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ORIGINAL CONTRIBUTION

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Time-dependent effect of clarithromycin on pro-inflammatory cytokines in CRS*

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Abstract

Background: The purpose of this study was to assess the time-effect of clarithromycin on the inflammatory response in chronic rhinosinusitis (CRS), to further explore the use of macrolides in cell culture as a model for CRS, and its action on the immune system.

Methodology: The time effect of clarithromycin on several cytokines was examined for IL-1β, IL-4, IL-5, IL-8 and GM-CSF. Samples prior and post-incubation were assessed, as well as samples collected 24h following removal of clarithromycin to determine if any immunomodulatory effect persisted. Cytokines were quantified using ProcartaPlex™ assays.

Results: Of the 5 cytokines assessed, only IL-1β and IL-8 production were significantly inhibited at 4h. Increased levels of IL-4 were observed at 72 hours of incubation and returned to near baseline levels after its removal. IL-8 showed the most time-dependent relationship with clarithromycin. No differences between the expression of IL-5 and GM-CSF were found.

Conclusions: The present work suggests a specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS. Moreover, the immunomodulatory effects of clarithromycin on the cytokines IL-4 and IL-8 varied depending on length of exposure to clarithromycin. Further studies to further establish the relationship between length of exposure and cytokine expression, and with additional "actors" in CRS pathophysiology should be considered. This may enable us in the future to determine appropriate duration of macrolide therapy in patients with CRS.

Key words: sinusitis, nose diseases, inflammation, cytokines, clarithromycin

Introduction

Despite its prevalence and impact on health, chronic rhinosinusitis aetiology remains incompletely understood [(1)]. Diagnosis is primarily based on clinical symptoms, with endoscopy or computerized tomography (CT) scans used to assist diagnosis by showing presence of mucosal changes and nasal polyps. This allows a division into phenotypes – CRSwNP and CRSsNP -, which frequently guides diagnosis, prognosis, and treatment. However, this classification does not reflect its heterogeneity in clinical presentation, pathology and therapeutic response [(1,2)]. The pathophysiological mechanisms underlying CRS involve cellular infiltrate of neutrophils, macrophages and proinflammatory cytokines associated with helper T cell type 1, 2 and 17 [(3)]. More, research has been taken to analyse the cytokine signatures associated with Th1, Th2 and Th17 inflammation to help us subtype according to endotype rather than phenotype [(4)]. A more robust expression of these cytokine markers could serve as an aid for guiding therapeutic decisions in the future. Recently, it has been suggested that several macrolides, such as erythromycin, azithromycin and clarithromycin, are effective for the treatment of respiratory diseases including CRS. Their action consists of inhibiting or killing pathogens and down-regulate pro-inflammatory mechanisms [(5,6)]. While long-term low dose macrolide therapy has been considered an option in the ma-

nagement of CRS, the evidence base for this remains uncertain and is the subject of an ongoing major trial [7]. The National Institute for Health and Care Excellence (NICE) has no clear guidance on dosage, duration of therapy or the patient groups most likely to benefit, based on an international consensus statement published in 2016 [8]. Systematic reviews published in recent years have concluded that macrolides may be effective in improving endoscopic and CT scores in CRS patients compared to baseline, but that effectiveness is likely to depend on appropriate patient selection [9–11]. The mechanism of their anti-inflammatory effect in CRS is an area of active research, with much of our understanding based on studies of relatively small sample sizes. A 2019 systematic review of 22 randomised controlled trials identified that, in CRS patients, macrolides may downregulate expression of pro-inflammatory cytokines IL-1β, IL-6, TNF-alpha and IL-8, amongst others [12], and that a decrease in Th2 cytokines were reported more frequently than a decrease in Th1. There, authors recommended further research to explore the exact mechanisms underlying the immunomodulatory effects of macrolides to optimise usage and identify appropriate patient groups who may benefit.

Evidence examining the relationship between time dependent macrolide exposure or concentration dependent macrolide exposure and cytokine responses in CRS is extremely limited. Two studies examining cytokine expression in mice following macrolide therapy suggested that different exposure times to macrolides may result in different changes in cytokine expression [13,14]. Duration of macrolide therapy may be a factor in responsiveness to macrolide therapy in CRS, but further studies are required to establish whether variation in expression of cytokines implicated in CRS occurs depending on duration of macrolide exposure.

