12} showed that patients who had a response after 3 months of treatment showed continued improvement in symptom scores, nasal nitric oxide and ciliary beat frequency after 12 months of macrolide. Our study therefore supports the use of relatively long-term courses of macrolide antibiotics in the treatment of chronic rhinosinusitis.

In order to assess any long-term benefit of the 12 week macrolide treatment, patients were assessed after a further 12 weeks following the cessation of treatment. The benefits of treatment were not sustained: SNOT-20 scores 12 weeks after treatment were similar to pre-treatment scores. Patients with low levels of IgE did show improved SNOT-20 scores but these failed to reach statistical significance (p=0.06). This finding suggests that treatments longer than 12 weeks are necessary or perhaps multiple courses of macrolide may be required to maintain the beneficial effect. Such treatments may create problems with side-effects and compliance, especially if the benefit is not marked. This issue has not been addressed in previous studies.

The observed benefits of macrolide treatment were only evident after 12 weeks of treatment. Such long-term treatment could lead to the emergence of resistant organisms.
In our study, middle meatal swabs performed before and after treatment failed to show any macrolide resistant organisms, however, the small number of patients examined precludes any firm conclusion regarding the likelihood of resistant organisms developing.

Previous authors have suggested that macrolide treatment may be most beneficial in those patients with low levels of IgE. This suggestion is partly based on the fact that macrolides exert an inhibitory effect on promoters of neutrophilic inflammation, such as interleukin-8. Suzuki et al in a study of 16 patients with chronic rhinosinusitis reported that symptomatic improvement correlated with low levels of IgE and with low eosinophil counts in peripheral blood, nasal smears and nasal mucosa. In the present study we performed a subgroup analysis on patients with low levels of IgE compared to those with high levels. Patients with low levels of IgE showed significant improvements in symptom scores, saccharine transit time, nasal endoscopic scoring and nasal lavage levels of interleukin-8. Patients with high IgE levels showed a significant improvement only in saccharine transit time. In addition the low IgE patients had a significantly improved final linear rating scale compared to the high IgE group. We believe that these findings provide further evidence that there is a subgroup of chronic rhinosinusitis patients who are more likely to benefit from macrolides and that levels of IgE may be a useful tool in selecting patients for treatment.

The findings of this study have raised a number of additional questions regarding the role of macrolides in clinical practice. As discussed above, the longevity of the treatment effect and the optimal duration of treatment are unknown. In addition, the actual benefit that patients experience with macrolide treatment needs to be weighed against the risks of side effects and the danger of development of resistant bacteria. It is worth noting in this study that although the linear rating scale did show significant improvements in patients receiving macrolide antibiotics, none of these patients reported that they were ‘completely improved’. Further studies with larger numbers of patients, further subgroup analysis and longer follow-up may help to answer these questions.
Chapter 5

DISCUSSION

In the preceding chapters we have reported the findings of our research into the anti-inflammatory effect of macrolide antibiotics in chronic rhinosinusitis. We have
performed a series of experiments that have investigated the effect of macrolides in vitro, ex vivo and in the clinical setting. We have aimed to clarify the molecular mechanism of action of macrolides and at all times have endeavoured to use models of chronic rhinosinusitis that resemble the clinical environment as much as possible. We have performed the first ever randomised, double-blinded, placebo-controlled trial of macrolides in the treatment of chronic rhinosinusitis in order to determine if the effect of macrolides noted in our laboratory work is present in treated patients. This progression from ‘bench to bedside’ is in keeping with our aim of providing scientific evidence that can be utilised by clinicians and applied in the treatment of patients with chronic rhinosinusitis.

In brief, we have shown that macrolides in vitro are capable of inhibiting the production of the pro-inflammatory cytokines IL-5, IL-8 and GM-CSF by whole sections of chronic rhinosinusitis mucosa. This inhibition was equal to that produced by the corticosteroid prednisolone. The expression of transforming growth factor-β was also reduced by treatment of chronic rhinosinusitis mucosa in vitro with both macrolide and clarithromycin. In contrast to previous studies we failed to show a reduction in the expression of the key pro-inflammatory nuclear transcription factor NF-κB.

Biopsies taken from patients treated with long-term, low dose macrolide showed reduced numbers of neutrophils after treatment. There was no change in the number of other inflammatory cells. These biopsies were also assessed for expression of TGF-β and NF-κB however a reduction in expression was not noted.

In our clinical trial we demonstrated significant improvements in several subjective and objective outcome measures in patients receiving roxithromycin compared to those receiving placebo. The symptomatic improvements were not present after 6 weeks of treatment and had largely resolved 3 months following the completion of treatment. Interestingly, we also showed significantly better outcomes in those patients with low levels of IgE compared to those with high levels.
A further discussion of these findings and a comparison to existing evidence in the literature is given in the following discussion along with identified weaknesses of our work and future directions.

