# Azithromycin in Cystic Fibrosis

*Pharmacokinetic and therapeutic aspects of maintenance therapy*

**Erik B. Wilms**

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**Promotiereeks HagaZiekenhuis**

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# Azithromycin in Cystic Fibrosis

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# Azithromycine bij cystic fibrosis

*Farmacokinetische en therapeutische aspecten van onderhoudsbehandeling*

*(met een samenvatting in het Nederlands)*

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# Chapter 1

# Introduction

# 1

# 1 Introduction

## AZITHROMYCIN MAINTENANCE THERAPY IN CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common inheritairy disease in caucasians with 1:32 being a carrier of the recessive gene causing CF. The calculated incidence of CF in newborns would be expected to be around 1:4100. The actual incidence is lower, currently in the Netherlands CF occurs in 1:4750 newborns [1].

CF was first described as a pathologic entity by the New York pathologist Dorothy Andersen in 1938 [2]. She named the pathology 'cystic fibrosis of the pancreas' due to the pathologic findings in the pancreas. In that time the symptoms of CF were described as: 'In those cases which survive the neonatal period the course is characterized by failure to gain weight, rather voluminous, sometimes frequent, stools, and the existence of a chronic respiratory infection' [3]. Elevated viscosity of pancreatic exocrine fluid results in stasis of pancreatic enzymes and damage to pancreatic tissue. Pancreatic insufficiency can already occur before a child is born. Pancreatic insufficiency can nowadays largely be overcome by the use of pancreatic enzymes around the meals, adapted nutrition and supplemental vitamins.

The genetic background of CF was found in 1989 after the gene for Cystic Fibrosis Transmembrane conductance Regulating protein (CFTR) was sequenced. The mutation involved was a deletion of phenylalanine from position 508 (Phe508del, delF508) [4-7]. The delF508 mutation can be found in about 70% of CF patients in the Netherlands. Nowadays worldwide about 1800 mutations have been detected and filed in the CF mutations database [7-9].

The CFTR protein is regulating chloride transport across membranes. CFTR protein is found in epithelial cells of lung, liver, pancreas, skin, digestive tract and genito-urinary tract. Functional CFTR moves negatively charged chloride and thiocyanate out of an epithelial cell. Positively charged sodium will follow in the same direction. Due to the excretion of chloride and sodium the extracellular mucus will attract water by osmosis and the viscosity will decrease which results in a more fluid mucus. CFTR is also found intracellular in the membranes of lysosomal vesicles [5,6,10].

In the skin mutated CFTR results in a reduction in sodium chloride transport in the sweat gland reabsorptive duct resulting in elevated salt content of sweat. Saltier sweat was one of the first signs linked to the pathology CF. Measuring the elevated salt content is used as a diagnostic tool [4,5,10].

Chronic, repetitive pulmonary infections and inflammation leading to end stage pulmonary disease, are the main cause of a reduced life expectancy. In early age Staphylococci are often involved in pulmonary infections. Later on, *Pseudomonas aeruginosa*, is involved and can colonize CF lungs and cause repetitive exacerbations of pulmonary infection. *P.aeruginosa* has distinct properties which enable the bacteria to grow in CF mucus and to colonize in the CF lung [11]. Chronic infection and inflammation lead to formation of sputum plugs, airway wall thickening, bronchiectasis and areas with reduced ventilation [12]. Finally, when lung function falls below a $FEV_1$ of 30% or other pulmonary complications develop, lung transplantation is the only remaining therapeutic option [12-16].

How the malfunctioning CFTR protein leads to deregulation of innate immunity in the lung and pulmonary infections is probably multi factorial. The first defense mechanism against inhaled pathogens is formed by the mucocilliary clearing system. The inability of mutated CFTR to excrete chloride leads to water absorption and a more viscous mucous layer of a reduced thickness hindering the mucocilliary movement of the mucous layer [20-22].

This also influences the excretion of bacterilytic enzymes (B. defensins, cathelicidins) from sub mucosal gland ducts which form a secondary defense against pathogens. Furthermore elevated viscosity also reduces the migration of neutrophils resulting in less effective bacterial removal. CFTR is also found in lysosomal vesicles of epithelial cells and macrophages and is involved in internal acification of the lysosomal vesicles. In CF this can result in an increase in cytokine release, in early cell death and in ceramide accumulation resulting in decreased bacterial killing. Increased cytokine production leads to inflammation and neutrophil attraction. DNA released from non vital cells increases viscosity of CF mucus and contributes to the development of a vicious circle. So a combined malfunctioning innate immune system leads to chronic infection and colonization of microorganisms which are adapted to the specific environment in a CF lung. This leads to a further disregulation of pulmonary immunity and to damage of pulmonary tissue [19-27].

## CURRENT TREATMENT OF CYSTIC FIBROSIS

Treatment is directed towards all symptoms of CF. Pancreatic dysfunction is treated with additional pancreatic enzymes. In case of diabetes, insulin therapy is instigated. Nutrition has to be adapted to the patients extra needs and fat soluble vitamins have to be supplemented. Physiotherapy is used to stimulate clearance of sputum and combined with mucolytic therapy (dornase alfa) and/or inhalation of hypertonic saline to stimulate mucus clearance [18].

Treatment of pulmonary infection is directed towards the pathogens involved. These can be isolated from sputum samples or from bronchial lavage fluid. Anti microbial therapy in younger patients is directed towards control of pulmonary exacerbations with *S. aureus* or other pulmonary pathogens. In case of first infection with *P.aeruginosa* an attempt to eradicate the pathogen has to be performed. Anti *P.aeruginosa* antibiotics like systemic ciprofloxacin and local colistin and tobramycin are used in eradication schemes with the purpose to delay chronic infection with *P.aeruginosa* [12-13, 18].

When a patient is chronically infected with *P.aeruginosa*, treatment has to be directed towards control of infection and inflammation. Maintenance treatment with inhaled antibiotics and macrolide maintenance therapy are indicated [4, 11-14, 18]. Exacerbations of pulmonary infection have to be treated with a course of systemic antibiotics, administered orally or intravenously. Combinations of aminoglycosides (tobramycin), cephalosporins (ceftazidim) and carbapenems (imipenem-cilastine or meropenem) are used in the treatment of exacerbations [12-14].

## HISTORY OF MACROLIDE MAINTENANCE THERAPY IN CHRONIC INFLAMMATORY LUNG DISEASE

Diffuse panbronchiolitis(DPB) is a chronic inflammatory condition of the lungs which was first recognized and described in Japan. DPB occurrence is largely restricted to Japan, in other countries only incidental patients have been reported. DPB has certain similarities with CF: chronic inflammation, neurophil and leucocyte infiltration in the lungs, sputum plugs, infection with *H. influenza* and *P.aeruginosa* and a continuous decline in lung function. In contrast to CF, in DPB no pancreas insufficiency and altered sweat electrolytes occur and no relation with disfunctioning CFTR protein is known. Although CF and DPB share part of the pulmonary complications they are two distinct diseases and do not share a common cause.

A remarkable amelioration in DPB patients was seen after courses of the macrolide antibiotic erythromycin. From 1991 the use of erythromycin in DPB is reported in Japanese literature. In 1991 the first report was made of a series of 19 patients that were treated for 2 month's with a daily dose of erythromycin of 400-600mg. 16 patients were relieved of dyspnea and productive cough and showed improvement on chest X-ray. In part of these patients quantification of erythromycin in serum and sputum showed concentrations below the minimal inhibitory concentration (MIC) for *P.aeruginosa* and *H. influenza*, which made the authors suggest the effect might be caused by a non antimicrobial mechanism [28].

In DPB erythromycin therapy is associated with a decline in production of pro inflammatory cytokines (Il-1, Il-6, Il-8, TNF-alfa) [29-33]. Erythromycin also inhibits the formation of leukotriene B4, the release of superoxide anions by neutrophils and a reduction in neutrophil count in broncho alveleolar lining fluid (BALF) [29]. In DPB the anti inflammatory effects result in fewer airway infections, improved airway function and an increase in survival. Therapy can be continued until the symptoms are resolved and centrilobular nodules can no longer be detected. Apart from erythromycin the beneficial effect of clarithromycin, roxithromycin and finally azithromycin in DPB have also been reported [33].

## MACROLIDE MAINTENANCE THERAPY IN CYSTIC FIBROSIS

Based on the experience in DPB and on reports about the influence of macrolides on the virulence of *P.aeruginosa* the possibility to use macrolides in CF patients with a *P.aeruginosa* infection were first mentioned in 1995 [34]. In 1998 the first report of a retrospecitive analyses of the use of maintenance therapy with azithromycin in children with end stage CF and colonized with *P.aeruginosa* was made by Jaffe [35]. Over a median treatment period of 0,6 year 6 out of 7 patients showed an increase in lung function compared to baseline(median increase $FEV_1$ : 11%). Based on these results 7 prospective, randomized placebo controlled trials were performed. Of these trials, 5 compared azithromycin therapy with placebo and 2 compared two azithromycin dosing regimens. All trials used change in lung function, compared to placebo, expressed as change in $FEV_1$ as primary outcome measure. Secondary outcome parameters were changes in FVC, number of exacerbations or time to first exacerbation, hospital admission rate, weight or body mass index(BMI), quality of life, effect of inflammatory markers and adverse effects [36-40].

In table 1 the key results of the primary outcome measure $FEV_1$ are summarized. In a recently updated review on behalf of the Cochrane Collaboration (2011) it was concluded that a consistent improvement in $FEV_1$ over 6 months was demonstrated. A mean difference of 3.97% (95% conf interval 1.74%-6.19%) was calculated using combined data of 4 studies (n=549 patients). The likeliness to be free of exacerbations during 6 months was 1.96 times higher in the patients treated with azithromycin [41]. The consistent improvement in respiratory function after 6 months of therapy has not been demonstrated for extended periods of time. Only a limited number of studies report data over a longer period of time. Data reported by Equi show the maximum increase in pulmonary function at 6 months and a decline in efficacy over 1 year, with a tendency towards equivalence between azithromycin and placebo [37, 41].

Sub analyses of only patients chronically infected with *P.aeruginosa* showed a larger increase in $FEV_1$: 4.7% (95% cI 1.2-8.2, p=0.009) [37]. In a second trial by Saiman in *P.aeruginosa* uninfected patients the difference in $FEV_1$ was not statistically significant (p=0.61) [40].

**Table 1:** *Key data of 5 prospective, randomized, placebo controlled trials of azithromycin maintenance therapy in CF patients. n t = number patients on active treatment, n c = number of patients control (placebo treatment), P.a. = P.aeruginosa nr or percentage patients colonized, $FEV_1$: forced expiratory volume in 1 second, % of predicted value, t = treatment period in months, CI = 95% confidence interval [36-40].*

| Ref | n t/n c | *P.a.* + (%) | Δ $FEV_1$ Compared to placebo | T Month | 95% CI | Dose < 40(36) kg | Dose > 40(36) kg |
|---|---|---|---|---|---|---|---|
| Equi 2002 | 41 cross over | 50% | 5.4% | 7 | 0.8-10.5% | 250 mg/d | 500 mg/d |
| Wolter 2002 | 30/30 | 83% | 3.9% | 3 | 1.5-6.3% | | 250 mg/d |
| Saiman 2003 | 87/98 | 100% | 5.0% | 6 | 3.4-6.6% | 250 mg 3x pw | 500 mg 3 x pw |
| Clement 2006 | 40/42 | 25% | 2.7% | 12 | -0.1-5.5% | 250 mg 3x pw | 500 mg 3 x pw |
| Saiman 2010 | 131/129 | 0% | 1.4% | 6 | -1.5-4.3% | 250 mg 3x pw | 500 mg 3 x pw |

Besides a better pulmonary function expressed as $FEV_1$, the FVC showed comparable results. Four trials, including the Saiman trial in *P.aeruginosa* uninfected patients, reported a significant decrease in need for antibiotic courses [36-40].

In all studies azithromycin was well tolerated. Gastro intestinal side effects (nausea and diarrhea) were reported with a higher incidence in the azithromycin treated patients. From the combined data of studies 1-4 a relative risk of 1.72 (95% CI, 1.3-2.2) for nausea and diarrhea was calculated [41]. In two studies audiometry was incorporated in the protocol [37,38]. Although some reversible changes were seen no differences between the treatment and placebo group were noted. Hearing loss is of great concern for CF patients pretreated with aminoglycosides and some cases of hearing loss in non-CF patients after azithromycin use have been reported [38].

It can be concluded that treatment with azithromycin results in an increase in lung function with an average difference at 6 months of 4.7% in *P.aeruginosa* infected patients. In patients not chronically infected with *P.aeruginosa* this effect on pulmonary function was not significant, but in non infected and in *P.aeruginosa* infected patients secondary parameters (number of antibiotic courses, number of hospital admissions) were favorable

in patients treated with azithromycin compared to placebo. In a detailed analyses of results of the trial by Saiman a relation between gain in lung function and secondary parameters like number of exacerbations could not be found and it was concluded that treatment with azithromycin resulted in benefits like a reduction in number of exacerbations regardless of the lung function response expressed as $FEV_1$ [43]. Only limited data about long term efficacy were reported, and in the data reported a tendency towards a reduced or no effect on pulmonary function was observed.

As a consequence of the outcome of these studies, maintenance treatment with azithromycin was incorporated in a Dutch guideline for the treatment of CF. The use of maintenance antimicrobial therapy using a combination of inhaled tobramycin or colistin and oral azithromycin is recommendated in patients chronically infected with *P.aeruginosa* [18].

## METHOD OF ACTION OF MACROLIDE MAINTENANCE THERAPY

The author of the first report of erythromycin use in DPB stated that the serum and sputum concentration of erythromycin were below the minimal inhibitory concentration (MIC) of *P.aeruginosa* and *H.influenza* and suggested an alternative mode of action [28]. Since then the results of a large number of experiments have been reported and part by part resolved the anti-microbial and anti-inflammatory mechanisms influenced by macrolides.

A sub MIC anti microbial effect towards *P.aeruginosa* has been documented in several reports. Iamamura showed that the mean bactericidal concentration (MBC) decreased when *P.aeruginosa* in the stationairy phase is exposed to sub MIC concentrations of azithromycin [44]. Tateda showed that sub MIC concentrations can be bactericidal to certain *P.aeruginosa* strains [45,46]. Additionally azithromycin appeared to interfere with the production of alginate biofilm [47,48]. Alginate producing mucoid strains of *P.aeruginosa* are more resistant to antimicrobial therapy [49]. Co administration of clarithromycin increased the susceptibility of *P.aeruginosa* for tobramycin in a in vitro biofilm model [49].

*P.aeruginosa* virulence depends on various cellular and extracellular factors and is in part controlled by quorum sensing. Azithromycin influences the production of quorum sensing factors and interferes with the production of several extracellular virulence factors thereby reducing *P.aeruginosa* virulence [50-54].

Apart from a sub MIC anti-microbial activity and reduction of virulence of *P.aeruginosa*, macrolides modulate several inflammatory pathways. Macrolides down regulate the production of pro inflammatory cytokines and interfere with neutrophil recruitment and tissue penetration [55-58]. Also chemotaxis and oxidative burst seem to be influenced [55,59,60].

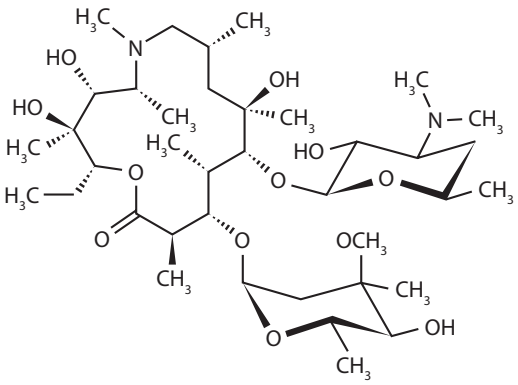
Besides the effects on *P.aeruginosa* and the anti inflammatory effects a reduction in prevalence of *S. aureus*, *H. influenza* and *S. pneumonia* was observed in patients on azithromycin maintenance therapy. This was associated with an increase in macrolide resistance in *S. aureus* [61-63].

Although a unique mechanism of action cannot be described, the combination of sub MIC anti *P.aeruginosa* effect, reduction of *P.aeruginosa* virulence and anti-inflammatory effects are likely to result in the clinical efficacy of macrolide maintenance treatment in CF and DPB.

## PHARMACOKINETICS OF MACROLIDES

Erythromycin is a metabolite of a strain of *Streptomyces erythreus* and is the prototype of the macrolide class of antibiotics. Azithromycin (deoxo-azamethyl-homo-erythromycin) and clarithromycin are the most widely used synthetic analogues with improved chemical, pharmacokinetic and microbiological properties. Clarithromycin (6-O-methylerythromycin) is chemically stabilized by the methylation of the lactone ring. Whereas clarithromycin and erythromycin consist of a 14 membered lactone ring, in azithromycin the ring was expanded with a methyl substituted nitrogen [64].