While macrolides may be effective in CRS, lack of understanding and guidance on appropriate antibiotic usage has resulted in unnecessary antibiotic prescribing. This raises concerns as this treatment can have long-term consequences by promoting antimicrobial resistance to antibiotics, with one study demonstrating an increase in proportion of macrolide resistant streptococci following macrolide therapy [5,15]. This risk appears to be greater in long term therapy compared to short term therapy [16]. Antimicrobial resistance is considered a significant threat to patients' safety in Europe [17] and promotion of appropriate antibiotic usage is part of the UK's 5-year antimicrobial resistance strategy [18]. Other concerns with macrolide usage include the risk of adverse effects, such as *Clostridium difficile* colitis and effects on cardiac conduction [19].

A better understanding of macrolides involved in CRS, such as their mechanisms of action and time dependent effects, could enable us to predict patient responses to macrolide therapy and enable a more personalised treatment [20,21]. For instance, it will help us understand whether high doses in the short-term are more beneficial than lower doses in the long-term [5,22].

In this study, clarithromycin was selected rather than erythromycin, since two reviews have raised concerns about its cardiac toxicity, especially in patients presenting a long QT interval [23,24]. Moreover, clarithromycin was selected rather than other macrolides due to its characteristic of therapeutic serum concentrations and high tissue concentrations [25], and has been described as having an immunomodulatory effect on respiratory diseases and also CRS [26–28], through the inhibition of neutrophilic inflammation and macrophage activation [12].

In summary, clarithromycin was preferred since erythromycin has poor tolerability, previous randomized controlled trial revealed poor efficacy with azithromycin administration and roxithromycin has limited availability in the UK. Clarithromycin, on the other hand, is readily available in the UK with a reasonable side-effect profile [25] and it is currently recommended as an option by EPOS and ENT-UK rhinosinusitis commissioning guidelines for the treatment of CRS patients in a secondary care setting [26–28]. Therefore, the aim of this work was to explore and compare the anti-inflammatory effect of clarithromycin in vivo on different inflammatory mediators, with direct correlation on different phenotypes of CRS.

Materials and methods

Ethics statement

All experiments were evaluated and approved by East Midlands - Leicester Central Research Ethics Committee. Due to methodological issues, the A549 cell line rather than patient samples were used to obtain the final results (see details below). All patient tissue samples initially used were disposed of according to principles of the Human Tissue Act 2004.

Cell culture

The cell line used in this work was A549 adenocarcinoma human alveolar basal epithelial cell line, which is used nowadays for both basic research and drug discovery. It was deemed as a good replacement for epithelial cells culture from CRS patients due to its similarity to the nasal mucosal tissue. The line was obtained from Chris Morrison's group (BMRC, University of East Anglia, United Kingdom) and cultured in Dulbecco's Modified Eagle's Medium - low glucose (Sigma Aldrich, Merck, Germany). This media was supplemented with 10% foetal bovine serum (FBS) and 1% of penicillin and streptomycin.

Cells were incubated under conditions of 5% CO2 and 37°C, and medium was changed daily. All cell culture work was performed under sterile conditions. The trypLE (a cell dissociation enzyme similar to trypsin) and phosphate buffered solution (PBS) were purchased from Thermo Fisher.

When the A549 cell line is cultured, a monolayer is formed, which becomes adherent to the base of the culture flask. This type of monolayer growth allows for the calculation and uniform

distribution of the cells within a 24-well plate.

Cell counts and viability

All cells freshly isolated, for tissue culture or cryopreservation were counted and assessed for viability, based on trypan blue dye exclusion. A small volume (10µl) of the sample was transferred to a well of a round bottom 96 well plate (Nunc) and diluted 1:1 with 0.4% trypan blue (Sigma-Aldrich). The cells were then counted using a hemocytometer (Neubauer) to enable a viability count under a light microscope (Olympus, Japan) using the x40 objective. Assessment was made according to cells' appearance under the microscope: cells with a phase bright appearance were viable cells, whereas cells staining densely with trypan blue were dead. The blue colour is due to the intake of trypan blue when cell membranes are disrupted. The percentage viability was calculated by counting numbers of live and dead cells.