5.1 Macrolides and cytokines

In the last decade there has been considerable interest in determining the molecular mechanism of the anti-inflammatory effect of macrolides. In general this work has been performed in vitro and has been performed on a variety of different tissue types and under differing experimental conditions. The vast majority of these in vitro studies have used individual cell line cultures from non-diseased sources. As an example of the importance of using accurate disease models, studies on animals have suggested that there is a difference between the effects of macrolide antibiotics on healthy animals and animal models of inflammation. For example, in healthy mice, a 28-day course of erythromycin resulted in increased interleukin-1 and interleukin-2 production\(^ {45}\). In contrast, roxithromycin decreased the production of interleukin-1 and TNF-\(\alpha\) in lipopolysaccharide-induced inflammation in the mouse\(^ {97}\). These observed differences between the effects of macrolides in healthy and disease models encouraged us to use diseased mucosal specimens rather than healthy tissue for our experiments.

Therefore, we developed a culture and analysis technique whereby whole tissue biopsies were taken from patients suffering from chronic rhinosinusitis. These were then cultured in vitro. The rationale behind this approach was to examine the effect of macrolides on a model that contained the full complement of resident cells and presumably with the usual complex interactions between those cells preserved.

**Interleukin-8**

Cytokine ELISA performed on cultured chronic sinusitis mucosa in our study revealed that clarithromycin and prednisolone both produced a significant dose-dependent reduction in IL-8 production. IL-8 is a potent neutrophil chemoattractant cytokine and has been shown to be one of the principal cytokines involved in chronic sinusitis\(^ {56}\). Our finding of inhibition of IL-8 production by clarithromycin confirms the work of several
previous authors. Kawasaki et al demonstrated that roxithromycin inhibited production of IL-8 by human bronchial epithelial cells\(^\text{42}\). IL-8 production by human nasal epithelial cells and exudative cells in the nasal discharge of patients with chronic rhinosinusitis was significantly reduced by \(10^{-5}\) M and \(10^{-6}\) M erythromycin\(^\text{98,101}\). Erythromycin and clarithromycin have been shown to reduce IL-8 release from human eosinophils in a dose-dependent fashion\(^\text{48}\). Similarly, Aoki et al\(^\text{2}\) reported that IL-8 release by T cells and transcriptional activation of the IL-8 gene was inhibited by erythromycin. The conclusion that can be drawn from the above findings and our own results is that macrolides are capable of inhibiting the production of IL-8 by a variety of cell types and therefore can help break the vicious cycle of neutrophil recruitment and further inflammation in chronic airway disease.

**Granulocyte macrophage-colony stimulating factor**

In our cytokine ELISA experiments we were also able to show that macrolides and prednisolone each produce a significant, dose-dependent reduction in GM-CSF production. GM-CSF is a pro-inflammatory cytokine and is associated with eosinophilic inflammation. Interestingly, unlike IL-5, which is predominantly elevated in allergic chronic sinusitis, GM-CSF receptor expression is greatest in non-allergic disease, suggesting that it has an additional role in the recruitment of neutrophils and/or macrophages\(^\text{51}\). Kawasaki et al\(^\text{42}\) also demonstrated that roxithromycin is capable of inhibiting production of GM-CSF in human bronchial epithelial cells. Sato et al reported that GM-CSF production by TNF-\(\alpha\) and IL-1\(\beta\) stimulated human lung fibroblasts was inhibited by erythromycin at doses of 100\(\mu\)g/ml but not at lower doses\(^\text{90}\). Our finding of reduced GM-CSF production following culture with macrolide was therefore the first time that this had been demonstrated in nasal mucosa and in mucosa in a diseased state.

**Interleukin-5**

Interleukin-5 is a pro-inflammatory cytokine associated with eosinophilic inflammation and its level of expression directly correlates with the degree of eosinophilia in allergic chronic sinusitis\(^\text{56}\). In comparison to IL-8 and GM-CSF, there is relatively little evidence
to suggest that macrolides are able to reduce IL-5 production. Studies on mice have revealed that the administration of roxithromycin results in decreased production of IL-5 by spleen cells and lung explants\textsuperscript{49,50}. In our experiments IL-5 production by chronic sinusitis mucosal specimens was shown to be reduced in a significant, dose-dependent fashion by clarithromycin and prednisolone. This finding was the first reported evidence of an inhibitory effect of macrolides on IL-5 production by human tissue.

**Comparing macrolide and corticosteroid**

Our ELISA results showed that prednisolone was also able to produce a significant reduction in IL-5, IL-8 and GM-CSF production. These results reflect the findings of previous authors. GM-CSF and IL-5 production has been shown to be significantly reduced in homogenates of nasal polyp tissue after treatment with prednisolone\textsuperscript{89}. Similarly, GM-CSF production by cultured nasal polyp epithelial cells has been shown to be significantly reduced by a variety of corticosteroids\textsuperscript{75}. Lennard et al\textsuperscript{56} demonstrated a significant reduction in IL-5 expression in the mucosa of patients with chronic rhinosinusitis treated with systemic corticosteroids but no significant reduction in IL-8 expression. Interestingly there was no significant difference between the reduction of cytokine expression produced by the macrolide clarithromycin and that by the corticosteroid prednisolone. This finding helps to illustrate the magnitude of the potential anti-inflammatory effect of macrolides in relation to that produced by a known potent anti-inflammatory agent, prednisolone.