*Azithromycin*



Erythromycin has a wide variation in oral bioavailability depending on the formulation used and the stomach content. Its $t_{1/2}$ in plasma is short (1-2 hr). Clarithromycin has a more favorable oral bioavailability ( 55%) and a $t_{1/2}$ in plasma of 2-6 hr. Azithromycin has an oral bioavailability of 37% with a $C_{max}$ at 2-3 hr. About 5-7% is excreted unchanged in urine but its kinetics are not dependent of renal function. Azithromycin is metabolized hepatically in about 10 metabolites which are believed to be inactive. A $t_{1/2}$ of about 70hr was measured in plasma, but low concentrations can be found in plasma up to 200 hr after dosing. Dosing schedules of 1500 mg in 3 days and in 5 days in adults and 30 mg/kg in children in 3 or 5 days have been used and incorporated in its summary of product characteristics (SPC) [64-66].

The pharmacokinetics of azithromycin are characterized by rapid distribution with cellular uptake in leucocytes and distribution into intracellular and interstitial compartments of tissues. In infectious conditions the tissue penetration is even better compared to non infectious tissue and is probably related to neutrophil recruitment. In lung tissue, epithelial lining fluid and saliva concentrations exceeding the plasma concentrations have been found [67-75].

## AIMS AND OUTLINE OF THIS THESIS

In the publications about clinical efficacy of azithromycin in CF pharmacokinetic data were lacking. The data available from non-CF treatment can only in part be extrapolated to the use in CF. Several questions have risen during the implementation of chronic macrolide therapy. At first: what are the consequences of chronic, continuous use of azithromycin? Given its long $t_{1/2}$ and excellent tissue penetration could this lead to accumulation in plasma, blood or in the target organ the lung? [75, 76]

As azithromycin is added to existing therapy and is chronically used together with a wide variation of other therapies we must be aware of the possibility of drug interactions. A specific combination suspected for drug-drug interaction is the combination with the DNA hydrolyzing enzyme dornase alfa. Does azithromycin, in the concentration range we found in sputum, interfere with the DNA hydrolyzing properties of dornase alfa? [77]

From the patient perspective description of pharmacokinetic data is of importance when these data can be used to optimize the dose and the dosing scheme [78,79].

A final remaining question from both patient and physicians perspective, was to gain information about long term efficacy, safety and the development of resistance in normally susceptible micro organisms.

This thesis is aimed to answer these questions and to contribute to the knowledge about the way azithromycin can best be dosed in azithromycin maintenance therapy in CF.

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# Chapter 2

# Quantitative determination of Azithromycin in plasma, blood and isolated neutrophils by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection

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# 2 Quantitative determination of Azithromycin in plasma, blood and isolated neutrophils by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection


## ABSTRACT

In this study, a high performance liquid chromatographic method with pre-column derivatization and fluorescence detection was optimised and validated for the quantification of Azithromycin (AZM) in plasma. Clarithromycin (CLM) was used as an internal standard. Pre-column derivatization was done with 9-fluorenylmethyloxycarbonyl-chloride. Recovery from blood and polymorphonuclear neutrophils (PMNs) isolated by gravety separation procedure was also assessed. Analytical separation was carried out using a C18 column as stationary phase and acetonitril-phosphatebuffer as mobile phase. Peak quantification was carried out by excitation at 267 nm and detection at 317 nm. A lower limit of quantitation of 0.042 ± 0.017 mg/l in plasma, 0.119 ± 0.065 mg/l in blood and 0.072 ± 0.036 mg/l in water was achieved. Linearity was assessed from 0 to 1.5 mg/l in plasma and blood and from 0-9 mg/l in water.

The analytical method was proved to be applicable in a pharmacokinetic study of AZM in a Cystic Fibrosis patient.

# 2 Quantitative determination of Azithromycin in plasma, blood and isolated neutrophils by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection

## INTRODUCTION

Azithromycin (AZM) is a 15 membered ring azalide antibiotic related to the macrolide antibiotics. AZM is active against a wide number of gram-positive bacterial pathogens like *Streptococci*, *Staphylococcus aureus*, *Propionibacterium acnes* and *Listeria monocytogenes*. Gram negative bacteria like *Haemophilus influenzae*, *Moraxella catarrhalis*, *Legionella pneumonia* and *Neisseria gonorrhoeae* are also susceptible to AZM. AZM is also active against bacteria that cause intracellular infections like *Mycobacterium avium* and *Chlamidia trachomatis* [1].

AZM exhibits a bacteriostatic effect on susceptible pathogens by interfering with RNA-dependent protein synthesis [2].

AZM is widely used for upper and lower respiratory tract infections, infections of skin and soft tissue and sexually transmittable infections of bacterial origin.

AZM is administered for a short period of time (usually three consecutive days) [1]. Accumulation of AZM in tissue and white blood cells (both polymorphonuclear neutrophils and monocytes) has been associated with a prolonged antimicrobial effect [3,4].

AZM is able to reduce or stabilize clinical symptoms of airway inflammation of patients chronically infected with *Pseudomonas aeruginosa*. A chronic infection with *Pseudomonas aeruginosa* often occurs in patients with diffuse panbronchiolitis and cystic fibrosis [5-7]. Although the minimal inhibitory concentration (MIC) for *Pseudomonas aeruginosa* is high (up to 128 mg/l) in vitro data suggest that long term exposure to sub-MIC concentrations are bactericidal [8]. To date the exact mechanism and the optimal dosing schedule for chronic administration of AZM in pulmonary infections with *Pseudomonas aeruginosa* remains unclear.

No pharmacokinetic investigations have been done so far to support chronic dosing in these pathologies. Investigations after short term administration (3 days) showed accumulation of AZM in white blood cells [4]. However, the amount of intracellular accumulation at chronic dosing is unknown. In order to quantify the amount of accumulation in PMNs, we adapted a separation technique to select PMNs by gravity separation [4]. For further pharmacokinetic studies the analytical method has to be suitable for quantification of AZM in blood, plasma, isolated PMNs.

Lack of UV absorption of macrolide antibiotics makes that fluorescence detection can only be used when macrolides are linked to a fluorescent compound. Otherwise, an alternative detection system, like electrochemical detection or mass spectrometry has to be applied [9,11-14]. We modified the method described by Sastre Torano and Guchelaar who used high-performance liquid chromatography with pre-column derivatization with 9-fluorenylmethyloxycarbonyl-chloride (FMOC-Cl) and fluorescence detection [10]. This method has also been used for the quantification of macrolide antibiotics in foods. Sastre Torano and Guchelaar validated this method to quantify 4 macrolide antibiotics (erythromycin, roxitromyin, AZM and clarithromycin) in serum within the same HPLC run. The lower limit of quantification of the described method was 0.09 mg/l for AZM and 0.2 mg/l for clarithromycin (CLM) in a 1 ml sample [10].

We optimised this method to be able to quantify AZM with a lower limit of quantification of 0.05 mg/l in a 0.5 ml sample and validated this method for plasma using CLM as an internal standard. We also determined the recovery, intra-assay variation and LOQ in blood and isolated PMNs.

## EXPERIMENTAL

### Materials

All chemicals and solvents were of analytical or HPLC grade and were used without further purification. AZM dihydrate salt (Pfizer Inc, New York, USA) and CLM (Abbott, Queenborough, UK) were used for spiking blanks. 9-fluorenylmethoxycarbonyl-chloride (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used for derivatization.

Methanol (Lichrosolv), acetonitrile (Lichrosolv), potassium dihydrogen phosphate, di-sodiumcarbonate anhydrous, diethyl ether and potassium hydroxide were purchased from Merck (Darmstadt, Germany).

Ficoll-Paque Plus (Amersham Biosciences, Uppsala Sweden) gravity separation medium, phosphate buffered saline (PBS) pH=7.4 (Mallinckrodt-Baker, Deventer, the Netherlands), Sodium Chloride 0.9% for irrigation and purified water for irrigation (both from Baxter Inc. Utrecht, the Netherlands) were used in the neutrophil separation procedure.

Flow cytometric analysis of isolated cell fraction was performed with anti-cd15 fluorescence probe (BD Biosciences, San Jose, USA).

### Instrumentation and chromatographic conditions

Chromatografic conditions were optimised towards the quantification of AZM with CLM as internal standard, using the settings published by Sastre Torrano and Guchelaar as a start. The concentration of acetonitril in the mobile phase, the column and flow rate were varied to optimise the quantification.

We used an Agilent HPLC system (Agilent 1100 series) which consisted of a Symmetry C18 column (4.6x100 mm, 3.5 microm; Waters WAT066265), and a Symmetry C18 pre-column (4.6x20mm, Waters WAT054225), a solvent delivery pump and degasser, an auto-injector, a column thermostat, and a fluorometric detector (λ excitation- 267 nm, λ emission 317 nm; peak width >0.2 min., photo multiplier gain 10) and a diode array detector (DAD). Chemstation integrating software was used (Agilent Inc, Palo Alto, Ca, USA).

The mobile phase we used in the validation process consisted of a mixture of 760 ml acetonitril and 240 ml 0.02 M phosphate buffer (pH 7.7). The phosphate buffer contained 0.65 g Potassium dihydrogen phosphate in 240 ml distilled water adjusted to pH 7.7 with potassium hydroxide 10% in distilled water.

An injection volume of 50 µl, a flow rate of 1.5 ml/min, a pressure of 115 bar and a column temperature of 28 °C were applied.

Differentiating cell counts were made with a Coulter Counter type Onyx (Beckman Coulter Inc, Pasadena, Ca, USA). Cell identification was performed with a flowcytometer FACS Calibur (BD Biosciences, San Jose, USA).

## Isolation procedure of PMNs

PMNs were isolated from 6 ml lithium-heparin venous blood samples. After a differentiating count of blood cells, 6 ml blood was diluted with 6 ml PBS. This was transferred on a 6 ml layer of Ficoll-Paque density separation medium and centrifuged for 15 min (1250 g) at 21 °C. The supernatant and Ficoll-Paque layer were removed and the cell pellet with PMNs and erythrocytes was incubated 15 min with 45 ml of NaCl 0.2 % at 2-6 °C in order to lysate erythrocytes. PMNs were isolated by centrifugation (5 min, 465 g) and resuspended in 6 ml PBS at room temperature. A cell-differentiating count was performed to determine the number of isolated PMNs. After centrifugation (5 min, 465 g) the supernatant was removed and the cell pellet was kept at -30 °C until determination of AZM.

## Sample treatment

Plasma and blood were obtained from lithium-heparin blood sampling tubes and kept at -30 °C until determination. Quantification of the amount of AZM in PMNs took place after thawing and addition of water until a volume of 1.2 ml. Sputum samples were homogenised after thawing by vortexing after addition of glass-pearls.

## Preparation of a calibration curve

A methanolic AZM stock solution (about 10 mg/50ml) was diluted to a work solution of 0.50 mg/50ml methanol. A calibration curve of 0.1 mg/l to 1.0 mg/l was made by adding 5 µl, 10 µl, 15 µl, 25 µl and 50 µl of the work solution to 0.50 ml of blank plasma. Additional methanol was added to the standards so each sample contained 50 µl of methanol.

For the determination of AZM in blood a standard curve in blank blood samples was made. For the determination of AZM in isolated PMNs a standard curve in water was made.

Standard curves were constructed by non-weighed linear regression.

## Sample extraction procedure

To 0.5 ml samples of plasma, blood and PMN a volume of 50 µl methanol was added. To the calibration curve and samples 100 µl of the internal standard CLM (1.0 µg/100 µl water) and 200 µl of a saturated di-sodiumcarbonate (0.4 mg/ml water, with a pH of approximately 11) were added in a 12 ml disposable glass tube and the solution was vortex-mixed for 2 s. A volume of 6 ml of diethyl ether was added to each tube. The tubes were shaken (200/min) for 15 min and centrifuged for 10 min (2550 g).

The organic layer was transferred into a disposable 12 ml glass tube and evaporated at 40 °C under a flow of $N_2$.

## Derivatization procedure

In order to optimize the derivatization procedure we varied the amount and concentration of FMOC-Cl and the water-acetonitril ratio. The following derivatization procedure was found to be optimal: The residue from the organic layer was dissolved in 100 µl of acetonitril and vortex-mixed for 20 s. The solution was centrifuged for 10 s (2550 g) and quantitatively transferred into a 2 ml reaction vial. A 100 µl of a freshly prepared FMOC-Cl solution (1 mg/ml in acetonitril), 100 µl water and 75 µl phosphate buffer (0.1 M phosphate, adjusted to a pH of 7.5 with 10 % potassium hydroxide) were added.

The reaction vial was incubated in a water bath at 40 °C for 40 min. After derivatization 50 µl of the solution was injected directly from the reaction vial into the HPLC system.

## Assay validation

The selectivity of the method was investigated for a number of drugs commonly used by patients with cystic fibrosis. Prednisolone (1 mg/l), ceftazidim (200 mg/l), omeprazole (1 mg/l), ibuprofen (30 mg/l), sulfamethoxazole (60 mg/l), trimethoprim (2 mg/l), itraconazole (2 mg/l) and fluconazole (6 mg/l) were investigated for interference with the assay.

Precision of the HPLC system, intra-assay variation, inter-assay variation, linearity, recovery, the lower limit of quantitation (LOQ), accuracy and the stability were determined as validation parameters of the analytical method for the assay of AZM in plasma.

LOQ was calculated from the calibration curve constructed by non-weighed linear regression. We defined LOQ at the y-ax intercept plus 3.3 times the standard deviation and extrapolated towards x.
Recovery was assessed with a 0.5 ml plasma and blood sample spiked with AZM. Recovery was also assessed from a blanc PMN suspension isolated from a 6 ml Li-heparin blood sample spiked with AZM after the isolation procedure. After the extraction procedure CLM was added as internal standard. All extraction steps were performed quantitatively. A methanolic solution of AZM with the same concentration served as reference. Recovery was defined as the percentage of the concentration in the methanolic solution determined in the sample.

Stability was assessed in derivatized extracts, stock solution and work solution kept at -20 °C, plasma, blood, PMN and sputum samples kept at -30 °C. The stock solutions and work solutions were compared with freshly prepared solutions. The plasma, blood, neutrophil and sputum samples were assayed with an appropriate time interval and compared to the initial results. Samples were defined as stable when the results were within 3 x the sd.

## Validation of neutrophil separation procedure

Recovery from a blank PMN suspension was assessed as described above.
Recovery and intra assay variation of the PMN separation procedure was assessed with blood incubated with a known amount of AZM and in samples from two patients using AZM.

AZM was quantified in PMNs and in all onther separation fractions and washing fluids. To 6 x 6 ml heparinised blood samples 140 µg AZM was added (3.89 µg/ml). Blood was incubated and gently shaken during 2 h at 37 °C. The incubated blood samples were pooled together and homogenised. The pool was split into 6 samples of 6 ml. Each sample was treated according to the PMN separation procedure. All washing fluids and the separation medium were harvested separately and frozen at -30 °C until assay. A mass balance was made after determination of the concentration and the volume of the sample. Purity of the cell selection was determined in both the PMN containing cell suspension by flow cytometric analysis after labelling with an anti-cd15 fluorescence probe.

From two patients, 4x6 ml Li-heparin blood each were separated as described in the isolation procedure of PMNs. AZM was determined in plasma and blood and in isolated PMNs and in all washing fluids and in the separation medium in four-fold. A mass balance of all fractions was made and compared to the concentration found in blood and plasma. The intra-assay variation was determined.

### Pharmacokinetic study

A pharmacokinetic study was conducted in a patient with cystic fibrosis who was treated with AZM 500 mg per day for at least 35 days. The study was approved by the medical ethical review board and written informed consent was obtained. Lithium-heparin sample tubes were used to obtain 2x6 ml venous blood per sample time. From 6 ml venous blood PMNs were isolated. Samples were taken at t=0, 1, 2, 3, 4, 6, 8, 74 and 220 h. AZM was determined in blood, plasma and PMNs. The amount in PMNs was expressed in amount of AZM in PMNs per 1 L blood.

## RESULTS AND DISCUSSION

### Optimizing chromatografic conditions

We increased the concentration of acetonitril in the mobile phase which resulted in a decrease in retention time. We used a C18 column packed with 3.5 µm particles instead of 5 µm particles in a shorter column and with a reduced flow. This resulted in a reduction in retention time and in a better signal-to-noise ratio leading to a lower detection limit.

### Optimizing derivatization

*Variation of pH:* An increase of the pH (>7.5) resulted in an increase in interferences. A reduction of the pH (<7.5) resulted in a reduction in AZM peak height. We, therefore, used a pH=7.5.