Cytokine selection

A literature search was conducted on MEDLINE OVID for systematic reviews published between the years 2000-2018 examining the immunopathology of CRS. Five cytokines were selected for inclusion in this study: IL-1β, IL-4, IL-5, IL-8, GM-CSF. Being an exploratory study, these five cytokines were chosen among numerous others related to CRS. Selection was based on the literature search and posteriorly, related to their particular action on distinctive CRS phenotypes. While IL-5 is widely studied and associated with eosinophilic CRSwNP, GM-CSF, IL-1β and IL-8 are known actors on neutrophilic CRS. IL-4 induces the Th2 differentiation and IgE class switching and was proven in our work to be associated greatly with CRS.

Clarithromycin

The pharmacy department of the James Paget University Hospital (Gorleston, United Kingdom) provided support for the work by providing the clarithromycin used in the experiments (Clarithromycin 500 mg powder for concentrate for solution for infusion, Hameln, Germany). Reconstitution of powder was performed according to manufacturer's instructions. The obtained solution of 50mg/mL clarithromycin was further diluted to 0.064mg/ml, using the same solution of PBS and DMEM used for establishment of the cell culture. This dilution was chosen as it corresponds to the minimum inhibitory concentration of clarithromycin [29].

Cell culture and clarithromycin treatment

To generate epithelial cell media, A549 cells, at an initial count of 0.05x106 cells, were grown in 24-well culture plates (Nunc) until confluent. Before treatment, supernatants were harvested from each well and stored at -80°C, to form our baseline samples. After incubation with 0.064mg/ml clarithromycin solution for 4, 12, 24, 48 or 72 hours, supernatants were collected and stored as before. The cells were then washed twice with PBS and media were replaced with fresh culture media solution to avoid direct effects of clarithromycin on cells survival. Supernatants were again gathered after an additional 24h of incubation and stored at -80°C until analysis.

Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were quantified for levels of IL-1β, IL-4, IL-5, IL-8 and GM-CSF using a human cytokine 5-plex panel (ProcartaPlex Mix&Match Human 5-plex, Invitrogen, MA, United States), according to the manufacturer's instructions. Detection threshold was 0.5 pg/mL. All data was collected and analysed using the Luminex 200 systema and Luminex XPONENT 3.1 Patch (Luminex Corporation, Austin, TX, United States). The median fluorescence intensity of the unknown sample was then converted into a value (pg/mL) based on the known cytokine concentrations of the standard curve using a 5-parameter regression formula.

Statistical analysis

Statistical analyses were performed using RStudio (version 1.4.1106, RStudio, Inc.). For continuous variables, results were expressed as means and standard deviation in box plots. Data distribution was tested for normality using the Shapiro-Wilk test. Comparisons were calculated by Kruskal-Wallis and BH-corrected P value for multiple comparisons. A p-value less than 0.05 was considered statistically significant. This section should provide enough detail to allow full replication of the study by suitably skilled investigators. Protocols for new methods should be included, but well-established protocols may simply be referenced. If applicable, info on ethics approval of either human or animal ethical committees should be stated here.

Results

Cell cultures

The trypan blue dye exclusion test was performed on the culture supernatants after incubation. There was no significant difference in cell viability between the treatment and control specimens (data not shown).

Effects of clarithromycin on in-vitro cytokine and chemokine expression

Clarithromycin in the dose used in these experiments did not exhibit significant effects on cell viability (results not shown). We started by analysing the effects of clarithromycin on in-vitro release of cytokines (GM-CSF, IL-1β, IL-4 and IL-5) and chemokine (IL-8) by A459 cells.

It was seen that these cells produced IL-1β, IL-4 and IL-8 even without further stimulation with LPS (Figures 1-5, incubation time 0). However, the concentrations of IL-5 and GM-CSF were

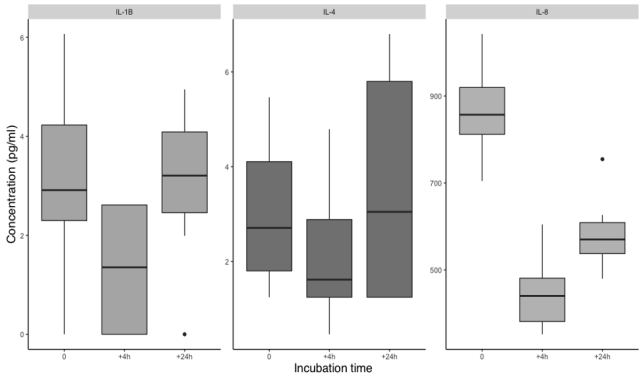

Figure 1. Incubation for 4 h - clarithromycin impact on pro- inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +4h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 8 independent experiments.