**Transforming growth factor-β**

Immunohistochemistry to determine the effect of clarithromycin and prednisolone on TGF-β expression was performed on the cultured chronic rhinosinusitis mucosal specimens. We found that TGF-β expression was significantly reduced by both clarithromycin and prednisolone. TGF-β is a potent chemoattractant for neutrophils, mononuclear cells and fibroblasts and greatly enhances extracellular matrix production. TGF-β has been shown to be present in nasal polyps and to a lesser extent chronic inflammatory mucosa but not in the nasal mucosa from disease-free controls\textsuperscript{14}. There is very little written in the literature regarding the possible effects of macrolide antibiotics
on TGF-β. The only such study found that the macrolide FK506 (Tacrolimus) is capable of blocking the TGF-β receptor in yeast cells\textsuperscript{106}. The possibility that macrolides are capable of altering the production or function of this important cytokine represents another mechanism by which macrolides may exert their anti-inflammatory effect.

**Nuclear factor-κB**

In chapter 2 we have reported the results of our experiments in which immunohistochemical analysis was performed on cultured mucosal specimens to determine if there was a reduction in expression of the key pro-inflammatory nuclear transcription factor NF-κB. NF-κB has been the subject of considerable interest in recent years, as it is thought to play a key role in up-regulating inflammation via its effect on the production of numerous cytokines. A number of previous studies have reported that macrolides do have an effect on NF-κB. Aoki and Kao\textsuperscript{2} demonstrated that erythromycin in vitro inhibited activation of NF-κB. Desaki et al\textsuperscript{16} showed that erythromycin inhibited the activation of the transcription factors NF-κB and AP-1 in human bronchial epithelial cells. Both of these factors are crucial regulators of interleukin-8 production and it is therefore possible that macrolides may produce decreases in IL-8 production by inhibiting the activation of these transcription factors. In contrast to the macrolide findings, at least 2 studies have reported that corticosteroids do not have an effect on NF-κB activation\textsuperscript{33,78}.

In our immunohistochemical analysis, neither clarithromycin nor prednisolone produced a significant reduction in activated NF-κB expression. A closer examination of the results however does reveal that 8 of the 10 macrolide-treated specimens had reductions in NF-κB expression compared to the control specimens. In comparison, only 6 of the 10 prednisolone-treated specimens had reduced levels of expression compared to the controls. It is tempting to speculate that if a greater number of specimens were examined, a statistically significant result would have been obtained in the macrolide-treated group. Alternatively, our findings may differ from those of previous authors because we used whole tissue specimens rather than individual cell lines or because previous studies have
largely used electrophoretic mobility shift assay rather than immunohistochemistry to
determine the degree of activation of NF-κB.

**In vivo effect on TGF-β and NF-κB**

In keeping with our aim of determining the effect of macrolide antibiotics in models of
inflammation resembling the clinical situation as closely as possible, we examined the
anti-inflammatory effect of macrolides in vivo. As described in chapter 3, patients with
chronic rhinosinusitis were treated with a 3-month course of clarithromycin and biopsies
were collected pre- and post-treatment. These biopsies were stained
immunohistochemically and the expression of markers specific for inflammatory cells,
TGF-β and NF-κB was determined. This in vivo study differs from the multitude of
previous studies that have investigated the in vitro effect of macrolides.

There have been a small number of in vivo studies previously published. Amongst these
are a study by Hamid et al in which patients with chronic rhinosinusitis were treated for
14 days with 500 mg of clarithromycin daily. Statistically significant reductions in IL-6,
IL-8, TNF-α, elastase and oedema score were observed. Another study involving
chronic bronchitis patients treated for 7 days with clarithromycin, reported reductions in
neutrophil activity, IL-8 levels and mucous viscosity.

In our study immunohistochemistry was performed on the pre- and post-treatment
biopsies to determine if there was an effect on the expression of NF-κB and TGF-β
following macrolide treatment in vivo. Our results showed no significant change in the
expression of either of these factors. This finding differs from some of our own in vitro
results as well as the results reported by previous authors as discussed above. The most
obvious reason for this discrepancy is the fact that this study was carried out in vivo and
is therefore inevitably associated with a number of confounding factors. For example, we
did not attempt to select out a particular group of patients who reported a clinical
improvement following treatment. Similarly, the subjects examined were quite diverse in
terms of the severity of their disease, their history of allergy etc. Evidence from the
clinical trial carried out in association with this project, suggests that patients with a
strong history of allergy and positive allergy markers tend not to respond well to macrolide treatment. If larger numbers of patients had been studied it may have been possible to analyse the immunohistochemical staining data in order to determine if there was a difference in the expression of these factors between patients who reported a response to treatment and those who did not or those with an allergy history and those without. An important aim of our clinical trial discussed in chapter 4 was to further clarify the response to macrolide treatment in these various subgroups. It should also be mentioned that the differences between in vivo and in vitro findings may actually represent a true difference between the effect of macrolides in the laboratory and in the clinical situation. If this was to be the case and the promising in vitro anti-inflammatory effect was not shown to also occur in vivo, the role of macrolides in the treatment of chronic rhinosinusitis would be doubtful.