*Variation of the acetonitril-water ratio:* The acetonitril-water ratio was varied from 300-75 to 100-175. An increase of the water content led to a smaller chromatographic front without interference with the strength of the AZM signal.

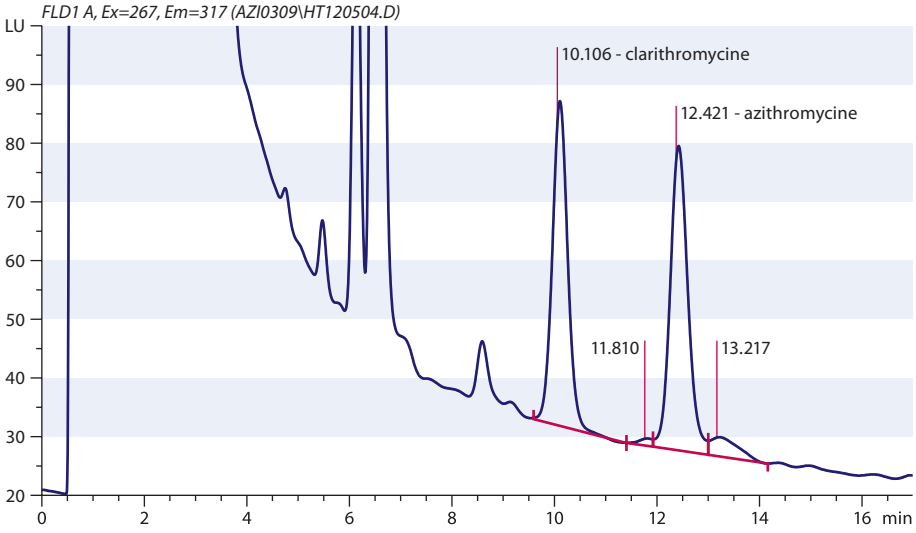
*Variation of the concentration of FMOC-Cl:* The concentration of FMOC-Cl was varied from 0.25 mg/l to 2.5 mg/l. An increase in the concentration of FMOC-Cl to 2.5 mg/l led to a wider front, more interferences and a small increase in AZM signal. Reduction to 0.25 mg/l led to better chromatographic separation (smaller front and less interferences) and a small reduction in AZM signal, which was equal to the reduction in CLM signal. We found an optimum at a concentration of 1 mg/l and an added volume of 100 µl.

We used a reaction time of 40 min. And a reaction temperature of 40 °C, which proved optimal in the method described by Sastre Torano and Guchelaar [10].

## Selectivity

A representative chromatogram and a representative chromatogram of AZM at 0.74 mg/l in plasma is shown in figure 1. The components prednisolone, ceftazidim, omeprazol, sulfamethoxazol, trimethoprim, itraconazol and fluconazol showed no interference with AZM and CLM. Ibuprofen (30 mg/l) showed some interference with CLM when detected with fluorescence detection. Detection with diode array showed no interference. Combined use of fluorescence detection for AZM and diode array detection for CLM can be used as an alternative in patients on high dosages of ibuprofen without loss of sensitivity. There were no interferences present in blank human plasma and isolated PMNs.

**Figure 1:** *A chromatogram of an extract from plasma spiked with AZM at a concentration of 0.74 mg/l and clarithromycin (internal standard) at a concentration of 1.0 mg/l.*




## Precision of the HPLC system

An organic solution of 0.3 mg/l AZM was injected six times. The coefficient of variation (vc) was 0.2 %.

## Intra-assay variation

Two spiked concentrations of AZM within the range of the calibration curve in plasma (0.317 mg/l and 0.634 mg/l) were assayed (each n=6) on the same day in the same run. The coefficients of variation were 0.4% and 1.2 % respectively. A spiked sample (0.063 mg/l, n=6) between the lowest sample of the calibration line and the LOQ was assayed with an intra-assay variation of 4.5%.

## Inter-assay variation

Samples, containing AZM (0.635 mg/l, n=6 and 1.182 mg/l, n=5) in plasma were assayed on different days. The coefficient of variation was 2.2 % and 2.8% respectively. In blood, the inter-assay variation of samples, containing AZM (0.295 and 0.739 mg/l) was 2.4 and 3.9%, respectively.

## Linearity

The calibration curve of AZM resulted in correlation coefficients of 0.999 (plasma, range 0 – 1.5 mg/l), 0.999 (blood, range 0 – 1.5 mg/l) and 0.999 (water, range 0 – 9 mg/l).

## Recovery

The recovery from plasma of 0.635 mg/l AZM was 103.0 % (n=6, vc=1.5 %) and of 1.0 mg/l CLM was 100.0 % (n=6, v.c.= 0.8 %).

The recovery from blood of 0.635 mg/l AZM was 94.5 % (n=6, vc=2.3 %) and of 1.0 mg/l CLM was 99.4 % (n=6, vc= 1.6 %).

Recovery of 2.0 mg/l AZM from a PMN suspension was 103.7 % (N=4, vc=3.4 %).

## Lower limit of quantitation

The lower limit of quantitation was 0.042 ± 0.017 mg/L (plasma, n=6), 0.119 + 0.065 mg/l (blood, n=6) and 0.072 + 0.036 mg/l (water, n=6). A chromatogram of blank plasma and plasma from a patient sample with 0.038 mg/l AZM are shown in figure 2.

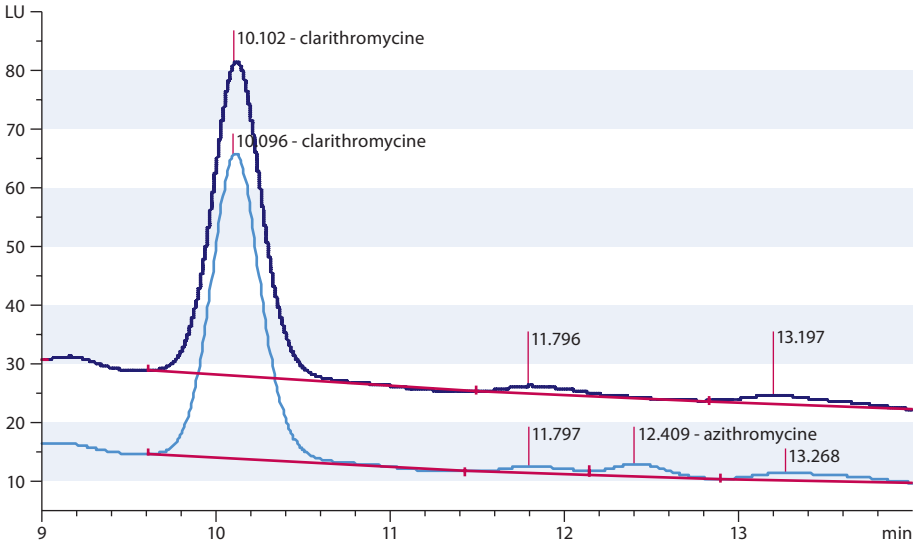
## Accuracy

Six different human plasma samples were spiked with AZM to a concentration of 0.317 mg/l and were calculated on a standard curve. The concentration found was 0.317 mg/l (n=6, vc=3.2 %).

## Stability

The derivatized extracts were stable for more than 24 h. The stock solution of 0.2 mg/ml of AZM in methanol kept at -20 °C was stable for at least 4 months. The work solution 0.01 mg/ml of AZM in methanol kept at -20 °C was stable at least 9 months.
The plasma and blood samples were at least 18 months stable at -30 °C. Isolated PMNs samples were at least stable for 4 months.

**Figure 2:** *Chromatogram of blank plasma (upper line) and plasma from a patient blood sample with 0.038 mg/l AZM (bottom line), which is just below the LOQ (0.04 mg/l). Clarithromycin (1 mg/l) was added to both samples as internal standard.*




## PMN separation procedure

The isolation procedure was performed six fold on a homogenised sample after addition of 23.35 µg AZM per 6 ml and a 2-h incubation period.

The homogenised blood sample contained 7.3 x $10^9$ PMNs per litre. With the separation procedure we harvested on average 3.7 $10^9$ ± 0.4 x $10^9$ PMNs per litre after the volume was adjusted to the starting volume (6 ml). On average, we could recover 51% (vc = 6%, n = 6) of the PMNs. Flow cytometric analysis showed a 91% content of PMNs in the cell pellet.

After determination of the volumes and the AZM concentration of all layers we could recover 88% (vc= 2.4%, n = 6) of the added amount of AZM. Intra-assay variation for the PMN selection and quantification process is calculated from the PMN fraction in which we found on average 0.42 mg AZM/l blood (vc 7.8%, n=6).

From the two patient samples, we isolated on average 52.3% of the PMNs (n=8, vc=15%).

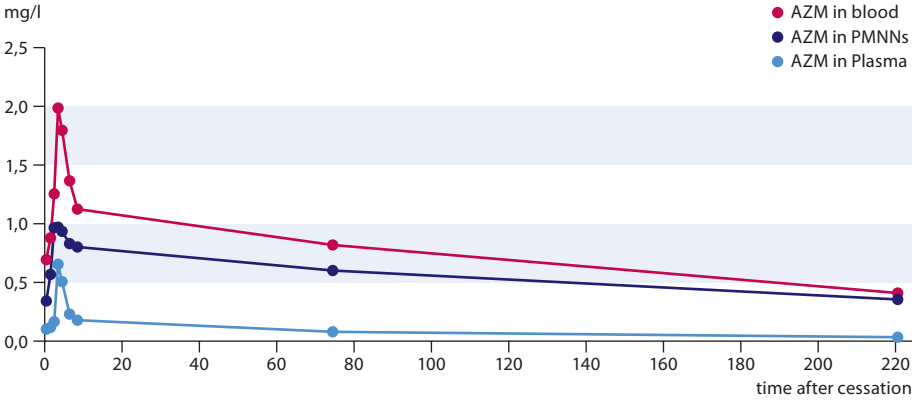
In patient 1, the AZM concentration in blood was 3.8 mg/l. The sum of the AZM content of all layers was on average 86.5% (vc=2.4%, n=4) of the amount detected in blood. In patient 2, we found an AZM level of 0.88 mg/l in blood and could recover 96.0% (vc=2.3%, n=4).

After correction for the incomplete PMN harvest, we found in the PMN fraction 1.35 mg/l blood (vc=4%, n=4) in patient 1 and 0,61 mg/l blood (vc=14%, n=4) in patient 2.

## Pharmacokinetic study

The analytical method and the PMN separation technique were applied to a Cystic Fibrosis patient after obtaining informed consent. Figure 3 shows the concentration-time curves of AZM in plasma (mg/l), in blood (mg/l) and in PMNs in 1 l blood (mg AZM in PMNs/l blood). We see a rapid decline in plasma concentration with a $t_{1/2\alpha}$=0.1 h. The $t_{1/2\beta}$ in plasma is 96 h, in blood 217 h and in PMNs 340 h. At t=220 h over 88% of the blood content of AZM is internalised in PMNs. The present detection method proved to be applicable in this pharmacokinetic study.

**Figure 3:** *Concentration of AZM in blood, plasma and polymorphonuclear neutrophils of a Cystic Fibrosis patient after chronic (>35 days) administration of AZM 500 mg per day and cessation of therapy during a 10 d sampling period.*




## DISCUSSION

We showed that AZM can be determined with HPLC and fluorescence detection after derivatization with FMOC-Cl in the range 0.05-1.5 mg/l. The assay was validated for use in plasma. Recovery, linearity, LOQ and inter-assay variation from blood, LOQ and intra-assay variation from isolated PMNs were also assessed. This makes the assay useful for pharmacokinetic research in our group of patients.

FMOC-Cl is able to form a stable ester-bond with the macrolide hydroxyl group. FMOC-Cl reacts also with the hydroxyl groups and with amino groups of other compounds and is used in amino-acid determination.

In order to achieve a reduction in LOQ with a smaller sample, we optimised the chromatographic conditions towards AZM using a different column and a higher water content in the acetonitril-water mobile phase compared to the method described before [10]. On molecular basis, we used an overage of FMOC-Cl compared to the molecular amount of AZM of about 500 times, calculated on an AZM concentration of 1 mg/l. Still, the added amount is critical: increase of the amount led to a small increase in AZM signal but to a higher increase in interferences, most probably due to FMOC-Cl reacting to hydroxyl- or amino- groups of other substances.

Because, an increase of the added amount of FMOC-Cl led to an increase of AZM signal, we could conclude that not all AZM was derivatized or an equilibrium between underivatized and derivatized AZM had not been reached. This did, however, not influence the validation parameters, probably due to a linear level of reaction between AZM and the internal standard (CLM).

The isolation and detection procedure for isolated neutrophils is difficult to validate completely. AZM is incorporated in PMNs. In order to validate the determination of incorporated AZM from PMNs we did both an ex-vivo uptake experiment and a determination in patients's sample. We quantified AZM in all separation and washing fluids. Ex-vivo we could retrieve 88% (vc=2.4%, n=6) of the AZM that was added to the blood sample. In the patients samples we could retrieve 87% (1 sample, n=4, vc=3.5%) and 96% (1 sample, n=4, vc=5.1%). This gives an identification that the separation method and the subsequent analysis were reliable.

We could recover 51% of the PMNs with a purity of 91%. In the isolation procedure we perform a differentiated cell count before and after the separation procedure in order to quantify the recovery in every separation run and to be able to correct for incomplete recovery of PMNs.

Ibuprofen showed some interference with the internal standard clarithromycin at a concentration of 30 mg/l. To overcome this interference the analyte could be analysed with a diode array detector instead of a fluorescence detector. When combined fluorescence and diode array detection is applied for, respectively, AZM and clarithromycin no loss in sensitivity occurred. However, in a sample obtained from a patient on ibuprofen no interference was seen due to a far lower plasma level of ibuprofen than anticipated.

We can conclude that the described HPLC method with pre column derivatization of AZM with FMOC-Cl is a valid method to determine AZM in plasma. The isolation method for PMNs, mass density separation with Ficoll-Paque separation medium, did not interfere with the assay. The assay proved applicable for pharmacokinetic research of AZM.

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# Chapter 3

# Pharmacokinetics of Azithromycin in Cystic Fibrosis patients

# Chapter 3.1

# Pharmacokinetics of Azithromycin in plasma, blood, polymorphonuclear neutrophils and sputum during long term therapy in patients with Cystic Fibrosis

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# 3.1

# 3.1 Pharmacokinetics of Azithromycin in plasma, blood, polymorphonuclear neutrophils and sputum during long term therapy in patients with Cystic Fibrosis


## ABSTRACT

Chronic therapy with the macrolide antibiotic azithromycin is widely practiced in the treatment of patients with cystic fibrosis (CF) and chronic lung infection with *Pseudomonas aeruginosa*. Azithromycin dosage is variable, based on published studies and not supported by pharmacokinetic data. This study describes the pharmacokinetics of the long term administration of AZM (500 mg per day) in CF patients. AZM concentrations were quantified in the plasma, blood, isolated polymorphonuclear neutrophils (PMNs) and sputum of 8 adult CF patients.

The AZM distribution $t_{1/2}$ was 0.1 hours in plasma. The (mean ± standard deviation) elimination $t_{1/2}$ was 102 ± 20 hours in plasma, 180 ± 68 hours in blood and 289 ± 166 hours in PMNs. The $C_{max}$ of AZM was 0.67 ± 0.31 mg/L in plasma and 2.01 ± 0.74 mg/L in blood, of which 1.44 ± 0.69 mg/L was found in PMNs. In sputum the concentration of AZM ranged from 12 and to 53 mg/L and was still detectable at concentrations in the range 4 to 27 mg/L 10 days after the last dose. On average, the concentration in PMNs was 2100 times the $C_{plasma}$ 24 h after dosing AZM.

These results confirm the accumulation of AZM in PMNs. The authors conclude that sputum levels are elevated far above plasma and blood concentrations. The long $t_{1/2}$ in blood and PMNs and the slow decrease in sputum levels indicate a less frequent dosing schedule (for instance once weekly) should be studied in future clinical trials of AZM in patients with cystic fibrosis.

# 3.1 Pharmacokinetics of Azithromycin in plasma, blood, polymorphonuclear neutrophils and sputum during long term therapy in patients with Cystic Fibrosis

## INTRODUCTION

Azithromycin (AZM) is a 15-membered ring azalide antibiotic related to the macrolide antibiotics. AZM exhibits a bacteriostatic effect towards susceptible pathogens by interfering with RNA-dependent protein synthesis [1].

AZM is generally administered for short periods of time, (usually during three consecutive days) (Product Information Zithromax® nr 70-5179-00-2 Pfizer Inc, NY, rev. 08-2003). Accumulation in tissues and white blood cells (both polymorphonuclear neutrophils and monocytes) has been associated with a prolonged effect [2,3].