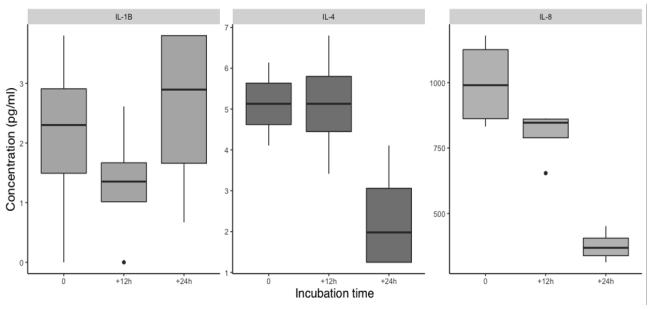

Figure 2. Incubation for 12 h - clarithromycin impact on pro- inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +12h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

low or below the detection threshold in each group and we were unable to analyse the potential immunomodulatory effect of clarithromycin.

After 4 hours of incubation with clarithromycin, there was a reduction in IL-1β and IL-4 concentrations. Removal of treatment increased levels closer to baseline, but these results were not statistically significant. Also, IL-8 concentrations significantly dropped after 4 hours of incubation with clarithromycin. This did not persist, and the IL-8 concentration increased after removal of clarithromycin. However, this continued to be a significant result compared with the baseline result (Figure 1).

To analyse if a longer incubation time with clarithromycin would cause similar patterns, cell cultures were incubated with treatment for 12 hours (Figure 2).

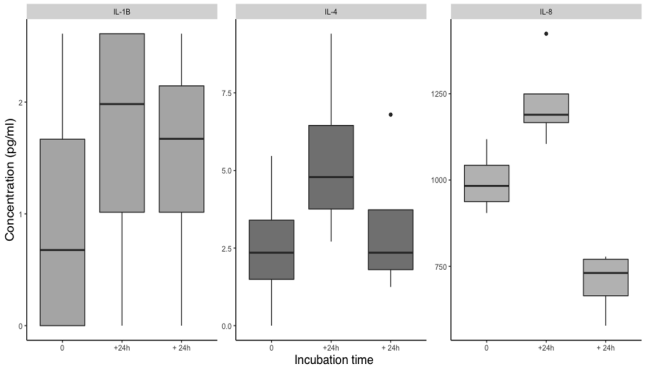

Figure 3. Incubation for 24 h - clarithromycin impact on pro- inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +24h), and 24 h after wash-out (incubation time + 24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

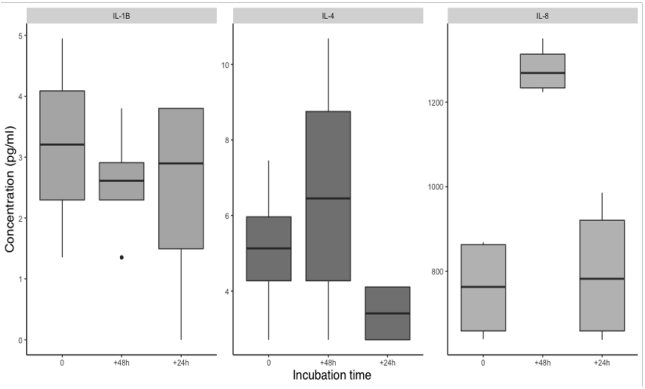

Figure 4. Incubation for 48 h - clarithromycin impact on pro- inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +48h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

Clarithromycin at 12h inhibited IL-1β production, but this was reversed 24h after removal of treatment, returning to values close to baseline levels. On the other hand, there was little difference in concentrations of IL-4 following incubation with clarithromycin. Twenty-four hours after removal of treatment, we could observe a slightly significant decrease on IL-4 levels (p=0.046). After incubation for 12 hours, the IL-8 concentrations reduced. After subsequent removal of clarithromycin, IL-8 concentrations reduced even further. This result was significant (p=0.0154) when compared to the other two samples (Figure 2). After 24 hours of incubation with clarithromycin, exposure led to an increase in IL-1β concentrations, which decreased fol-