5.2 Macrolide effect on inflammatory cells

Neutrophils
In our in vivo study, chronic rhinosinusitis patients were treated with 3 months of clarithromycin. In the post-treatment biopsies from these patients there was a statistically significant reduction in neutrophil count. This finding of reduced neutrophil numbers after macrolide treatment is in keeping with the known effects of long-term, low-dose macrolides.

Persistent purulent discharge and paranasal sinus effusion containing high numbers of emigrated neutrophils is one of the hallmarks of chronic sinusitis. Interleukin-8 is a potent neutrophil chemoattractant cytokine that has been shown to be elevated in the mucosa of patients with chronic sinusitis. In vitro studies have demonstrated that interleukin-8 is secreted from nasal epithelial cells and also from the exudative cells in the nasal discharge of patients with chronic sinusitis. This interleukin-8 production establishes a positive feedback loop in which emigrating neutrophils secrete interleukin-8 that in turn produces further neutrophil emigration. Macrolides are capable of inhibiting the production of interleukin-8 as we have shown in our in vitro experiments and may
therefore reduce mucosal neutrophil numbers in patients with chronic rhinosinusitis, as was demonstrated in our in vivo study. Further evidence of the inhibitory effect of macrolides on interleukin-8 production and therefore in turn, neutrophilic inflammation, was shown in our clinical trial in which there was a statistically significant reduction in interleukin-8 levels in the nasal lavage of patients treated with roxithromycin.

Therefore, from the evidence that we have produced in the experiments described in this thesis, we believe that one of the key mechanisms by which macrolides exert their clinical effect is through the inhibition of neutrophilic inflammation. We have shown an effect in vitro on the production of interleukin-8 and also a reduction in IL-8 levels in nasal lavage fluid in patients receiving a 3 month course of macrolide. We have also shown in vivo, that as would be expected from the inhibition of interleukin-8 production, there is a reduction in neutrophil numbers in the mucosa of patients following treatment with macrolide.

**Eosinophils**

In contrast to the abundant evidence supporting the anti-neutrophilic effect of macrolides, there is some debate as to the effect on eosinophils. In our in vitro study we showed that clarithromycin produced a dose-dependent reduction in the production of the eosinophilic cytokines interleukin-5 and GM-CSF. This reduction was equal to that seen with prednisolone treatment of the cultured mucosal specimens. Previous in vitro studies have suggested that macrolides may have an anti-eosinophilic effect. Sato et al.\(^{90}\) examined the effect of erythromycin on human lung fibroblasts. Erythromycin significantly reduced eosinophil chemotactic activity and suppressed production of the eosinophil chemotactic cytokines GM-CSF, eotaxin and RANTES. It was postulated that these effects may have relevance to the use of macrolides in the treatment of bronchial asthma. Matsuoka et al.\(^{66}\) reported that clarithromycin significantly inhibited the expression of the adhesion molecules ICAM-1 and VCAM-1 in synovial (fibroblast-like) cells. Both molecules have been shown to play an important role in the extravasation of eosinophils into tissue in models of airway inflammation.
In the clinical trial described in chapter 4 we reported significantly improved outcomes in patients with low levels of immunoglobulin E. Whilst IgE is not absolutely sensitive or specific for allergy, this finding does suggest that those patients without significant atopy respond better to macrolide treatment. The evidence concerning the effect of macrolides on eosinophilic inflammation in vivo is somewhat conflicting in the literature. Konno et al\textsuperscript{50} examined the effect of roxithromycin on a mouse model of asthma. Roxithromycin treatment for 21 days inhibited the appearance of the eosinophilic cytokine interleukin-5 in lung extracts and also decreased the bronchial responsiveness to metacholine. Amayasu et al\textsuperscript{1} treated a group of 17 asthmatic patients with 8 weeks of clarithromycin or placebo. A double-blinded, randomised, crossover design was used. Patients treated with clarithromycin showed significantly decreased blood and sputum eosinophil counts and sputum eosinophil cationic protein counts compared with placebo. In addition, patient symptoms and bronchial responsiveness to metacholine were reduced. In a similar study Shoji et al\textsuperscript{91} showed that roxithromycin treatment for 8 weeks significantly decreased asthmatic patients’ symptoms, serum eosinophil count and eosinophil cationic protein and sputum eosinophil count and eosinophil cationic protein. It is possible that these promising findings of improved airway function and reduced inflammatory parameters in asthmatic patients may in fact be due to the treatment of superimposed infections rather than a direct anti-inflammatory effect and therefore, these findings should be regarded with some reservation.