Long term administration of AZM is able to reduce or stabilise clinical symptoms of airway inflammation in patients chronically infected with *Pseudomonas aeruginosa* in patients with diffuse panbronchiolitis and cystic fibrosis [4-7]. In vitro, the minimal inhibitory concentration (MIC) of AZM for *Pseudomonas aeruginosa* is as high as 100 mg/L [8]. *Pseudomonas aeruginosa* is considered 'resistant' to AZM on the basis of MIC determination. Sub-MIC concentrations (2 mg/L in strain $PAO_1$), however, did inhibit the formation of factors promoting *Pseudomonas aeruginosa* biofilm formation ('quorum sensing factors') [8-10]. The biofilm protects the bacteria against the immune system of the host and the penetration of antibiotics. Three recent randomised clinical trials in patients with CF used dosing schedules ranging from 250 mg 3 times a week to 500 mg once daily. AZM therapy reduced the number of respiratory exacerbations, improved lung function or reduced the rate of decline in lung function [4,6,7].

To date, no pharmacokinetic studies have been published to support long term dosing schedules of AZM in patients with CF. An optimal dosing schedule for chronic administration of AZM in pulmonary infections caused by *Pseudomonas aeruginosa* remains unclear.

In pharmacokinetic studies after short term use of AZM in healthy subjects/volunteers it was found that plasma concentrations decline rapidly ($t_{1/2}$ = 3 h) during the distribution phase but in the elimination phase low plasma concentrations could be detected for an extended period ($t_{1/2}$ = 30-93 h) [11-14]. In isolated polymorphonuclear neutrophils (PMNs) accumulation of AZM and an extended $t_{1/2}$ >200 h was found [2,3,11,12].

In CF, PMNs accumulate in the airways and contribute to the destruction of airway tissue by the release of proteases [8,15]. CF patients suffer from a chronic pulmonary infection, which results in an elevated PMN count and accumulation of PMNs in airway tissue. This might influence AZM kinetics and distribution in patients with CF.

Therefore, we decided to determine AZM in plasma, isolated PMNs and whole blood, during a sampling period long enough to detect a terminal $t_{1/2}$ of approximately 200 h. In addition, we determined the concentration of AZM in sputum samples.

The main objective of this study was to describe the pharmacokinetics of AZM and blood-cell distribution during a 10-day period after chronic use of AZM, 500 mg once daily, in patients with CF. Furthermore, we sought to calculate the degree of enrichment in PMNs during chronic use of AZM and to evaluate the relationship between AZM concentrations in plasma, blood, PMN and sputum.

## PATIENTS AND METHODS

Adult patients with CF were recruited from the Leyenburg Hospital Adult Cystic Fibrosis Centre. Patients who had received AZM 500 mg once daily for more than 35 days and had chronic *Pseudomonas aeruginosa* infection (confirmed with at least 2 positive cultures in the last 6 months) were included in the study. The study protocol was approved by the institutional review board and patients gave written informed consent before study participation.

### Drug Administration and Sampling

AZM was administered 500 mg once daily during at least 35 days (Zithromax® tablets 500 mg, Pfizer, Capelle a/d IJssel, the Netherlands) before the 10-day sampling period. AZM therapy was ceased at the end of the first day of the 10-day sampling period and restarted after completion of the study on day 10.

Venous lithium-heparinised blood (119 IU li-heparin/7 ml tube, VacutainerTM Becton-Dickinson, Alphen a/d Rijn, the Netherlands) samples were collected at each time point. On day 1 the samples were collected prior to administration and 1, 2, 3, 4, 6 and 8 hours after the last dose of AZM.

A single sample was also collected between day 4 and 7 and on day 10. All expectorated sputum was collected during 24 hours on the day before initial blood sample collection, before the sample drawn at day 4-7 and before day 10.

### Isolation Procedure of Polymorphonuclear Neutrophils and Bioanalysis of AZM

PMNs were isolated from lithium-heparinised venous blood samples. A differential blood cell count was made. To 6 ml blood 6 ml phosphate buffered saline (PBS, pH 7.4, Mallinckrodt-Baker, Deventer, the Netherlands) was added. The diluted blood-PBS mixture was transferred into a separation tube with a 6 ml layer of Ficoll-Paque Plus density separation medium (Amersham Biosciences, Uppsala, Sweden) and centrifuged for 15 min (1250 G) at 21 °C. The supernatant and Ficoll-Paque layer were removed and the cell pellet with neutrophils and erythrocytes was incubated during 15 minutes with 45 ml of NaCl 0.2 % at 2-6 °C in order to lyse the erythrocytes. PMNs were isolated by centrifugation (5 min 465 G) and resuspended in 6 ml PBS. A differential cell count was performed to determine the number of isolated neutrophils. After centrifugation (5 min 465 G) the supernatant was removed and the cell pellet was kept at -30 °C until determination of AZM.

Detailed specifications and validation of the methodology of cell isolation and AZM quantification in blood, plasma and PMNs are described elsewhere [16]. A brief description of the method and the key performance parameters follows: A high-performance liquid chromatographic method with pre-column derivatization and fluorescence detection was used for the quantification of AZM in blood, plasma, isolated PMNs and sputum. Clarithromycin (CLM, Abbott, Queenborough, UK) was used as an internal standard and AZM dihydrate salt (Pfizer Inc, New York, USA) was used as reference.
Pre-column derivatization was performed using 9-fluorenylmethyloxycarbonyl-chloride (Sigma-Aldrich, Zwijndrecht, the Netherlands). Analytical separation was carried out using a C18 column as stationary phase and a mixture of 760 ml acetonitril and

240 ml 0.02 M phosphatebuffer (0.65 g potassium dihydrogen phosphate in 240 ml water adjusted to pH 7.7 with potassium hydroxide 10%) as mobile phase. Fluorimetric detection of the analytes was used for quantification (λ-excitation 267 nm and λ-emission 317 nm).

Calibration curves in plasma and blood (0-1.5 mg/L) were used for determination in plasma and blood. A calibration curve in water (0-9 mg/L) was used for determination in sputum and the PMN fraction.
Plasma, blood, PMNs and sputum samples were kept at -30 °C until determination.
Quantification of AZM in PMN samples was performed after thawing and the addition of distilled water to a total volume of 1.2 ml. AZM was quantified in duplicate in 0.5 ml aliquots in plasma, blood and PMNs.
The lower limit of quantitation of AZM was 0.042 ± 0.017 mg/L in plasma, 0.119 ± 0.065 mg/L in blood and 0.072 ± 0.036 mg/L in water. Linearity was assessed from 0-1.5 mg/L in plasma and blood and from 0-9 mg/L in water.
Recovery and intra-assay variation (expressed as %CV) from plasma at 0.635 mg/L AZM was 103.0 % (n=6, %CV=1.5 %) and of 1.0 mg/L CLM was 100.0 % (n=6, %CV= 0.8 %).
The recovery from blood of 0.635 mg/L AZM was 94.5 % (n=6, %CV=2.3 %) and of 1.0 mg/L CLM was 99.4 % (n=6, %CV= 1.6%). Inter-assay variation at 0.635 mg/L (n=6) and 1.182 mg/L (n=5) in plasma were 2.2 % and 2.8 %, respectively. In blood the inter-assay variation at 0.295 mg/L (n=6) and 0.739 mg/L (n=6) were 2.4% and 3.9%, respectively.
Intra-assay variation of the combined PMN separation procedure and AZM quantification was 4% (n=4) at 1.35 mg/L and 14% (n=4) at 0.61 mg/L.

## Validation of the Bioanalysis of AZM in Sputum

Quantification of AZM in sputum was performed after thawing and vortex mixing of the sputum samples using glass pearls. No liquefying agents were used. For each sputum sample two volumes (50 and 250 µl) were assayed in duplicate. After addition of 200 µl of a saturated di-sodium carbonate (0.4 mg/ml in water, with a pH of approximately 11) an extraction with diethylether (both from Merck, Darmstadt, Germany) was performed. The concentration was calculated for each sample volume producing results within the range of the calibration curve (0-9 mg/L).

To validate the extraction and bioanalysis in sputum a reference curve prepared in AZM-free sputum from patients with CF was compared to a reference curve in water in a concentration range of 0,94 – 9.4 mg/L. CLA 4.6 µg per 250 µl sample was added as internal standard. The recovery, linearity, intra-assay variation and LLQ were determined.

## Pharmacokinetic Data Analysis

The concentration of AZM was determined in plasma, blood and PMNs. The amount quantified in the cell pellet was divided by the cell count (in the final washing fluid) and corrected for loss of PMNs. The amount of AZM in PMNs has been expressed as the amount of AZM in PMNs in 1 L of blood. Also the amount of AZM per PMN was calculated and subsequently divided by the mean cell volume of human PMN (334 femtoliter) [17], providing an estimation of the concentration of AZM in the PMN.

Pharmacokinetic data analyses were performed with MW\Pharm (version 3.50, Mediware, Groningen, the Netherlands) [18]. Plasma, blood and PMN concentration data points were weighted against the variation of the calibration curve.

# RESULTS

## Patients

Eight patients with CF (5 males, mean age, 29.6 (range 22-40) years; mean weight of 67 (range 57-75) kg) were included in the study. All patients met the inclusion criteria and had received AZM 500 mg once daily for more than 35 days before inclusion. Concomitant medication for CF included: oral omeprazole (n=5), prednisone (n=5), pancreatic enzymes (n= 5), vitamins A, D, E and K (n=6), ursodeoxycholic acid (n=3), ibuprofen (n=2), calcium carbonate (n=1), nystatin (n=1); intravenous tobramycin (n=2), meropenem (n=3), ceftazidim (n=1), piperacillin/tazobactam (n=4), colistin (n=6); and the following inhaled therapies: salbutamol (n=7), tobramycin (n=2), hypertonic saline (n=4), dornase-α (n=5), colistin (n=5) and corticosteroids (n=8).

## Validation of the bioanalysis of AZM in Sputum

Comparing the reference curve in water and in cystic fibrosis sputum a correlation coefficient of 0.999 was determined (concentration range 0.94-9.4 mg/L). A blank sample fell within the 95% confidence interval of the calibration curve. LOQ was 0.03 mg/L in water and 0.33 mg/L in sputum. Recovery from sputum spiked with 4.68 mg/L AZM was 95% (n=8, %CV =3.1%). No interferences with the assay were seen in AZM-free sputum from patients with CF.

## Pharmacokinetics of AZM

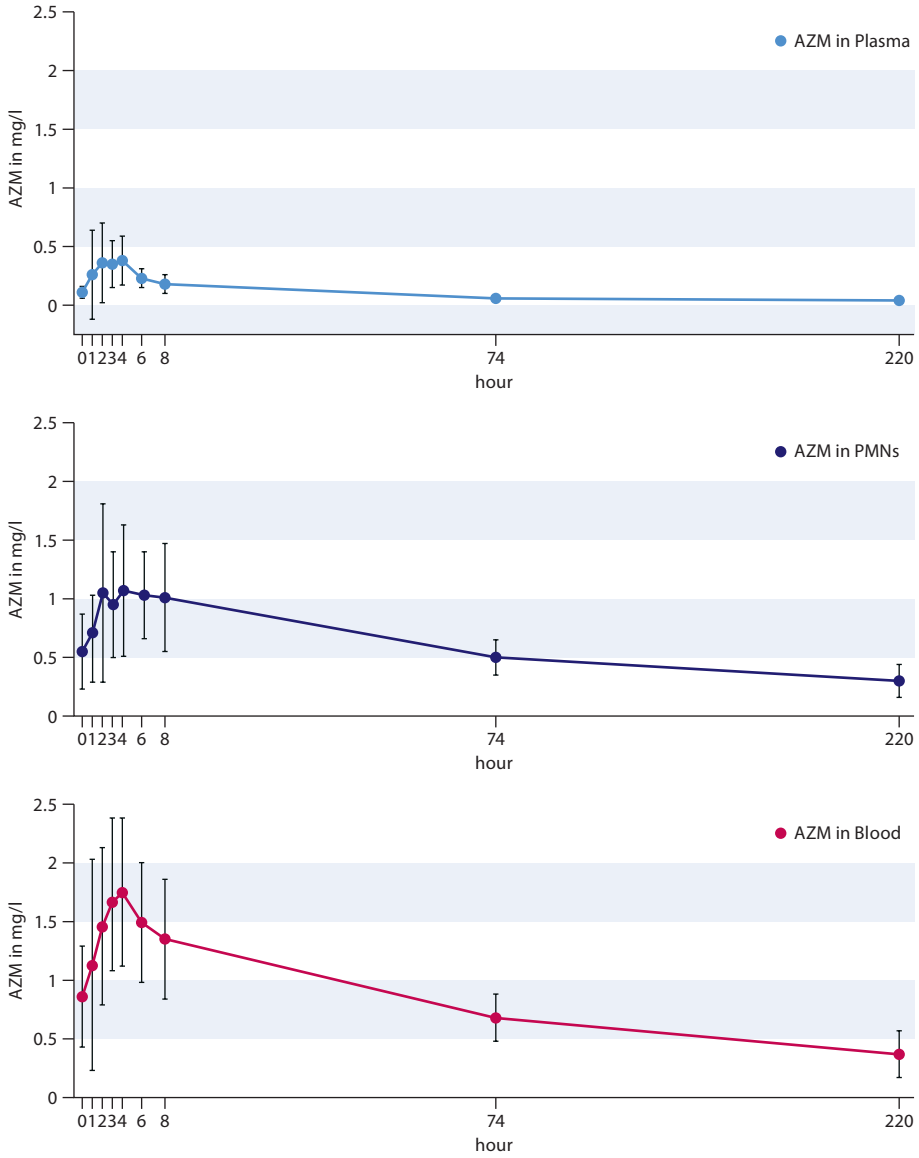
AZM kinetics were best described with a two compartment model. This was characterised by a short distribution half-life in plasma (mean and standard deviation: 0.1 h ± 0.02 h) and an extended elimination half-life ($t_{1/2\beta}$) in plasma (102 h ± 20 h), blood (180 h ± 68 h) and in PMNs (289 h ± 166 h).

The key pharmacokinetic data are summarized in Table 1. The combined patient concentration - time curves of AZM in plasma, blood and PMNs (mean ± standard deviation) are depicted in Figure 1.

**Table 1:** *Key mean pharmacokinetic data of AZM in plasma, blood and PMNs after > 35 days of 500 mg once daily in CF patients (n=8). Data are means ± standard deviations or percentages. Concentration in PMNs is expressed as the amount of AZM detected in isolated PMNs per 1 L blood.*

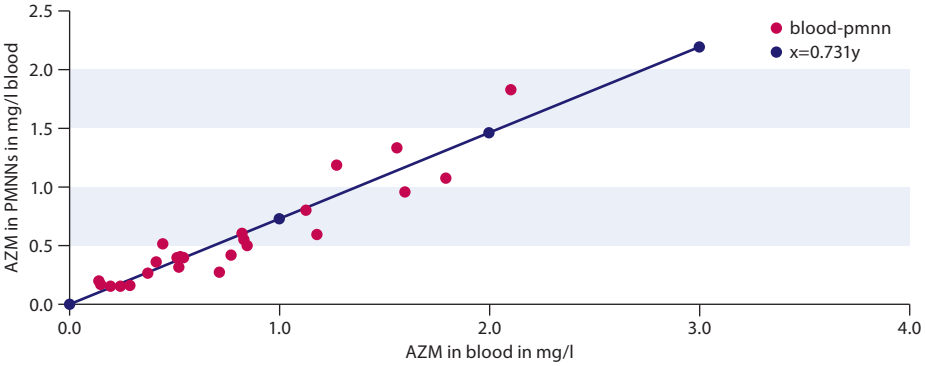
| AZM | Plasma mg/L mean (n=8) ± sd | PMN mg/L mean (n=8) ± sd | Blood mg/L mean (n=8) ± sd | PMN (%) | Plasma (%) |
|---|---|---|---|---|---|
| $C_{max}$ | 0.67 ± 0.31 | 1.44 ± 0.69 | 2.01 ± 0.74 | 72% | 33% |
| $t_{max}$ | 3 ± 1.6 h | 4 ± 0.4 h | 3 ± 1.1 h | | |
| $C_{min}$ t=0 h | 0.11 ± 0.05 | 0.55 ± 0.32 | 0.86 ± 0.43 | 64% | 13% |
| $C_{min}$ t=220 h | 0.04 ± 0.02 | 0.3 ± 0.14 | 0.37 ± 0.2 | 81% | 11% |
| $t_{1/2\ \alpha}$ | 0.1 ± 0.02 h | - | 2.9 ± 1 h | | |
| $t_{1/2\ \beta}$ | 102 ± 20 h | 289 ± 166 h | 178 ± 68 h | | |
| $AUC_{0\text{-}24\ h}$ | 5.3 ± 1.4 mg.h/L | 18.5 ± 8.9 mg.h/L | 27.8 ± 10.6 mg.h/L | | |

**Figure 1:** *AZM time concentration profiles during a 10-day sampling period after long term (>35 days) use of AZM 500 mg per day. Average value (n=8, ± SD) of AZM in plasma (upper part), in PMNs (middle part), and in blood (bottom part) are presented. Concentration in PMNs was expressed as amount quantified in isolated PMNs per liter blood.*



The linear relationship between AZM concentrations in all blood and in all PMNs samples was described by a slope of 0.731 and an intercept of ≈ 0. Correlation (r) between AZM concentrations in blood samples and PMN samples from t = 8 hours until t = 220 hours was 0.94 (n=23, p<0.001). During this interval an average of 73% of the AZM present in blood could be found in PMNs (Figure 2).