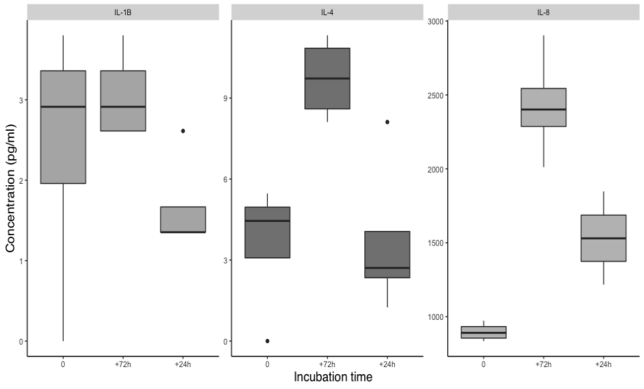

Figure 5. Incubation for 72 h - clarithromycin impact on pro- inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +72h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

lowing removal of clarithromycin. None of these results were significant (Figure 3). After longer exposures of 48 and 72 hours, clarithromycin decreased Il-1β concentration, and the effect persisted following removal. These results were also not statistically significant (Figure 4 and 5).
After 24h, 48h and 72h incubation periods, IL-4 concentrations increased after treatment and decreased following removal of clarithromycin (Figure 3, 4 and 5). This was statistically significant in the 72 hours incubation group (p=0.03, Figure 5).
IL-8 expression levels followed the same trend as IL-4, with its concentrations increasing after treatment and decreasing once clarithromycin was removed (Figure 3, 4 and 5). At 24h and 48h of incubation time, levels after treatment were significantly increased compared with baseline and after washout, whereas at 72h of incubation, all group medians were significantly different from each other.

KEY POINT: A specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS was observed.

Discussion

Our results demonstrate reduced expression of IL-1β after incubation with clarithromycin, although only significant at 4 hours. In cell sample groups which had been exposed to clarithromycin for 24, 48 or 72 hours, concentrations of IL-1β did not return to baseline levels, and the reduction of IL-1β persisted, most noticeably at 72 hours of incubation. IL-4 expression following incubation with clarithromycin and following removal varied, with IL-4 expression reducing after incubation for 4 hours but increasing following exposure for 24, 48 and 72 hours. Incubation with clarithromycin for 4 hours and 12 hours resulted in a reduction of IL-8 levels, this effect persisted on removal of clarithromycin after incubation for 12 hours. As with IL-4, after incubation with clarithromycin for 24, 48 and 72 hours there was an increase in IL-8 expression, with reduction to near baseline levels following removal.

Limitations

Future studies should aim to overcome the limitations present in this study. These include small sampling due to financial constraints. Studies using larger sample sizes may produce less variation in their results and therefore produce more representative results. Other limitations include the choice of the cell line. Ideally, studies should use isolated cells from healthy and CRS patients, however here, we were unable to create a derived stable cell line from patients' samples in the available time. Nonetheless, the A549 cell line can be an appropriate choice, provided that cytokine levels of resting cells are assessed prior to any testing. Also, stimulation might need to be considered if resting levels of mediators are below detection, as seen for IL-5 and GM-CSF. Future studies should also build upon our study and examine additional mediators implicated in CRS to give a broader idea of which cytokines have time dependent responses to clarithromycin. This will be useful in developing duration of therapy recommendations, but also selecting appropriate patients for macrolide therapy.

Interpretations

Macrolides are important therapeutic options in the treatment of many chronic inflammatory diseases due to their immunomodulatory effects, and therefore they can be clinically effective in CRS [12]. However, little is known about how macrolides affect specific pathophysiological features of CRS. In this study, the anti-inflammatory activity of clarithromycin and its immunomodulatory function was investigated, as well as, to assess the duration of its effects. Therefore, this macrolide was administered for 4, 12, 24, 48 and 72 hours, and mediators' levels were measured before and after incubation, and after a washout period of 24h.
The goal of this study was to evaluate the action of clarithromycin on the selected cytokine levels – GM-CSF, IL-1β, -4, -5 and -8. Previously, Courcey et al. [30] performed a similar experiment using nasal epithelial cells that were stimulated in order to assess cytokine concentrations. The study design is identical to the one adopted on this project, so their protocol was tested. Unfortunately, we were unable to obtain primary sinonasal epithelial cells in culture and an immortalised respiratory cell line was privileged to carry out this work. Currently, there are no immortalized nasal cell lines from either "normal" or patients suffering from CRS. As such, we investigated the possibility of using currently available cell lines that could mimic the upper respiratory cells. Previous studies established primary nasal

fibroblast cultures, while others used immortalized lower respiratory epithelial cell lines, such as A549 or BEAS-2B given the similarities between the upper and lower airways [31]. The A549 adenocarcinoma human alveolar basal epithelial cell line is used nowadays for both basic research and drug discovery, and was widely available at our laboratory. Given its similarity to the nasal mucosal tissue [31], it was privileged to act as a replacement of epithelial cells culture from CRS patients.