Fewer studies have investigated the effect of macrolides on eosinophilic inflammation in chronic rhinosinusitis. MacLeod et al\textsuperscript{64} treated 25 chronic rhinosinusitis patients with clarithromycin 500mg twice daily. Significant reductions were seen in patient symptoms, oedema score and EG2 (a marker of eosinophil activity). A decrease, which did not reach statistical significance, in total eosinophil count was also noted. It should be noted that the study was not placebo-controlled and that the dose and duration of macrolide treatment was not the long-term, low-dose protocol used in most studies investigating the anti-inflammatory effect of macrolides. Other investigators have suggested that macrolides are not effective in the treatment of chronic rhinosinusitis when there is a significant component of eosinophilic inflammation. Iino et al\textsuperscript{38} performed an
immunohistological study of inflammatory cells in the paranasal mucosa of chronic rhinosinusitis patients treated with long-term, low-dose macrolide. They noted that the ratio of IgA-positive cells to IgE-positive cells significantly decreased after therapy and concluded that macrolides are capable of suppressing the inflammatory response except in cases of IgE and eosinophil-dominated inflammation. Similar conclusions were drawn following a study that investigated the prognostic factors that influence response to macrolide therapy in chronic rhinosinusitis. Sixteen patients were treated for 2-3 months of low-dose macrolide. Patients with normal levels of IgE showed a significantly higher symptomatic improvement rate than those with elevated IgE levels. The symptomatic improvement rate was inversely correlated with eosinophil counts in blood, nasal smear and nasal mucosa. The authors concluded that macrolide therapy is not indicated for patients with atopy or eosinophilia.

Weaknesses of the laboratory experiments

The most obvious weakness of our in vitro and in vivo experiments was the small number of patients examined. For example, in our in vitro immunohistochemical experiment, we showed a reduction in expression of NF-κB in 8 of the 10 patients but this reduction was not statistically significant. It is possible that with a larger number of patients examined, there would have been a significant reduction as has been shown in previous studies using different tissue types and techniques.

As has been mentioned in the introduction, patients with chronic rhinosinusitis represent a relatively heterogeneous population. It would have been ideal to obtain mucosal biopsies from a much larger number of patients as this would have allowed an examination of various subgroups of patients. For example, in our clinical trial, patients were more likely to respond to macrolide treatment if they had lower levels of serum IgE. It would have been an interesting and worthwhile exercise to examine the difference in the effect on inflammatory parameters between those with a normal and with an elevated serum IgE. Similarly, patients who responded subjectively to macrolide treatment
compared to those who did not could have been compared to see if there was a difference in macrolide effect on inflammatory parameters.

We used immunohistochemistry for several of our analyses. The staining was performed on formalin-fixed paraffin sections and this proved to be a dilemma on occasions. In particular, we were unable to adequately stain the sections for eosinophil cationic protein, a marker of eosinophil activation. The results obtained from staining with this marker would have provided a valuable insight into the effect of macrolides on eosinophils, which are known to play an important role in chronic sinusitis and nasal polyposis\textsuperscript{74}. With the benefit of hindsight it may have been better to use frozen sections rather than formalin-fixation, as some of the antibodies used in the experiments were more effective when used with frozen sections according to the manufacturers.

As mentioned in chapter 2 and 3, immunohistochemistry was used to determine the degree of expression of activated NF-\(\kappa\)B. The majority of previous studies examining NF-\(\kappa\)B have used electrophoretic mobility shift assay. In future studies we would recommend the use of EMSA rather than immunohistochemistry as the latter produces results that are not easy to accurately quantify.

Our in vitro experiments involved culturing mucosal specimens for 24 hours in macrolide, prednisolone or control. This is a rather artificial situation as it is known that the effect of macrolides in the clinical scenario takes weeks if not months to become apparent. Previous studies in the literature have almost all used similar short durations of treatment in their in vitro experiments and there is a potential danger in assuming that the effect of macrolide seen in vitro will also be present in vivo with longer courses of treatment.

5.3 Clinical trial

Study design
Evidence-based medicine is becomingly increasingly important in the practice of medicine. To enable clinicians to make informed decisions about treatment for their patients, there is a necessity for correctly designed clinical trials in the literature. The ‘gold-standard’ of clinical trials is a double-blinded, randomised, placebo-controlled trial. Despite the abundance of in vitro and open-label studies of macrolides in the literature, there was yet to be such a trial prior to that which was performed as a part of this thesis. Our aim therefore was to perform the first double-blinded, randomised, placebo-controlled trial of long-term, low-dose macrolides in the treatment of chronic rhinosinusitis.

We recruited patients diagnosed with chronic rhinosinusitis and randomised them to receive placebo or roxithromycin for 3 months. As discussed in chapter 4, a wide variety of subjective and objective outcome measures were performed on the patients before and after treatment. In order to obtain information about the time to onset and longevity of any possible macrolide effect, patients completed symptom and quality of life questionnaires at the mid-point of their treatment and 3 months following its conclusion.