**Figure 2:** *Correlation between AZM in blood (x-as) versus AZM in PMNs. The combined data from 8 patients receiving AZM 500 mg per day chronically (> 35 d) were used. The data from t=8 hours till t=220 hours after the last dose of AZM were used to calculate a correlation coefficient of 0.94 and a fraction of 0.73 (73%) of AZM in blood incorporated in PMNs.*




In 5 patients, sputum samples were obtained at 3 time points: 0 to 24 hours before, and 96 to 120 hours and 196 to 220 hours after the last dose. In 2 patients, sputum samples were obtained at 2 time points, and 1 patient could only produce sufficient sputum during 1 sampling period. Concentrations of AZM in sputum up to 53 mg/L were found (Table 2).

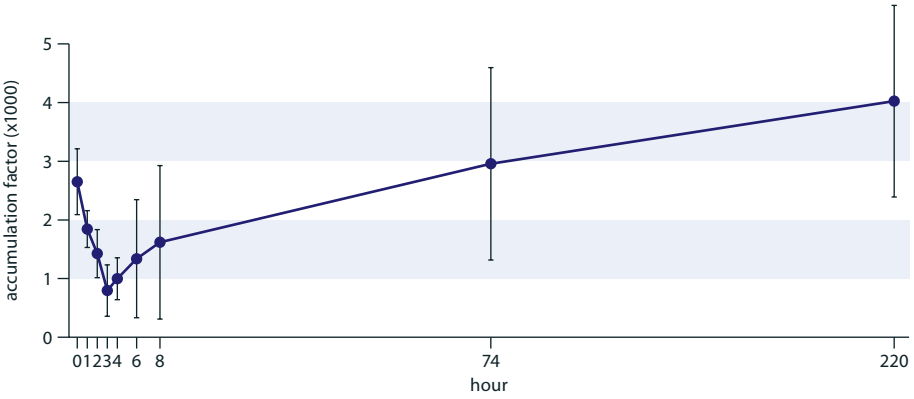
The calculated intracellular concentration in PMNs and the PMN to plasma ratio is summarized in Table 2. The maximum intracellular concentration calculated (mean of combined patient data at t=8 hours) was 305 ± 82 mg/L.

**Table 2:** *AZM PMN to plasma ratio and concentration in sputum. Data are means ± standard deviations or numbers. The concentration in PMNs is calculated from the amount per PMN divided by the mean cell volume of human PMN (334 femtoliter). The concentration of AZM in sputum was determined in a sample from a 24 h collection of spontaneously produced sputum.*

| | **AZM in plasma mg/L mean (n=8) ± sd** | **AZM in PMNs mg/L mean (n=8) ± sd** | **PMN to plasma concentration ratio** | **AZM in sputum mg/L mean ± sd** |
|---|---|---|---|---|
| $C_{max}$ plasma | 0.67 ± 0.31 | 300 ± 125 | 448 | - |
| $C_{min}$ t=0 h | 0.11 ± 0.05 | 231 ± 71 | 2100 | 32 ± 16 (n=7) |
| C t=96-120 h | - | - | - | 17 ± 9 (n=6) |
| $C_{min}$ t=220 h | 0.04 ± 0.02 | 118 ± 41 | 2950 | 20 ± 11 (n=7) |

The PMN to plasma ratio was not constant, as is depicted in the PMN to plasma ratio versus time curve in Figure 3.

**Figure 3:** *PMN/plasma ratio versus time curve (mean ± SD). The PMN/plasma ratio was calculated from the concentration in PMNs and the concentration in plasma. The concentration in PMNs was calculated from the amount per PMN divided by the mean cell volume of human PMN (334 femtoliter).*




For sputum the correlation between pharmacokinetic parameters and concentration of AZM in the first sample (0-24 hours) was calculated. There was no correlation between the concentration of AZM in sputum and the following parameters: area under the curve (AUC) 0-24 h, maximum AZM concentrations and pre-dose AZM concentrations in plasma, blood and PMNs (in all $r < 0.45$; $p > 0.2$). There was a weak correlation between $t_{1/2\beta}$ in blood and sputum concentrations between 0-24 hours ($r = 0.89$; $p < 0.01$).

## DISCUSSION

The pharmacokinetics of AZM in blood are primarily determined by the accumulation and prolonged retention of AZM in PMNs. The elimination half-lives in plasma, blood and PMNs were comparable to the data found by Wildfeuer et al [3] after short term (3 days) administration in healthy subjects, as was the $C_{max}$ in plasma (0.67 ± 0.31 mg/L vs 0.64 ± 0.27 mg/L). The mean $C_{max}$ in blood was substantially increased to 2.0 ± 0.7 mg/L after chronic use, versus ± 1.0 mg/L after 3 days of AZM therapy.

We found a mean concentration of 305 ± 82 mg/L in PMNs at t = 8 hours. This value was substantially higher compared to the 119 ± 31 mg/L that Wildfeuer found at t = 6 hours after 3 days dosing of 500 mg per day in healthy volunteers. Comparing these results, we have to be aware that the methodology was different. Wildfeuer calculated the intracellular concentration after determination of the intracellular water space. We used an average volume of the PMN. Taking the long half-life of AZM in PMNs into account, we can assume a steady-state situation had not been established after 3 days of dosing AZM, which may explain our higher intracellular concentration.

The concentration ratio between AZM in PMN and plasma in our study was between 450 and 2950 to 1. Mandell and Coleman [19] found a concentration ratio of 517 in an *in-vitro* experiment and Wildfeuer found a concentration ratio between PMNs and plasma of 177 at 3 hours and 1814 at 120 hours after administration of 500 mg per day for three days [3]. The decline and rise of the concentration ratio is more or less reciprocal to

the plasma concentration of AZM and not a constant factor, not even at steady state. The V-shaped pattern of the concentration ratio versus time is predominantly determined by the variation in plasma concentrations during the absorption and distribution phase and describes the lag-time between resorption in plasma and uptake in PMNs. Whether this difference is due to saturation of transport or just the time needed to establish a new equilibrium between internal and external concentration remains unknown. Other explanations, such as an increased loss at higher intracellular concentrations during the process of PMN separation, cannot be excluded.

During the absorption and distribution phase there was no correlation between the concentration in blood and in PMNs. However, for samples drawn 8 hours and later (after the absorption and distribution phase) the correlation coefficient was 0.94 and, on average, 73% of AZM present in blood could be retrieved from PMNs. This correlation is sufficient to relate PMN concentration directly to whole blood concentration of AZM.

Leakage of AZM from PMNs during the separation procedure could have influenced our results. In this study we used an analytical assay and separation method for PMNs, the details of which have previously been described [16]. Using this method we were not able to demonstrate leakage of AZM from PMNs during the isolation process. From the amount measured in isolated PMNs, after correction for incomplete PMN isolation, we found on average 87% of the AZM in the PMNs and plasma fraction (compared to blood, calculated from all patient samples). Part of the difference between blood concentration and the sum of plasma and PMN concentration could be explained by the accumulation of AZM in other lysosome containing blood cells, such as monocytes. Therefore, leakage from PMNs in the isolation process may have been less than the difference between 87% and 100% and did not influence our findings.

At $C_{max}$, the sum of the concentration in plasma and in PMNs (average of combined patient data) was > 100%. This can only be explained by the variation found in the selection and quantification of PMNs and the analytical method.

The intracellular concentration in PMNs was calculated from the amount per PMN and the mean volume of a PMN. This would reflect the real concentration if AZM was distributed through the entire PMN. From the results of Hand and Gladue we can conclude that the dibasic molecule AZM ($pK_a$=8.1 and 8.8) is merely concentrated in the acidic inner environment of intracellular lysosomes [20-22]. The difference in pH is the most probable driving force towards accumulation in intracellular lysosomes [20,21,23,24].

Finally, we were interested in the amount of AZM at the site of infection: the lung. Sampling techniques which could be applied were: bronchial alveolar lavage (BAL), sampling spontaneously coughed up sputum and sampling sputum after stimulation of secretion. The advantage of a BAL sample is its origin from the alveolar space. The disadvantage of a BAL sample is its unknown dilution factor and the fact that BAL is an unpleasant and distressing procedure for our group of patients. Sputum is more easily available and is formed not only in the alveolar space but throughout the entire lung. We assumed a preferable relation between sputum and the concentration in the lung without stimulation of sputum secretion and sampled sputum which was produced by spontaneous coughing during a 24-hour period.

In the sputum of patients with CF, profound retention of neutrophils is found [24]. The elevated concentration of AZM in sputum can, in our opinion, be explained when we see PMNs as the transporting vehicle for AZM towards the lung. From our results, we cannot conclude whether AZM in sputum is associated with PMNs, bound to other sputum constituents, or is freely soluble.
This begs the question as to what sputum AZM concentration is of clinical significance in the treatment of a chronic infection with *P. aeruginosa*. Teteda et al [10] found inhibition of quorum sensing factor production at concentrations of 2 mg/L in the *P. aeruginosa* strain PAO1. Takeoka et al [9] found a MIC of 100 mg/L towards both mucoid and non-mucoid strains of *P. aeruginosa*, and his findings suggest an increase of *P. aeruginosa* phagocytosis by PMNs after exposure to sub MIC concentrations of AZM. Tateda et al [10] found a reduction of the viability of *P. aeruginosa* after exposure for 48 hours to AZM at a concentration of 0.5 mg/L.

To be able to relate the concentrations we found in sputum (20-54 mg/L) to the concentrations at which AZM has been shown to influence *P. aeruginosa* growth in *in-vitro* experiments, more information about the way in which AZM is incorporated in sputum and its ability to penetrate *P. aeruginosa* biofilm should be gained. Our results clearly demonstrate that sputum is a reservoir with a relevant elevated AZM content.

We found no correlation between AUC of AZM in plasma, blood and PMNs and the concentration of AZM in sputum in the sample collected at t = 0 until t = 24 hours. Absence of a correlation between plasma and sputum concentrations may indicate that transport from blood to sputum is not described by a linear relationship as is the case in passive diffusion. According to our hypothesis, that AZM is transported to the lung through PMNs, other variables may influence sputum concentrations: the number of PMNs in blood, the number of PMNs migrating into the lung, the amount of sputum produced and the amount of sputum excreted will all have influence on the concentration of AZM in sputum.

We conclude that chronic administration of AZM leads to higher accumulation in PMNs compared to short term use. Furthermore, we conclude that sputum concentrations are elevated far above concentrations found in plasma and blood. We suggest that a less frequent (for instance once weekly) dosing regimen be compared with daily dosing regimens to assess clinical efficacy, pharmacokinetics, and accumulation in sputum of AZM.

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# Chapter 3.2

# Pharmacokinetics and sputum penetration of Azithromycin during once weekly dosing in cystic fibrosis patients

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3.2

# 3.2 Pharmacokinetics and sputum penetration of Azithromycin during once weekly dosing in cystic fibrosis patients


## ABSTRACT

In this study we examined pharmacokinetics, systemic exposure and sputum penetration of Azithromycin (AZM) in CF patients on chronic daily AZM therapy after changing to an once weekly dosing scheme.

Eight adult CF patients using AZM 500 mg per day were changed to a once weekly dose of 1000 mg during 3 months. Once per month sputum and blood samples were collected. AZM was quantified in blood plasma and polymorphonuclear neutrophils.
The cumulative weekly dose was reduced with a factor 3.5 (7 x 500 mg vs 1 x 1000 mg weekly).This led to a reduction in area under the curve (AUC ± SD) of 2.5±0.8 in plasma, 2.8±0.9 in blood, 2.3±1.1 in PMNs and to a reduction in average sputum concentration of 3.0 (±1.5).

At 1000 mg once weekly reduced but still substantial concentrations were achieved in PMNs and in sputum. A tendency towards less than linear reduction of systemic exposure was found. In order to calculate and propose an optimal dosing scheme we need to establish a relation between exposure levels and clinical efficacy.

## INTRODUCTION

Chronic use of azithromycin (AZM) reduces or stabilizes clinical symptoms of airway inflammation associated with chronic infection of *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF). Clinical studies demonstrating this effect have been performed with different dosing schedules ranging from 250 mg – 500 mg 3 times per week to 250 mg once daily). All randomized controlled trials comparing AZM with placebo have shown a significant advantage in $\Delta FEV_1$ and a reduction in number of exacerbations in patients on AZM [1-6].

We have previously reported pharmacokinetic data for AZM after chronic administration of 500 mg daily. AZM was quantified in plasma, blood, isolated polymorphonuclear neutrophils (PMNs) and sputum of 8 adult CF patients and demonstrated an extended elimination $t_{1/2}$ in plasma (102 ± 20 h), blood (180 ± 68 h) and in PMNs (289 ± 166 h) [7].
In blood we found a $C_{max}$ of 2.01 ± 0.74 mg/l at $T_{max}$ of 3 ± 1.1 h of which 1.44 ± 0.69 mg/l (72%) was found in PMNs. In sputum the concentration was between 12 and 53 mg/l (immediately after the last dose) and was still between 4 and 27 mg/l 10 days after the last dose. On average, the concentration we found in PMNs was 2100 times the $C_{plasma}$ 24 h after the last dose.
We concluded that accumulation in PMNs is high and that the $t_{1/2}$ in PMNs and in sputum is long enough (289 h) to explore a dosing interval of 1 week, assuming a relationship between concentration in PMNs or sputum and the clinical efficacy of AZM [7].

The key mode of action of AZM in CF patients remains unclear. Both a sub-MIC anti Pseudomonas effect due to interference with Pseudomonas biofilm formation or a non antimicrobial anti-inflammatory effect have been hypothesised to lead to a reduction in decline of lung function [8-11]. Several investigators have published results of in-vitro experiments towards the mode of action and the corresponding concentrations of AZM. Tateda *et al* found inhibition of quorum sensing factor production at concentrations of 2 mg/l in the *Pseudomonas aeruginosa* strain PAO1 and a reduction of the viability of *Pseudomonas aeruginosa* after exposure of 48 h of AZM in a concentration of 0.5 mg/l [8,9]. Takeoka et al found a MIC of 100 mg/l towards both mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. Moreover this study also suggested an increase of *Pseudomonas aeruginosa* phagocytosis by PMNs after exposure to sub MIC concentrations of AZM [10].

The sputum levels we found in our previous study exceed the levels at which quorum sensing signalling is reported to be influenced but do not reach MIC levels towards *Pseudomonas aeruginosa* [7]. Based on our previous findings at a dose of 500 mg per day, we postulate that sputum levels will remain at a level high enough to retain the proposed anti Pseudomonas and/or anti-inflammatory activity during a dosing schedule with an extended dosing interval.

The weekly dose selected in the current study was primarily based on equivalence to the lowest cumulative weekly dose administered in a randomised controlled trial and secondly a weekly dose of 1000 mg is generally well tolerated in other patient groups (i.e. prophylactic treatment in HIV-infected patients and the treatment of *Chlamidia trachomatis* infection) [12,13].

The primary aim of this study was to describe pharmacokinetic data and exposure in blood, PMNs and sputum of a once weekly dosing schedule in CF patients already on chronic once daily AZM treatment and to compare data of both dosing schedules. A secondary target is to describe the tolerability of the once weekly dosing schedule. The clinical efficacy of such a change in dosing regimen was not a part of this study.

## PATIENTS AND METHODS

### Patients

8 Adult patients with CF were recruited from the Adult Cystic Fibrosis Centre at the Haga Teaching Hospital. Patients receiving AZM 500 mg once daily for at least 35 days with chronic *Pseudomonas aeruginosa* infection (confirmed with at least 2 positive cultures in the last 6 months) were eligible to the study. The study protocol was approved by the regional medical ethical review board and patients gave written informed consent prior to study participation.