Literature research was performed for this study to select the mediators of interest - IL-1β, -4, -5, -8 and GM-CSF. Recent studies have clearly demonstrated that in addition to anti-bacterial effects, macrolides may also have anti-inflammatory effects. Previous research has shown that reduction of IL-1β may be a potential mechanism of macrolides [32]. Our results also demonstrate reduced expression of IL-1β after incubation with clarithromycin, although only significant at 4 hours. IL-1β is associated with neutrophilic CRS and clarithromycin may impair production or secretion of these cytokines. This may result in reduced neutrophil accumulation in the sinus mucosa, thereby reducing the inflammation underlying CRS. This may be of particular use in Asian CRSwNP populations, with research suggesting a tendency towards neutrophilic inflammation in Chinese patients [33]. Multiple studies in Asian populations have described patients with neutrophilia as difficult to treat despite treatment with endoscopic sinus surgery and poor response to corticosteroid treatment compared to patients with predominantly eosinophilic inflammation [34–36]. Macrolide therapy may be an alternative or adjunctive treatment option in these patient groups.

In cell sample groups which had been exposed to clarithromycin for 24, 48 or 72 hours, concentrations of IL-1β did not return to baseline levels, and the reduction of IL-1β persisted. This was most noticeable in the samples which had been incubated with clarithromycin for 72 hours. Although these results are not statistically significant, the trend highlighted an area for future research. There is no existing research examining the relationship between IL-1β and time dependent clarithromycin exposure. If found to be statistically significant in studies with larger sample sizes, this may give us an indication that longer durations of treatment may result in longer term suppression of pro-inflammatory cytokine IL-1β. This may help us answer the question of whether short term high dose macrolide therapy or long-term lower dose therapy is most appropriate in relevant patient groups and direct appropriate guidance for clinicians.

When investigating IL-4 levels and clarithromycin exposure, the data obtained demonstrated a reduction in IL-4 after 4 hours, with concentration of IL-4 returning to above baseline levels upon removal. At 12 hours, there was little change when clarithromycin was introduced, while removal resulted in a reduction in IL-4 concentration. After incubation with clarithromycin for 24, 48 and 72 hours, however, IL-4 concentrations increased and returned to near baseline levels upon removal. The increase from baseline and decrease following removal of clarithromycin was significant ($p<0.1$).

Existing research suggests that macrolides may reduce levels of IL-4 [12]. While incubation with clarithromycin for 4 hours demonstrated a reduction in IL-4, this was not statistically significant, and incubation for other times demonstrated an increase in IL-4 following addition of clarithromycin, significant after incubation for 72 hours ($p<0.01$). However further research with larger sample sizes should be conducted to determine whether the trend observed at 24 and 48 hours is significant. As IL-4 has been associated with epithelial barrier dysfunction in CRS [37], and epithelial barrier dysfunction has been implicated as a pathological mechanism in CRS, IL-4 is an appealing drug target. If future studies find that IL-4 expression is significantly increased following clarithromycin exposure for 24, 48 or 72 hours, defining optimum duration of treatment or development of other therapies to target IL-4 may be important.

After 4-hour incubation with clarithromycin, IL-8 concentrations were significantly decreased compared to baseline concentrations ($p<0.05$). Following removal of clarithromycin, the levels increased, although not to baseline levels, and this result was not statistically significant.

Similarly, exposure to clarithromycin for 12 hours also reduced IL-8 levels, although not significantly. Concentrations of IL-8 were further reduced following removal of clarithromycin, and this was significant ($p<0.01$). This may suggest that incubation with clarithromycin for 12 hours is sufficient for the initial reduction to persist. After 24, 48 and 72 hours of exposure to clarithromycin, IL-8 levels appeared to increase, and removal reduced IL-8 concentrations close to baseline levels. The reduction following removal was significant at 24 hours, and the increase in IL-8 and reduction following removal was also significant at 48 and 72 hours ($p<0.01$). These results support Shinkai et al.'s research examining macrolide antibiotics in COPD [38]. They found that IL-8 levels decreased over 6 hours and then increased at 12-72 hours after exposure to clarithromycin and found similar results with azithromycin at 24 and 48 hours, although in our study incubation for 12 hours was associated with an initial reduction in IL-8. This could be due to a severe depletion of Il-4 and IL-8, which on its turn increases Th1/Th2 ratios, favouring the expression of these cytokines during a prolonged exposition to treatment. With the removal of macrolide, ratios would return to normal with cytokine expression returning to normal levels. Further research should focus on examining this result for longer time periods to examine whether this trend continues and enable us to determine whether macrolides are appropriate for patients with neutrophilic predominant CRS.