**Placebo vs Roxithromycin**

We have shown statistically significant improvements in a range of outcome measures in patients treated with roxithromycin. These improvements were seen in saccharine transit time, nasal endoscopic scoring and SNOT-20 scoring at the conclusion of treatment. A decrease was seen in levels of interleukin-8 in nasal lavage fluid but this reduction was not statistically significant. There was no statistically significant improvement in olfactory function testing, peak nasal inspiratory flow and nasal lavage for fucose and α2-macroglobulin. In contrast, patients receiving placebo did not show any changes in their post-treatment outcome measures.

Importantly, this is the first time that long-term, low-dose macrolide has been shown to be effective in the treatment of chronic rhinosinusitis in a placebo-controlled trial. Our findings support those of previous authors who have reported clinical improvement in patients in open-label trials\textsuperscript{12,34}.
Patients with low vs high IgE

One of the principal aims in our clinical trial was to determine if there was a subgroup of patients who are more likely to respond to macrolide treatment. A previous study by Suzuki et al\textsuperscript{100} reported significantly improved symptoms in those patients with normal levels of IgE. Improvement was also seen to inversely correlate with levels of eosinophilia in blood, nasal smear and nasal mucosa. In our clinical trial we showed that patients with low levels of IgE had statistically significantly improved outcomes compared to those with elevated levels. Patients with low levels of IgE showed significant improvement in saccharine transit time, nasal endoscopic scoring, SNOT-20 scoring at the conclusion of treatment and IL-8 levels in nasal lavage fluid. In contrast, patients with elevated levels of IgE showed improvement only in saccharine transit time. Our findings are in keeping with previous studies and with the known mechanisms of action of macrolides as discussed above. We believe that this finding of improved outcomes in patients with low levels of IgE may prove to be a useful screening tool in the assessment of patients who may be suitable for macrolide treatment.

Onset of effect

The clinical effect of macrolide treatment is known to be delayed. Previous studies have suggested that prolonged courses are necessary in the treatment of chronic airway inflammation\textsuperscript{12,34}. These studies are in keeping with the results of animal experiments. Erythromycin has been shown to produce an anti-inflammatory effect in rat peritonitis. This effect was maximal after 28 days of treatment\textsuperscript{70}. Similarly, roxithromycin had minimal effect after 5 weeks of treatment in lipopolysaccharide-induced inflammation in mice but at seven weeks there was a dramatic suppression of interleukin-1\textbeta\textsuperscript{97} and TNF-\textalpha production.

In our clinical trial we aimed to confirm the need for a prolonged course and also wished to determine the longevity of any beneficial effect. To fulfil this aim, we asked patients receiving roxithromycin or placebo to complete the SNOT-20 questionnaire at the mid-point of their treatment and 12 weeks following the conclusion of treatment. No
significant improvements were seen in the SNOT-20 scores after 6 weeks of treatment. This is in contrast to the significant improvement seen in patients after receiving 12 weeks of roxithromycin. This finding confirms the need for a prolonged course of macrolide if it is to be trialled in a chronic rhinosinusitis patient.

Patients enrolled in the clinical trial also completed the SNOT-20 questionnaire 12 weeks after their treatment had concluded. The significant improvements seen in the roxithromycin-treated patients after 12 weeks of treatment, was no longer present 12 weeks following the completion of treatment. In the subgroup of patients with low levels of IgE there was an improvement in SNOT-20 scores 12 weeks following treatment, however, this improvement failed to reach statistical significance (p=0.06). Previous studies have not examined the question of the longevity of the clinical effect of macrolides despite this being an important question. If patients are to be given prolonged courses of macrolide antibiotics it is important for the physician and the patient to be aware that the effect seen at the conclusion of treatment may not last for a prolonged period. This may necessitate multiple or even longer courses of macrolide and may lead to problems with bacterial antibiotic resistance, compliance and side-effects.

**Magnitude of the clinical effect**

In our clinical trial we reported that patients receiving roxithromycin had significant improvements in their symptom and quality of life scores. However, it should be noted that this improvement does not necessarily mean that patients felt that they had been ‘cured’. In fact, on the final response scale, not 1 of the 29 patients who received roxithromycin reported that their symptoms had ‘completely improved’. Instead, the improvements seen were more subtle, patients reported some benefit in certain symptoms without experiencing complete relief. This is an important finding that physicians prescribing macrolides should make their patients aware of. Patients should have realistic expectations about the magnitude of any clinical benefit that they may experience. This benefit then needs to be weighed against the risks, cost and inconvenience of the treatment in order to decide whether macrolides are indicated.
Weaknesses of the clinical trial

A power analysis was performed prior to the clinical trial being commenced and on the basis of that analysis we aimed to recruit 50-60 patients. This aim was achieved, however, a larger trial with more patients would have been beneficial. As discussed in the introduction, patients with chronic rhinosinusitis represent a heterogeneous population and thus a larger cohort of patients would have allowed further analysis of different subgroups. For example, we did not differentiate between patients who had previous surgery or not. It would have been useful to perform such an analysis as this would have enabled us to come up with some additional guidelines as to which patients are more or less likely to respond to macrolide treatment. Similarly, in our subgroup of patients with low levels of IgE we showed an improvement in SNOT-20 scores 12 weeks after the conclusion of treatment but this improvement failed to reach statistical significance. It is possible that with a larger group of patients (there were only 14 in the low IgE group) there may have been a significant result.