### Drug Administration and Sampling

AZM was administered as 500 mg tablets (Zithromax® tablets 500 mg, Pfizer, Capelle a/d IJssel, the Netherlands) once daily for at least 35 days prior to recruitment in the once weekly study. Upon enrolment, blood samples were collected immediately before and 3 hour after the final dose of 500 mg AZM once daily. Sputum was collected during 24 hours. Hereafter the dosing regimen was changed to 1000 mg once weekly and continued throughout the 3 month study period. Blood and sputum samples were collected after 1, 2 and 3 months. Spontaneously produced sputum was collected during a 24 h period preceding the blood collection. After the 3-month study, patients returned to 500 mg AZM daily.

At each time point blood samples were collected immediately before the weekly dose of AZM and 3 hours after dosing. AZM was given in the form of two 500 mg tablets which were swallowed with a glass of water.

Venous lithium-heparinised blood (119 IU li-heparin/7 ml tube, VacutainerTM Becton-Dickinson, Alphen a/d Rijn, the Netherlands) samples were collected at each time point.

Adverse effects were documented at each visit as a measure of drug tolerability.

### Isolation Procedure of Polymorphonuclear Neutrophils and bioanalysis of AZM

PMNs were isolated from lithium-heparinised venous blood samples. Before the separation a differential blood cell count was made. To 6.0 ml of blood 6.0 ml of phosphate buffered saline (PBS, pH 7.4, Mallinckrodt-Baker, Deventer, the Netherlands) was added. The diluted blood-PBS mixture was transferred into a separation tube with a 6.0 ml layer of Ficoll-Paque Plus® density separation medium (Amersham Biosciences, Uppsala, Sweden) and centrifuged for 15 min (1250 G) at 21 °C. The supernatant and Ficoll-Paque Plus® layer were removed and the cell pellet with neutrophils and erythrocytes was incubated during 15 min with 45 ml of NaCl 0.2 % at 2-6 °C in order to lyse the erythrocytes. PMNs were isolated by centrifugation (5 min 465 G) and resuspended in 6.0 ml PBS. A differential cell count was performed to determine the number of isolated neutrophils.
After centrifugation (5 min 465 G) the supernatant was removed and the cell pellet was kept at -30 °C until assay of AZM.

Detailed specifications and validation of the methodology of cell isolation and AZM quantification in blood, plasma and PMNs are described elsewhere [14].

In brief, a high-performance liquid chromatographic method with pre-column derivatization and fluorescence detection was used for the quantification of AZM in blood, plasma, isolated PMNs and sputum. Clarithromycin (CLM, Abbott, Queenborough, UK) was used as an internal standard and AZM dihydrate salt (Pfizer Inc, New York, USA) was used as reference. Pre column derivatization was performed using 9-fluorenylmethyloxycarbonyl-chloride (Sigma-Aldrich, Zwijndrecht, the Netherlands). Analytical separation was carried out using a C18 column as stationary phase and a mixture of 760 ml acetonitril (Merck, Darmstadt, Germany) and 240 ml 0.02 M phosphatebuffer (0.65 g potassium dihydrogen phosphate (Merck, Darmstadt, Germany) in 240 ml water adjusted to pH 7.7 with potassium hydroxide 10%) as mobile phase. Fluorimetric detection of the analytes was used for quantification (λ-excitation 267 nm and λ-emission 317 nm).

Calibration curves in plasma and blood (0-1.5 mg/l) were used for determination in plasma and blood. A calibration curve in water (0-9 mg/l) was used for determination in sputum and the PMN fraction.

Plasma, blood, PMNs and sputum samples were kept at -30 °C until determination. Quantification of AZM in PMN samples was performed after thawing and the addition of distilled water to a total volume of 1.2 ml. AZM was quantified in duplicate in 0.5 ml aliquots in plasma, blood and PMNs. Sputum was homogenized by vortex mixing (after addition of glass pearls) and a 50 µl and 250 µl aliquot was diluted with distilled water till 0.5 ml and extracted according to the procedure described above. The two samples volumes were used to asses homogeneity of the sputum sample.

The lower limit of quantitation of AZM was 0.042 ± 0.017 mg/l in plasma, 0.119 ± 0.065 mg/l in blood and 0.072 ± 0.036 mg/l in water. Linearity was assessed for 0-1.5 mg/l in plasma and blood and for 0-9 mg/l in water [14].

Recovery and intra-assay variation (expressed as %CV) from plasma at 0.635 mg/l AZM was 103.0 % (n=6, CV=1.5 %) and of 1.0 mg/l CLM was 100.0 % (n=6, %CV= 0.8 %). The recovery from blood of 0.635 mg/l AZM was 94.5 % (n=6, %CV=2.3 %) and of 1.0 mg/l CLM was 99.4 % (n=6, %CV= 1.6%). Inter-assay variation at 0.635 mg/l (n=6) and 1.182 mg/l (n=5) in plasma were 2.2 % and 2.8 %, respectively. In blood the inter-assay variation at 0.295 mg/l (n=6) and 0.739 mg/l (n=6) were 2.4% and 3.9%, respectively [14].

Intra-assay variation of the combined PMN separation procedure and AZM quantification was 4% (n=4) at 1.35 mg/l and 14% (n=4) at 0.61 mg/l [14].

## Pharmacokinetic calculations

The concentration time points were processed with the pharmacokinetic modelling program MW/Pharm version 3.50 (MediWare, Groningen, Netherlands) [15].

For estimatation of pharmacokinetic parameters, the method of D-optimal sampling time was chosen, adjusted to practice. Based on the pharmacokinetic parameters determined in our previous work, D-optimal sampling times were determined according to the method of D'Argenio [7,16]. The sample time points, immediately before dosing and 3 hours after dosing were planned during the nearest scheduled visit to the outpatient clinic.

Pharmacokinetic parameters $t_{½}$, Cl/F, $V_d$/F and $C_{max}$ for individual subjects were estimated by fitting the model to the data. We calculated the level of exposure per interval expressed as Area Under the (concentration-time) Curve (AUC). AUC data were normalized to 7 days of exposure for both dosing regimens. Average values with standard deviation were calculated of $t_{½}$, Cl/F, $V_d$/F and $C_{max}$.

## RESULTS

### Patients

Eight CF patients (4 male, 4 female) with a mean age of 29.3 year (range 22-47 year), mean weight of 61.5 kg (range 51-84 kg) and a mean Body Mass Index of 21.0 (range 17-24 kg/m$^2$) participated in the study. All patients were chronically infected with *Pseudomonas aeruginosa* and had received AZM 500 mg once daily for more than 35 days.

### Tolerability

Six patients reported no change in side-effects. Two patients experienced an increase in transient nausea starting shortly after intake of 1000 mg AZM and lasting 3-6 hours. One of these patients continued the dosing schedule of 1000 mg weekly. For the second patient the weekly dose was split in 500 mg once daily on two consecutive days during the 3$^{rd}$ (last) month of the study. This patient's data of the 3$^{rd}$ month were excluded from the pharmacokinetic calculations.

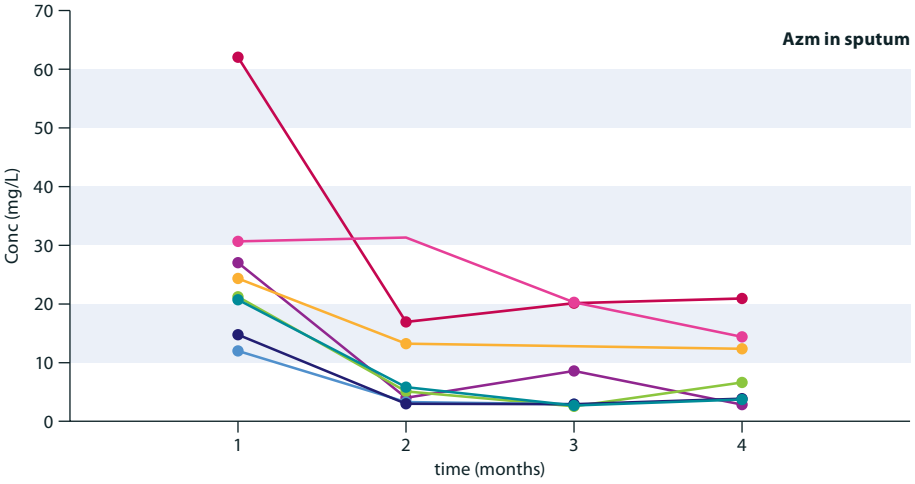
### Pharmacokinetics of AZM

The AUC per week in plasma, whole blood and PMNs are summarized in table 1. The individual sputum concentrations are depicted in figure 1. One patient could not produce a sputum sample at the 3$^{th}$ visit after 2 months.

**Table 1:** *Pharmacokinetic data of AZM 500 mg/d and 1000 mg/week in 8 CF patients*

| Sample: | Plasma | Plasma | Blood | Blood | PMNs | PMNs |
|---|---|---|---|---|---|---|
| Regimen: | 500 mg/d | 1000 mg/w | 500 mg/d | 1000 mg/w | 500 mg/d | 1000 mg/w |
| $C_0$ (mg/l)(sd) | 0.09 | 0.023 | 0.56 | 0.26 | 0.72 | 0.31 |
| | (0.03) | (0.027) | (0.24) | (0.20) | (0.61) | (0.30) |
| $C_3$ (mg/l)(sd) | 0.35 | 0.77 | 1.30 | 1.57 | 1.31 | 0.58 |
| | (0.14) | (0.53) | (0.47) | (0.92) | (0.76) | (0.52) |
| $C_{max\ estimated}$ (mg/l)(sd) | 0.35 | 1.10 | 1.31 | 2.15 | 1.31 | 1.31 |
| | (0.14) | (0.54) | (0.47) | (1.07) | (0.76) | (0.86) |
| $V_d$/F (l)(sd) | 4790 | 4286 | 1365 | 1820 | 398 | 2025 |
| | (625) | (1028) | (298) | (1714) | (89) | (3088) |
| Cl/F (l/h)(sd) | 45.7 | 37.0 | 5.7 | 5.1 | 12.1 | 6.6 |
| | (7.5) | (7.4) | (2.5) | (2.3) | (7.7) | (4.9) |
| $AUC_{0\text{-}168h}$ (h*mg/l)(sd) | 38.5 | 15.2 | 130.0 | 53 | 165.6 | 76.9 |
| | (19) | (4.6) | (51) | (29) | (106.7) | (30.0) |
| $T_{1/2\ ß}$ (h)(sd) | 73.6 | 136 | 194 | 254 | 32.7 | 180 |
| | (9.9) | (164) | (79) | (239) | (23.4) | (224) |

**Figure 1:** *Individual sputum concentrations of azithromycin (AZM) during dose regimen 1 (500 mg once-daily; day 1) and dose regimen 2 (1000 mg once-weekly; after 1, 2 and 3 months). AZM concentration was determined in spontaneously produced sputum collected on the day before administration of the weekly dose of AZM.*




In sputum we found an average concentration of 26.6 mg/l (sd=15.6; min: 12 mg/l, max 62 mg/l) at a dose level of 500 mg/day. At 1000 mg AZM once weekly we found an average concentration of 9.6 mg/l (sd=7.1; min 2.5 mg/l, max 20.9 mg/l) in sputum collected between 144-168 hours after dosing.

Calculated on a weekly basis we reduced the dose with a factor 3.5 (7 x 500 mg vs 1 x 1000 mg weekly). This led to a reduction in AUC (± SD) of 2.5±0.8 in plasma, 2.8±0.9 in blood, 2.3±1.1 in PMNs and to a reduction in average sputum concentration of 3.0 (±1.5). Reduction in AUC was less than expected in plasma and PMNs (one-sample t-test, $p<0.05$) but differed not statistically significant in blood ($p>0.05$) and in sputum concentration ($p>0.25$).

## DISCUSSION

This pharmacokinetic study was performed to obtain pharmacokinetic data during a once weekly dosing schedule of AZM in adult Cystic Fibrosis patients. Our main objective was to describe the level of exposure in blood (expressed as AUC) and the concentration in sputum. The results show an almost linear relation between the dose and the AUC in blood and the concentration in sputum. A weekly dose of 1000 mg is related to a reduced but still substantial concentration in sputum. The concentration in sputum is remarkably stable during the 3 consecutive monthly visits. Our pharmacokinetic parameters were based on the samples drawn 2 and 3 month after start of once weekly dosing. On the basis of the half life in sputum and PMNs we assumed that a steady state equilibrium was not achieved within 5 times $t_{1/2}$. In most patients however we see a stable concentration in sputum with little difference between the samples after 1, 2 and 3 months.

Although several dosing schemes with a lower weekly dose have been investigated in CF(i.e. 250-500mg 3 times per week) our patients were still using 500 mg per day, a dose level registered for chronic use in HIV patients [13].
In this study we intended to use a weekly equivalent for a dose schedule which was proven effective in a randomized clinical trial. We chose 1000 mg for the once weekly dose.
The lowest dose proven effective in a randomized clinical trial was 250 mg 3 times weekly in patients <40 kg and 500 mg 3 times weekly in patients >40 kg. As all of our patients were > 40 kg, our dose per kg bodyweight was lower than in the study of Saiman [4].

We found that 6/8 patients tolerated a change in dosing regimen from 500 mg per day to 1000 mg once weekly without change in adverse events. In two patients nausea was increased. These results reflect the tolerability in patients already tolerating 500 mg per day and cannot be extrapolated to AZM naïve patients.

We quantified AZM in sputum which was obtained by spontaneous coughing up during 1 day. Compared to samples obtained by broncho alveolar lavage (BAL) our method of sampling was less stressful for our patients and correction for dilution was not necessary. On the contrary we cannot exclude some dilution with saliva. AZM is excreted in saliva but concentrations are comparable to serum and do not reach the concentrations we found in sputum and therefore does hardly influence our findings [17].

Comparing the pharmacokinetic data before and after the change in dosing regimen, a reduction in $C_0$ in plasma, blood and PMNs was observed. The estimated $C_{max}$ was doubled in plasma but remained similar in PMNs. In PMNs the $C_0$ was reduced in the weekly regimen in accordance with the expected $t_{1/2}$.

In all matrices an increase in $t_{1/2}$ was found at weekly dosing. We attributed this effect to the presence of a third compartment which influences the terminal $t_{1/2}$. In weekly dosing the influence of a third compartment on the terminal $t_{1/2}$ is more pronounced than in once daily dosing and could explain the increased $t_{1/2}$ and decreased clearance. We hypothesise that PMNs act as a third compartment due to their AZM accumulation and uptake and release characteristics. In interpretation of $t_{1/2}$ data we must remind that the sampling time was 168 h after the previous dose which is within 1 x $t_{1/2}$. For a more accurate determination of terminal $t_{1/2}$ sampling over 4-5 x $t_{1/2}$ is necessary. We assume that the $t_{1/2}$ we determined reflect an underestimation of the true terminal $t_{1/2}$ which is extremely long due to uptake, release and reuptake in neutrophils. These data agree with in-vitro studies which have demonstrated rapid uptake into neutrophils and slow release from neutrophils [18].

In this study the systemic exposure was expressed by the AUC. Reducing the weekly dose with a factor 3.5 lead to a reduction in AUC of 2.5 in plasma, 2.8 in blood and 2.2 in PMNs. These differences are statistically significant in plasma and PMNs but - regarding the large interindividual variation in AUC - clinically of little relevance.

This finding is comparable to the differences in exposure level found by Amsden [19].
He compared administration of 1500 mg at once with 500 mg per day for three days.
He found an increase in AUC in serum which did not reach statistical significance but tended towards a higher level of exposure after a single dose ( $AUC_{0-\infty}$ 1500 mg as single dose: 13.1 (3.02-20.6 ) mg.h/l; as three 500 mg doses 11.2 (2.98-24.5) mg.h/l [19].
These findings can be the result of the uptake, release and reuptake characteristics of AZM which are more pronounced at a longer dose interval as explained above.

Alternatively we hypothesise that these findings might be caused by changes in bio-availability of AZM. In our pharmacokinetic model we assumed the bio-availability was identical during both dosing regimens. An increase in bio-availability when AZM is given as 1000 mg dose however, could explain the relative increase of the AUC after switching to 1000 mg weekly. An increase in bio-availability could be caused by enzyme saturation of P-glycoprotein (which acts as an efflux pump in intestinal cells) since AZM is an substrate for P-glycoprotein [20].

This study demonstrates a mean reduction in sputum concentration of AZM by a factor 3.0±1.5 mg/l after conversion to once weekly dosing, which is in the same magnitude as the reduction in AUC plasma (2.5±0.8 mg/l) and blood (2.8±0.9 mg/l). This reduction occurred within the first month and remained stable during the course of the study in 7 out of 8 patients. The variation in sputum concentration within these 7 subjects was remarkably low during the three monthly visits.
On the other hand, a substantial inter individual variation was observed which could not be related to any clinical parameter or co-medication.