No conclusions can be drawn from the results of this study relating to the relationship between IL-5 and time dependent clarithromycin exposure. Many of our samples had IL-5 concen-

trations below the detection threshold of our assay. Previous studies have demonstrated a decrease in IL-5 in response to macrolide therapy [12], and therefore, future studies examining IL-5 in this cell line may need to consider stimulation of the A549 cells with TNF-α or Interferon-γ [42]. Other cell lines might also prove to be more adequate in such conditions than the one used in this study.

Many of the samples tested for GM-CSF yielded undetectable results. Previous studies examining GM-CSF in lung cancer and inflammation in airway epithelium have detected little or no GM-CSF in resting A549 cells [43,44]. Future studies examining GM-CSF and time dependent clarithromycin exposure using A549 cells as a model for CRS will need to stimulate the cells with IL-1β and use more samples to provide meaningful results [45]. While GM-CSF was detected in a small number of samples, these levels were low and differences between groups were not found to be statistically significant.

One previous study has considered the relationship between time dependent exposure to macrolides and GM-CSF levels while examining how macrolide antibiotics modulate ERK phosphorylation and cytokine production in patients with chronic obstructive pulmonary disease (COPD) [38]. These researchers found that clarithromycin increased GM-CSF at 48 hours. This is an unexpected result, given that other research has proposed that clarithromycin may reduce levels of GM-CSF [46], suggesting time dependent exposure may have a role. Examining the expression of GM-CSF in response to different time lengths of clarithromycin exposure warrants further research. GM-CSF is proved to be elevated in asthma [12]. Given that CRS is thought to share some of the pathophysiological mechanisms of asthma in the upper airways and that GM-CSF is elevated during symptomatic exacerbations in patients with CRS [47], more research based on the recommendations from this study may provide useful information which could be used to guide management. Clarithromycin in the doses used in these experiments did not exhibit significant effects on cell viability. These findings outline a specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS.

Generalisability

This study showed changes in the cytokine expression profile associated with exposure to clarithromycin in one particular cell line – A459. At the outset our underlying assumption was that different patients will have different patterns of cytokine expression and different changes in cytokine expression profile when exposed to clarithromycin. Further studies on samples of upper respiratory nasal mucosa from different individuals may produce a different pattern of cytokine change, so unnoticeable cytokines in our study should not automatically be excluded from further studies.

The results which have emerged, combined with suggestions from previous research examining macrolides in other airways diseases warrant further investigations into the time dependent effects of macrolide antibiotics in CRS. This is an area which has been little investigated but may enable development of appropriate guidance for macrolide prescribing in CRS patients.

Conclusions

This study demonstrates that the effect of clarithromycin exposure on the cytokines measured here varies over time. Several interesting patterns have emerged in this study. While some cytokines presented no real change, or no obvious pattern of change; others showed apparent trends which were not statistically significant. Lastly, others displayed significant changes. Regarding the lasting effect of clarithromycin, we could note two different patterns: a reversal of the clarithromycin effect or a prolongation of the effect. This could indicate that macrolides may have a long-lasting effect on immune mediators beyond the time that therapy is completed, as well as showing a time-dependent effect.

Determining appropriate duration and dose of macrolide therapy is essential to enable clinicians to balance risks with minimal treatment duration for persistent anti-inflammatory effect. As existing research proposes that mechanisms of actions of different macrolides may vary between each other [20], studies examining other time dependent immunomodulatory effects on these cytokines with other macrolide antibiotics should also be conducted. This would explore whether the effects observed are exclusive to clarithromycin or also present in other macrolides.

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Authorship contribution

AC.P. and J.G. verified the analytical methods. Z.S. carried out the literature review. AC.P. and Z.S. carried out the experiment. AC.P. and Z.S. wrote the manuscript with support from C.P and J.G. C.P. and J.G. helped supervise the project. C.P. conceived the original idea. All authors discussed the results and contributed to the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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