One of the principal concerns regarding the use of long-term, low-dose macrolides in the treatment of chronic rhinosinusitis is the development of resistant bacteria. Macrolide-resistance is a worldwide problem and is related to the rate of consumption in the community. Resistance develops via 2 mechanisms. The first involves the $\text{mef}$ gene and results in an increase in the minimum inhibitory concentration to between $1 \mu g/ml$ and $32 \mu g/ml$. The second mechanism involves the $\text{erm A/M}$ gene which alters the drug’s target binding site, increases the MIC to $>64 \mu g/ml$ and renders macrolides ineffective. The majority of the clinical trials investigating the immunomodulatory effect of macrolides have failed to investigate the development of resistant organisms. A Japanese study of patients with diffuse panbronchiolitis receiving macrolide failed to show the development of resistant organisms however, this study involved only a small number of patients. In our trial we did perform pre- and post-treatment swabs of the middle meatus for microscopy, culture and sensitivity. No macrolide-resistant bacteria were noted after macrolide treatment however, once again, a weakness of our trial was that our patient numbers were far too small to make any meaningful observations. A large-scale trial with careful microbiological assessment and large numbers of patients will be required to answer this important question. In the meantime, the decision to use macrolides should be
based on the possible benefit to the patient versus the possible risk of side-effects and development of resistant bacteria. It may be that the clinical improvement seen in patients receiving macrolides may be such that they no longer require such frequent short-term courses of antibiotics from their primary physician and therefore that the overall risk of antibiotic resistance may fall.

An additional weakness of our clinical trial is the relatively short duration of follow-up. Unlike previous studies, we did assess patients 12 weeks following the completion of treatment, however, longer follow-up with perhaps additional courses of macrolide would have been a worthwhile endeavour as this is likely to be the clinical practice in the treatment of such patients. Similarly, it would have been interesting to assess any additional improvements that may have become apparent with courses longer than 12 weeks.

**Comparing different macrolides**

In the experiments performed as part of this thesis we used clarithromycin for our laboratory experiments and roxithromycin for our clinical trial. Both of these agents are new generation 14 membered-ring macrolides. As mentioned in chapter 4, roxithromycin was used in the clinical trial as large amounts were required and it was considerably less expensive and more readily available than clarithromycin. Previous authors have examined the efficacy of different macrolide agents in terms of clinical effect and in vitro anti-inflammatory effect. These studies have been performed in Japan and largely published in the Japanese literature but have been quoted in articles published in English. Kita in 1995 treated 71 patients with clarithromycin or erythromycin for 3 months and noted no clinically significant differences between the 2 groups. Tanaka showed no difference in clinical outcomes in patients treated with 6 weeks of clarithromycin or roxithromycin. In contrast, Hashiba et al reported that more patients receiving clarithromycin 200mg twice daily showed clinical improvement than those receiving erythromycin 200mg three times daily. In vitro studies support the findings of these clinical trials. Kohyama et al reported equivalent reductions in IL-8 production by human eosinophils after treatment with erythromycin or clarithromycin. Suzuki et al
showed that IL-8 production by nasal epithelial cells was equally reduced by treatment with erythromycin, roxithromycin and clarithromycin\textsuperscript{101}.

The studies mentioned above suggest that each of the 14 membered-ring macrolides are equally effective in terms of their anti-inflammatory and clinical effect. The new generation macrolides such as clarithromycin and roxithromycin have the advantage of improved bioavailability and reduced gastrointestinal side effects compared to erythromycin.

5.4 Future directions

Much work remains to be carried out in order to further clarify the mechanisms of action and the clinical role of macrolide antibiotics in the treatment of chronic airway inflammation. The effect of macrolides on pro-inflammatory cytokines and inflammatory cell populations requires further investigation. Many of the cytokines proven to be involved in the pathogenesis of chronic sinusitis are yet to be examined. In addition, the effect of macrolides on bacterial biofilm formation, inflammatory cell apoptosis and bacterial virulence needs to be determined.

Of particular interest would be future studies that examine the effect of macrolides in disease models and in ex vivo specimens following macrolide treatment. Such studies would provide valuable information about the effects of treatment in the diseased state rather than in the somewhat artificial environment of the laboratory. Studies to investigate the time course of the anti-inflammatory effect of macrolides may also provide an insight into the apparent delayed onset of clinical benefit.