In 1 patient no stabilisation in sputum concentration was reached. In this patient the decline in sputum concentration partially correlated with a decline in pre-dose AZM blood levels which occurred in this patient after 2 months instead of direct after the first month. We do not know whether this finding was due to non adherence to the dosing scheme or to changes in patient characteristics (resorption, sputum production).

We collected sputum samples on the day before dosing 1000 mg. These samples represent trough levels. From our previously reported data on sputum clearance of AZM we can calculate that the concentration on day 1 will be 30-40 % higher compared to the concentration on day 5-10 [7]. Whether these sputum concentrations remain within the therapeutically effective range for all patients remains to be established.

These pharmacokinetic data can be seen in relationship with the clinical results of a randomised clinical trial comparing 250 mg AZM once daily and 1200 mg AZM once weekly (personal communication S.Bell). These dosing schedules comprise a difference in cumulative weekly dose with a factor 1.5 (1750 mg versus 1200 mg). Taking our results into account, this will result in a reduction in systemic exposure and in sputum concentration with a factor 1.5, or slightly less.

We conclude that a single dose of 1000 mg AZM weekly is generally well tolerated by most CF patients. A trend towards less than linear reduction in systemic exposure was observed. At 1000 mg once weekly reduced but still substantial concentrations were achieved in PMNs and in sputum. In order to calculate and propose an optimal dosing scheme we need to establish a relation between exposure levels and clinical efficacy.

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# Chapter 4

# Absence of inhibition of DNA hydrolysing capacity of dornase alfa by azithromycin in vitro at clinical representative concentrations of azithromycin, dornase alfa and DNA

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4

# 4 Absence of inhibition of DNA hydrolysing capacity of dornase alfa by azithromycin in vitro at clinical representative concentrations of azithromycin, dornase alfa and DNA


## ABSTRACT

Azithromycin and dornase alfa are used concomitantly in patients with cystic fibrosis and are both found in substantial concentrations in sputum. In a study in 1996, inhibition of the DNA hydrolysing capacity of dornase alfa by azithromycin was reported (Ripoll, 1996) [1]. The present study investigated if inhibition occurred at clinical representative sputum-levels of azithromycin, dornase alfa and DNA.

The hydrolysing capacity of dornase alfa (present at 10 mg/L, 0.2 mg/L and 0.04 mg/L) on the kinematic viscosity of a DNA solution (2.6 g/L), in the presence and absence of azithromycin (50 mg/L and 100 mg/L) was determined.

Azithromycin 50 mg/L did not affect the hydrolysing capacity of dornase alfa. Azithromycin 100 mg/L, which is above the concentration usually found in sputum, showed a minor inhibitory effect.

In an in vitro experimental setting, azithromycin did not inhibit the DNA hydrolysing capacity of dornase alfa at clinical representative sputum concentrations.

# 4 Absence of inhibition of DNA hydrolysing capacity of dornase alfa by azithromycin in vitro at clinical representative concentrations of azithromycin, dornase alfa and DNA

## INTRODUCTION

Patients with cystic fibrosis (CF), especially those chronically infected with *Pseudomonas aeruginosa*, frequently use dornase alfa and azithromycin concurrently. Dornase alfa is administered by inhalation and acts directly on the mucus by hydrolysing free deoxyribonucleic acid (DNA) that is present at an elevated concentration in the mucus of CF patients. Free DNA originates from necrotic neutrophils that migrate to the lung because of a chronic pulmonary infection. DNA is able to form a hydrogel and increases mucus viscosity. By hydrolysing DNA, dornase alfa reduces the viscosity and elasticity of CF mucus and thereby improves sputum and bacterial clearance [2].

The macrolide azithromycin is used to reduce deterioration of lung function in CF patients chronically infected with *P. aeruginosa*. Azithromycin has immunomodulatory and antimicrobial properties [3,4]. Elevated concentrations of azithromycin, far above those found in plasma and blood, have been found in CF sputum (20-56 mg/L) of patients on maintenance therapy with oral azithromycin [5]. After short-term dosing of azithromycin in non-CF patients, the concentration of azithromycin was measured in lung tissue samples (8.9 ± 2 mg/kg) and bronchial washing (0.8 ± 0.07 mg/L); both exceeded the concentration in plasma [6]. A relation between sputum and lung tissue concentration during maintenance therapy has not been studied.

In two prospective, controlled studies, the efficacy of azithromycin alone or in combination with dornase alfa has been studied. Equi and co-workers found a reducing effect of azithromycin on dornase alfa in patients using dornase alfa; however, this subgroup comprised too few patients to allow a statistically significant conclusion [3]. In contrast, Saiman and co-workers showed that CF patients taking azithromycin and dornase alfa concomitantly had similar benefits from azithromycin as CF patients who did not use dornase alfa [4].

In an earlier in vitro experiment, Ripoll and co-workers found an inhibitory effect of azithromycin on the hydrolysing activity of dornase alfa [1]. In their experiments, however, a DNA concentration far below the concentration found in CF sputum was used.

The results of Ripoll and the conflicting, inconclusive data from the clinical trials of Equi and Saiman stress the importance of gaining more knowledge about the nature and extent of an interaction between azithromycin and dornase alfa [1,3,4].

In the present study, inhibition of dornase alfa by azithromycin at clinically representative concentrations of azithromycin, dornase alfa and DNA in an in vitro setting was determined experimentally. DNA solutions are able to form hydrogels with elevated viscosity directly related to the concentration and the average DNA chain length. The change in viscosity of a DNA solution was used to evaluate the hydrolysing capacity of various concentrations of dornase alfa alone and in the presence of various concentrations of azithromycin.

## METHODS

Linear genomic DNA sodium salt (PN 1,626; source salmon testis; Sigma-Aldrich, Missouri, USA) was dissolved in a 20 mM Hepes buffer containing 70 mM sodium chloride (NaCl), 3 mM calcium chloride dihydrate ($CaCl_2.2H_2O$) and 2 mM magnesium chloride hexahydrate ($MgCl_2.6H_2O$). To aid dissolution of DNA, the solution was stirred for 24 hours at 4 °C. DNA concentration in this solution was 2.6 g/L and pH was 7.4.

Azithromycin dihydrate salt (Pfizer, Capelle a/d IJssel, the Netherlands) was dissolved in 50% (volume ratio) ethanol to give a stock concentration of 5 mg/mL. Azithromycin was added to the DNA solution to obtain final concentrations of 50 mg/L and 100 mg/L. Blank experiments were performed by adding azithromycin and its solvent (50% ethanol) to the DNA solution in the absence of dornase alfa. The azithromycin-DNA solution was gently mixed and incubated for 10 minutes. Subsequently, dornase alfa (Pulmozyme, Roche, Woerden, the Netherlands) was mixed with the azithromycin-DNA solutions. The final dornase alfa concentrations were 10 mg/L, 0.2 mg/L and 0.04 mg/L (1 mg equals 1,000 'Genentech' units). Immediately after mixing, 3.0 mL was transferred to a calibrated capillary microviscosimeter (type 53710/1; nr 909718, $K = 0.009584\ mm^2/s^2$, Schott Geräte, Hofheim, Germany) with optoelectronic outflow time measurement; the outflow times were determined at three or four consecutive time points. The microviscosimeter was placed in a water bath at 36 °C. The kinematic viscosity ($mm^2/s$) was calculated by multiplying the outflow time (s) with the instrument constant K ($mm^2/s^2$).

The average of two series of three to four measurements was used to determine the inhibitory effect of azithromycin. A Student t-test was performed to assess statistical significance of the outcome.

## RESULTS

Kinematic viscosity of the 2.6 g/L DNA solution was 2.07 ± 0.05 mm2/s (v ± sd). In the absence of azithromycin, dornase alfa 10 mg/L reduced the viscosity of the DNA solution within the timeframe of the first measurement (within about five minutes). The capacity of dornase alfa to lower the viscosity of the DNA solution was not inhibited by 50 mg/L azithromycin. However, azithromycin 100 mg/L caused a very small, but statistically significant, inhibitory effect on the viscosity-lowering capacity of dornase alfa ($p = 0.001$ versus control). The results are summarised in Table 1.

**Table 1:** *Kinematic viscosity of DNA solutions, alone, with azithromycin, and azithromycin and dornase alfa.*

| | Kinematic viscosity (v) in $mm^2/s$ ± sd | Remaining viscosity % ± sd |
|---|---|---|
| DNA solution (2.6 g/L) | 2.07 ± 0.05 | 100 ± 3 |
| + azithromycin 50 mg/L | 1.94 ± 0.04 | 94 ± 2 |
| + dornase alfa 10 mg/L + solvent azithromycin (=control) | 0.75 ± 0.001 | 36.0 ± 0.05 |
| + dornase alfa 10 mg/L + 50 mg/L azithromycin | 0.75 ± 0.005 | 36.0 ± 0.3 |
| + dornase alfa 10 mg/L + 100 mg/L azithromycin | 0.78 ± 0.01* | 38.0 ± 0.5 |

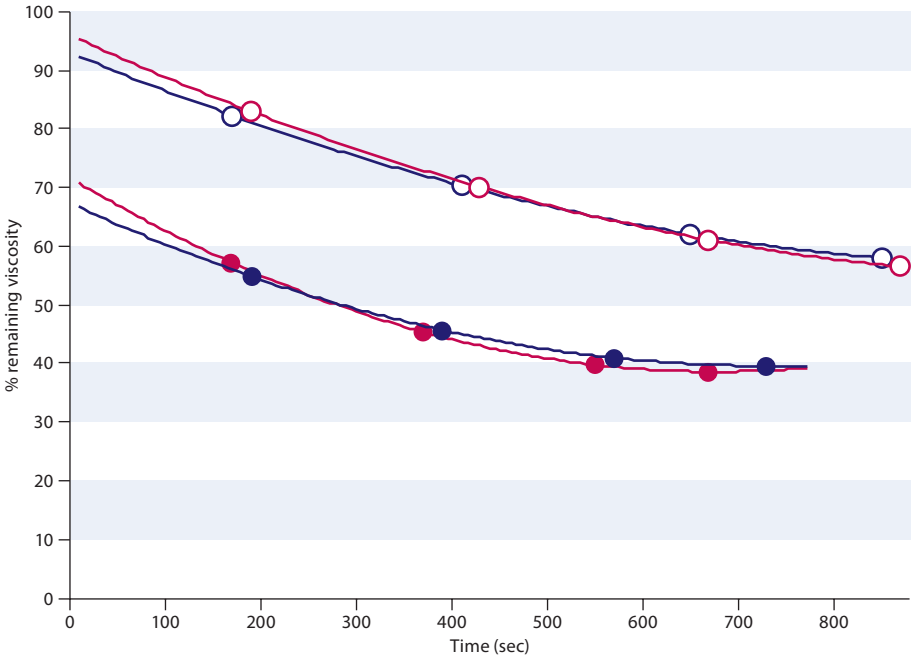
*Kinematic viscosity was determined with a capillary microviscosimeter.*

** Statistical significance of the difference between dornase alfa 100 mg/L and control was achieved (p = 0.001; Student's t-test)*

# 4 Absence of inhibition of DNA hydrolysing capacity of dornase alfa by azithromycin in vitro at clinical representative concentrations of azithromycin, dornase alfa and DNA

Additional tests were carried out in an attempt to elucidate whether or not the inhibitory effect of azithromycin was dependent on the dornase alfa concentration. Therefore, dornase alfa concentrations, which caused a hydrolysing process that continued during the time course of the measurements, were used. Dornase alfa, 0.2 mg/L and 0.04 mg/L, was tested in the presence and absence of 50 mg/L and 100 mg/L azithromycin.
The decline in viscosity was not affected by azithromycin in either concentration.
In Figure 1, representative curves of the decline in viscosity as a function of time are shown.

**Figure 1:** *The remaining kinematic viscosity as a function of time of a DNA solution with azithromycin 50 mg/mL and azithromycin 100 mg/mL*




DNA solution (2.6 g/L) with 0.2 mg/L dornase alfa in the presence ( ● ) and absence ( ● ) of 50 mg/L azithromycin
DNA solution (2.6 g/L) with 0.04 mg/L dornase alfa in the presence ( ○ ) and absence ( ○ ) of 100 mg/L azithromycin.

## DISCUSSION

Inhibition of dornase alfa activity by azithromycin could result in a clinically relevant, unwanted drug-drug interaction. Ripoll and co-workers evaluated the effect of increasing concentrations of azithromycin (0 to 77 mg/L) on the potency of 20 mg/L dornase alfa (Pulmozyme, Roche) to hydrolyse DNA in a solution that contained 5 mg/L genomic DNA derived from human peripheral blood leukocytes [1]. The dornase alfa activity was inhibited in a concentration-dependent way with almost complete inhibition at a concentration of 77 mg/L azithromycin. Similar results were found with erythromycin and roxithromycin [1]. To quantify hydrolysis of DNA, Ripoll measured UV absorbance (at 260 nm), which increases when DNA is disrupted by, for example, DNAses such as dornase alfa (hyperchromic effect) [1, 7].

In CF sputum however, an average DNA concentration of 2.6 g/L is present and after inhalation the dornase alfa concentration in CF sputum is up to 10 mg/L [8, 9]. Consequently, the DNA concentration used in the study of Ripoll (5 mg/L) was not representative for the concentrations usually found in CF sputum. Besides this, the pH of the DNA solution was 8.0 while CF sputum has a pH of about 7.4 [8] or less (unpublished observations). In an attempt to mimic the clinical situation in CF sputum, inhibition experiments were performed in a more concentrated DNA solution (2.6 g/L versus 5 mg/L) and at a lower pH (7.4 versus 8.0). The pH could be of importance as it could have an effect on the solubility of azithromycin, a dibasic salt with pKa values of 8.74 and 9.45 [10].

A solution of salmon DNA was used as a model for the human DNA molecules present in CF sputum. Salmon DNA and human DNA in CF sputum are both linear and sensitive towards hydrolysis by dornase alfa [11,12].

DNA is able to form polymers with a complex rheological behaviour including elasticity and thixotropy [12]. Capillary viscometers are highly accurate and sensitive, and measure relative viscosities of Newtonian fluids (without elasticity and thixothropy) with errors of less than 0.1% [13]. The DNA solution of 2.6 g/L used in the present study had a viscosity of about 2 $mm^2.s^{-1}$, which is threefold that of water at 36 °C. In this viscosity range, DNA solutions show neither elasticity nor thixotropy, but are almost Newtonian; measurement of capillary outflow time could be used to follow changes in viscosity related to the depolymerisation of the substrate [11-13]. This technique is also used to measure accurately the enzymatic activities of other depolymerising pharmaceuticals such as hyaluronidases [12].

The time required to decrease the viscosity to half the initial value decreases by a factor of about five when increasing the dornase alfa concentration from 0.04 mg/L to 0.20 mg/L. This indicates that in this range the hydrolysing capacity of dornase alfa towards the salmon DNA solution is roughly proportional to its concentration.

In contrast to the work of Ripoll, the present study showed only minor or no effects of azithromycin on the dornase alfa activity, which could not be explained by the differences in DNA source or analytical technique. Addition of 100 mg/L azithromycin showed a very small level of inhibition of the capacity of dornase alfa to decrease the viscosity of the DNA solution. Although statistically significant, it was thought that the inhibitory effect was unlikely to be relevant because of its minimal magnitude and also because it has only been found at a concentration of azithromycin of 100 mg/L, which is above the levels usually found in CF sputum [5]. In addition, inhibition of hydrolysing capacity was not detected in the additional experiments with 0.2 mg/L and 0.04 mg/L dornase alfa.

The absence of an inhibitory effect of azithromycin even at the reduced dornase alfa concentrations could indicate that azithromycin does not inhibit dornase alfa by binding to the enzyme but, more likely, by binding to the substrate, i.e. DNA. In the work of Ripoll, the azithromycin to DNA concentration ratio was much higher than in the present study. Therefore, it could be possible that in the Ripoll study the negatively charged DNA backbone was covered by azithromycin, which is positively charged at pH 8, and thereby prevented dornase alfa from binding effectively to DNA. This could also explain the minor inhibitory effect found with 100 mg/L azithromycin in the present study.

## CONCLUSION

Azithromycin did not inhibit the hydrolysing capacity of dornase alfa in an in vitro experimental setting with clinically representative sputum azithromycin, dornase alfa and DNA concentrations.

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# Chapter 5

# Long term effects of Azithromycin maintenance therapy

# Chapter 5.1

# Long term effects of azithromycin maintenance treatment on pulmonary function in pediatric cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa*

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# 5.1

# 5.1 Long term effects of azithromycin maintenance treatment on pulmonary function in pediatric cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa*


## ABSTRACT

Azithromycin is used as maintenance treatment in cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa (P. aeruginosa)* for reducing the frequency of pulmonary exacerbations. Effect of long term use of azithromycin on pulmonary function decline is not clear.