There are even more questions to be answered in regards the clinical role of macrolide treatment. Firstly, there are issues of side-effects and safety. The side-effect profile in short-term macrolide treatment is well-established but the effects of long-term, low-dose treatment is relatively unknown. Also, there may be issues of emerging microbial antibiotic resistance as a result of using sub-minimum inhibitory concentration doses.
The optimum duration of treatment and the incidence of relapse after treatment has been completed remain uncertain. Future studies should continue to identify prognostic factors that favour macrolide therapy, such as the low levels of serum IgE that we have described. This area in particular requires further investigation so that in the future, treatment can be rationally targeted towards those patients who will benefit most. This will allow for judicious prescribing and reduce the rates of treatment failure and side effects. These questions and others that arise will need to be answered in a series of clinical studies.

5.5 Conclusion

In this work we have performed a series of experiments which have investigated the anti-inflammatory effect of macrolide antibiotics from ‘the bench to the bedside’. We have made a contribution to the increasing body of evidence concerning the mechanism of action of macrolides. Importantly, we have also performed the first ever double-blinded, randomised, placebo-controlled trial of macrolides in the treatment of chronic rhinosinusitis and hope that the results of this trial will assist physicians in making decisions regarding the treatment of their patients. Several questions remain unanswered and we hope that in the future we will be able to continue to contribute to the research in this exciting field.
BIBLIOGRAPHY


Appendix A

Materials

Buffers

EDTA solution
- 10mM, pH 7.5
- EDTA Disodium Salt, Sigma Chemical Co. (St. Louis, USA)

Tris-buffered saline solution
- 50mM, 0.9% saline, pH 7.6
- Tris-Ultra pure, Merck (Darmstadt, Germany)

Phosphate-buffered saline solution
- ph 7.4

Phosphate-buffered saline solution + 30% sucrose

Tris-HCl solution
- 50mM, pH 7.6

Antibodies

Mouse anti-human NFkB p65 subunit monoclonal antibody
- Chemicon (Temecula, USA)
Mouse anti-human Transforming Growth Factor B₁, B₂ and B₃
- Research and Diagnostic Systems Inc. (Minneapolis, USA)

Mouse EG2 anti-human ECP/EPX monoclonal antibody
- Pharmacia and Upjohn Diagnostics AB (Uppsala, Sweden)

Rabbit immunoglobulin fraction – negative control
- DAKO (Carpinteria, USA)

Mouse immunoglobulin – negative control
- DAKO (Carpinteria, USA)

Rabbit biotinylated anti-mouse immunoglobulin
- DAKO (Carpinteria, USA)

Swine biotinylated anti-rabbit immunoglobulin
- DAKO (Carpinteria, USA)

Rabbit anti-human CD3 (T-cell) monoclonal antibody
- DAKO (Carpinteria, USA)

Mouse anti-human CD8 (T-cell) monoclonal antibody
- DAKO (Carpinteria, USA)

Mouse anti-human CD20cy (B cell) monoclonal antibody
- DAKO (Carpinteria, USA)

Mouse anti-human CD68 (Macrophages) monoclonal antibody
- DAKO (Carpinteria, USA)

Tissue culture
Formalin
- 10% neutral buffered
- Sigma Diagnostics (St. Louis, USA)

Prednisolone
- Sigma Chemical Co. (St. Louis, USA)

Clarithromycin
- Abbott Australasia (Sydney Australia)

RPIMI Medium 1640
- Life Technologies (Rockville, USA)

Nunclon™ 24 and 96 well plates
- Nalge Nunc International (Denmark)

**Immunohistochemistry**

Streptavidin ABComplex/Alkaline phosphatase
- DAKO (Carpinteria, USA)

Fast Red Substrate System
- DAKO (Carpinteria, USA)

Liquid DAB+ large volume substrate-chromogen solution
- DAKO (Carpinteria USA)
Streptavidine/Horseradish peroxidase
   - DAKO (Carpinteria, USA)

Vectastain ABC Kit Elite
   - Vector Laboratories (Burlingame, USA)

Superfrost Plus™ slides
   - Menzel Glaser (Germany)

Supermount™ permanent aqueous mounting medium
   - Biogenex (San Ramon, USA)

Mayer’s Haematoxylin
   - Sigma Diagnostics (St. Louis, USA)

Triton X-100
   - Sigma Diagnostics (St. Louis, USA)

Dutch Jug instant skim milk powder
   - Bonlac Foods Ltd. (Melbourne, Australia)

Cryoform™ embedding tissue medium for frozen tissue sections
   - International Equipment Company (Needham, USA)

**ELISA and protein assay**

Bradford Stock Solution (Protein assay dye reagent concentrate)
- Bio-Rad Laboratories (Richmond, USA)

Complete™ Protease inhibitor cocktail

- Roche Diagnostics (Sydney, Australia)

Cytoscreen human IL-5 immunoassay kit

- Biosource International (Camarillo, USA)

Cytoscreen human IL-8/NAP-1 immunoassay kit

- Biosource International (Camarillo, USA)

Cytoscreen human GM-CSF immunoassay kit

Biosource International (Camarillo, USA)