The objective was to evaluate the long term effect of azithromycin on pulmonary function and compare these results to a non-*P.aeruginosa* infected cohort.

We included 74 cystic fibrosis patients, treated at the CF centre of the University Medical Centre Utrecht, who were chronically infected with *P. aeruginosa* and who started azithromycin maintenance treatment between 1998 and 2005. We analysed decline in forced expiratory volume in 1 second percentage of predicted ($FEV_1$%) before and after start of azithromycin treatment, using linear mixed effects models. We also compared $FEV_1$% decline in our study cohort with a non-pseudomonas colonized cohort, not treated with azithromycin maintenance therapy (n=60).

Mean follow-up after start of azithromycin was 7.6 (SD 1.9) years. One year after start $FEV_1$% was + 1.71 % compared to baseline, after 18 months +0.78 % and after 24 months $FEV_1$% was back to baseline (-0.19%) During the following years mean annual change in $FEV_1$% was - 2.26 %, which was comparable to the decline in the non-pseudomonas, non-azithromycin cohort.

Initiation of azithromycin therapy is associated with a temporary improvement of pulmonary function. After the first year patients with *P. aeruginosa* and azithromycin have a pulmonary function decline comparable to patients without *P. aeruginosa* and azithromycin.

# 5.1 Long term effects of azithromycin maintenance treatment on pulmonary function in pediatric cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa*

## INTRODUCTION

Cystic Fibrosis (CF) lung disease is characterized by chronic pulmonary infections leading to end stage lung disease [1,2]. Up to 80 percent of adult CF patients are infected chronically with *Pseudomonas aeruginosa (P. aeruginosa)* [3]. In the past years, macrolide antibiotics have been added to antipseudomonal antimicrobial treatment in patients with CF because of their immunomodulatory and anti microbial properties. In mouse models, azithromycin (AZM) has shown to suppress quorum sensing induced virulence factors, to impair biofilm formation and to enhance bacterial clearance [4-7]. Furthermore azithromycin suppresses inflammatory mediators in the lung [7,8] and interferes with various immunomodulatory mechanisms [6,9].

Since 1998, several studies have demonstrated beneficial effects of azithromycin maintenance therapy on pulmonary function in patients with CF[10-13] and maintenance therapy has become standard care [14]. Guillot et al. (2006) [15] described a significant reduction in the decline of pulmonary function in *P. aeruginosa* colonized populations while no significant change was found in non *P. aeruginosa*-colonized patients [12]. A recent study of Saiman et al also failed to show beneficial effects on $FEV_1$% in non *P. aeruginosa*-infected patients 16.

Data on the effects of long-term use of azithromycin are lacking [14,17] and to date, follow-up studies are limited to a follow-up of 4 years [1,9,10,13]. Recent studies suggested an increase in the $FEV_1$% in the first year [18], but it is not clear whether this effect is sustained in the following years. A retrospective study of Tramper-Stranders et al. [12] demonstrated an increase in $FEV_1$% in the first year, but a decline in the following three years. In a retrospective cohort study we evaluated the long term effects of azithromycin maintenance therapy on pulmonary function and compared these results with a non *P. aeruginosa* infected cohort not using azithromycin.

## METHODS

Introduction of maintenance therapy with azithromycin was started in the CF- centre Utrecht (Department of Pediatrics, University Medical Centre Utrecht) in 1998.
A retrospective cohort study was performed on all patients with chronic *P. aeruginosa* infection who started azithromycin maintenance therapy between 1998 and 2005.
Chronic *P. aeruginosa* infection was defined according to the Leeds criteria [19].
Patients had to have at least one reliable pulmonary function measurement before start of azithromycin therapy and follow-up had to be at least 3 years to be included.
Patients who had a lung transplantation during our follow-up were excluded. Dosage of maintenance azithromycin therapy was 5 to 10 mg/kg once daily. All measured pulmonary functions during 3-monthly visits and annual check-ups during the use of azithromycin maintenance therapy were included. Date of birth, gender, gene mutation, and age at start of azithromycin were included as potential confounders in the database.

### Pulmonary function (outcome measure)

$FEV_1$ was measured using Jaeger Mastercreen and Zan pulmonary function devices.
$FEV_1$ was expressed as percent of predicted for sex and height ($FEV_1$%).Mean pulmonary function was measured in the period of one year before start of azithromycin and change before start of treatment was compared with changes after start of treatment.
Outcome can be described as total percentage of decline after x years.

### Control cohort

In the absence of *P.aeruginosa* infected patients without azithromycin maintenance therapy (since azithromycin is standard care), we compared pulmonary function change in our study patients with all non-pseudomonas infected CF patients from our centre in the same period, of whom pulmonary function data were available. Patients who had a lung transplantation during our follow-up were excluded.

### Statistical analysis

Data are represented as mean values and standard-deviations. Linear mixed effects models were used to evaluate the effect of azithromycin maintenance therapy on pulmonary function decline during our study period. The outcome measure was change in $FEV_1$% since start of treatment. Random intercept and a random effect per patient for duration of azithromycin therapy (linear and quadratic) was included in this model in order to take into account correlation of patients and measurements within patients. Several variables were added to the model. These included fixed effects for duration of azithromycin treatment and age.
The fit of the models was compared using the likelihood ratio test under maximum likelihood estimation. The final model, presented here, was estimated using restricted maximum-likelihood.
For the comparison with a non-pseudomonas infected cohort, linear mixed effects model analysis was used to determine significant difference in decline between both cohorts.
To examine the difference between the cohorts, two models were compared. The first model including fixed effects for duration of azithromycin treatment, type of cohort (pseudomonas/azithromycin or non-pseudomonas) and age. An interaction between type of cohort and duration of azithromycin treatment was added to the second model. Both models included random effects for patient and duration of azithromycin treatment. Best fit was determined as described above.

## RESULTS

Patient characteristics are summarized in Table 1. We included 74 *P. aeruginosa* positive patients who started azithromycin maintenance therapy between 1998 and 2005. Mean age at time of therapy initiation was 10.9 years (standard deviation [SD] 3.8); 46% was male. Mean $FEV_1$% before start of study was 82.5 (SD 19.4)%. Mean duration of azithromycin use in this study cohort was 7.6 years. The non-pseudomonas CF control cohort had a similar age, 55.0% was male. Mean $FEV_1$% at start of follow-up was 88.9 (21.3)%. Mean duration of follow-up was 5 years.

**Table 1:** *Patient characteristics of our study cohort (CF pseudomonas colonized) and non-pseudomonas colonized CF patient cohort.*

| | **Pseudomonas colonized cohort n=74** | **Non Pseudomonas colonized study group n=60** |
|---|---|---|
| Mean age at start[1] in years (SD) | 10.91 (3.83) | 10.10 (6.06) |
| Mean follow-up in years (SD) | 7.6 (1.9) | 5 |
| Mean $FEV_1$ before start study (SD)[2] | 82.5 (19.4) | 88.9 (21.3) |
| Male, n (%) | 46.0% | 55.0% |
| Gene mutations | | |
| Homozygous ΔF508 | n=54 (73%) | n=33 (55%) |
| Heterozygous ΔF508 | n=11 (14.86%) | n=21 (35%) |
| Others | n=9 (12.16%) | n=6 (10%) |

Mean pulmonary functions per year of use of azithromycin therapy are shown in Table 2. In the first year after start of azithromycin mean annual pulmonary function was 3.0 % higher compared to the year before start ($p<0.001$). In the second year $FEV_1$% started to decline.

**Table 2:** *Mean values of FEV1% (SD) per year before and after start of azithromycin maintenance therapy.*

| **Year of use azithromycin*** | **Mean pulmonary function (SD)** |
|---|---|
| Year before start | 81.32 (19.06) |
| First year after start | 84.33 (20.19) |
| Second year after start | 81.96 (20.05) |
| Third year after start | 79.30 (20.22) |
| Fourth year after start | 75.52 (21.36) |
| Fifth year after start | 73.93 (21.87) |
| Sixth year after start | 70.12 (23.23) |
| Seventh year after start | 67.53 (23.69) |
| Eight year after start | 59.39 (23.00) |

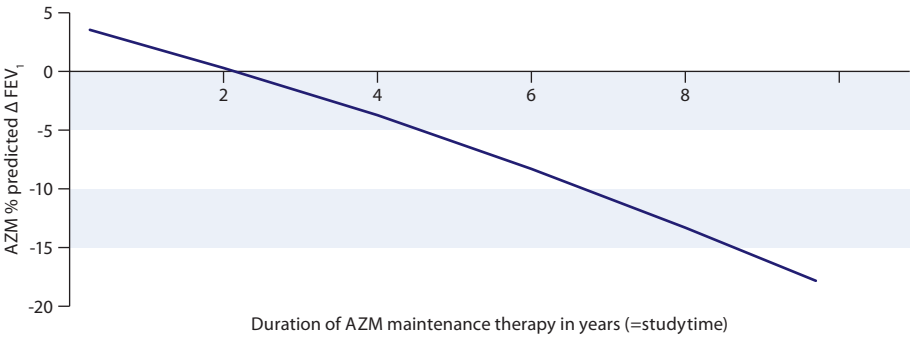
** Year -1: year before start of azithromycin maintenance therapy, year 0: year of start azithromycin maintenance therapy, year 1: second year after start of azithromycin maintenance therapy, etc.*

To analyze how the rate of decline in pulmonary function changed after start of azithromycin we performed a mixed model analysis. The final model included a linear and quadratic fixed effect for duration of azithromycin treatment and age. All pulmonary data were corrected as to pulmonary data of the year before start of azithromycin (year -1). Parameters of the final equation are described in table 3. Cumulative change in pulmonary function after start of maintenance therapy is shown in Figure 1. One year after start of maintenance therapy $FEV_1$% was +1.71% higher compared to baseline, after 18 months this was +0.78%, after 24 months $FEV_1$% was back to baseline (-0.19%). During the following years mean annual change in $FEV_1$% was -2.26 %.

**Table 3:** *Multilevel model for long-term pulmonary function changes during maintenance therapy with azithromycin.*

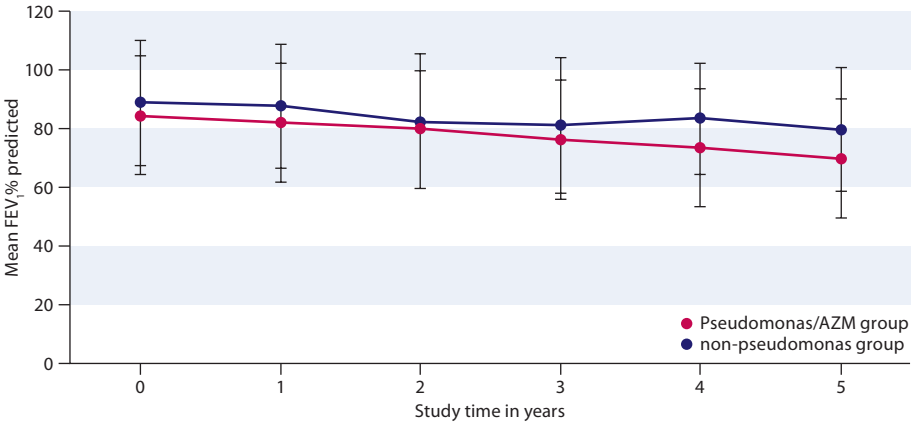
| Fixed coefficients | b (SE) | P |
|---|---|---|
| Intercept | -4.43 (1.53) | < 0.004 |
| Duration azithromycin maintenance treatment | -1.84 (0.56) | < 0.002 |
| Duration azithromycin maintenance treatment$^2$ (quadratic equation) | -0.05 (0.07) | 0.456 |
| Age | 7.50 (1.02) | < 0.001 |

**Figure 1:** *Modelled cumulative change in $FEV_1$% over time in azithromycin treated, P. aeruginosa positive CF patients. Y-axis shows decline in delta $FEV_1$% predicted and X-axis shows duration of azithromycin use in years.*




To assess whether rate of decline in the *P. aeruginosa* positive azithromycin treated cohort is comparable to *P. aeruginosa* negative patients without azithromycin treated at the same centre and at the same period we compared both cohorts during a period of 5 years (2002-2007). The rate of decline between the two cohorts was comparable and linear mixed effect analyses did not detect a statistically significant difference between these two cohorts. The possible confounders gender and gene mutation showed no significant effect (Figure 2).

**Figure 2:** *Mean $FEV_1$% of P.aeruginosa infected group using azithromycin over study period compared with a non-pseudomonas infected cohort.*




## DISCUSSION

This is the first study to address long term effects of azithromycin on pulmonary function in patients with CF over a period of almost 8 years. Although pulmonary function improves in the first year after start of azithromycin, decline continues after the first year with an average of 2.26 % per year, based on our mean study time of 7.6 (SD 1.9) years. This percentage is comparable with non *P. aeruginosa* - non azithromycin children of the same age [1,9,10,11,13,18].

A major limitation of this study is the retrospective character. Since azithromycin is standard care it is hard to perform a placebo controlled, prospective trial to determine the long-term effects of azithromycin on pulmonary function. As second best we compared pulmonary function decline before and after initiation of azithromycin treatment in our study cohort and compared decline in pulmonary function within a cohort of CF patients without azithromycin and pseudomonas infection. Data on exacerbations and additional antibiotic treatment are lacking in this study. De Boer et al. recently demonstrated that CF patients with frequent exacerbations appear to experience an accelerated decline in pulmonary function, and they have an increased 3 year risk of death or lung transplant [20]. It has also been shown that pediatric CF patients had an increase in exacerbation frequency after cessation of azithromycin maintenance treatment (both high and low dose) [21]. Therefore not only pulmonary function decline is important to consider as effect parameter of azithromycin use but also exacerbation frequency.

However, until now, no studies with a follow-up of more than 4 years are available. Our study, showing an initial increase in $FEV_1$% followed by a decrease thereafter, is in line with other studies. Previously, Clement et al [22] showed that the maximal difference between azithromycin and control was found after 6 months of therapy: a mean difference of 5.58% (-1.98-13.58). At 1 year however the difference was -2.8% (-10.04-4.44) pointing at a gradual decline in efficacy. Van den Berg et al [23] pointed out that the observed decline in pseudomonas colonized patients is comparable to patients without chronic *P. aeruginosa* infection. Our study also confirms earlier data of Tramper-Stranders et al who described a follow-up of 3 years [12].

In that and other reports a significant rise in macrolide resistance of *S. aureus* was documented in patients on azithromycin [12,24,25]. Also an increased incidence of non-tuberculous mycobacteria in azithromycin treated patients has been suggested [26]. It might be speculated that the initial beneficial effects of azithromycin on pulmonary function are compensated by the detrimental effects of *S. aureus* and non-tuberculous mycobacteria later on.

We find no differences in the rate of decline in our *P. aeruginosa* positive cohort (treated with azithromycin) and our *P. aeruginosa* negative cohort (not treated with azithromycin). To our knowledge, there are no long-term trials comparing pulmonary function decline between pseudomonas colonized cohorts with and without azithromycin treatment. It is unclear whether the lack of difference between both cohorts points to a lack of detrimental effects of *P. aeruginosa* in the current era of inhaled antibiotics, or to compensation of these effects by azithromycin.

Based on our findings, advantages and disadvantages of the use of azithromycin maintenance therapy should be weighed. Azithromycin has clear beneficial effects in the first years after start. After two or more years this effect is less obvious and antimicrobial resistance might be an upcoming problem. Future studies should make clear whether stop of azithromycin after several years of treatment results in deterioration of pulmonary function. Studies should also focus on the effects of alternating on-off regimen of azithromycin, as is usual in the treatment with inhaled antibiotics. Maybe this could overcome the upcoming resistance of *S. aureus* while saving the suppressing effects on *P. aeruginosa*.

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# Chapter 5.2

# Antimicrobial surveillance and decline in pulmonary function in adult Cystic Fibrosis patients with and without chronic pulmonary infection with *Pseudomonas aeruginosa*

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*Submitted*

# 5.2

# 5.2 Antimicrobial surveillance and decline in pulmonary function in adult Cystic Fibrosis patients with and without chronic pulmonary infection with *Pseudomonas aeruginosa*


## ABSTRACT

Since 2000 CF patients infected with *P.aeruginosa* were treated with azithromycin maintenance therapy.

We retrospectively compared microbiological data of 1997 (the last year before introduction of azithromycin maintenance therapy) and 2007. We also analyzed pulmonary function data from 2000 up to and including 2007 and analyzed patients infected with *P. aeruginosa* with those without a *P. aeruginosa* infection.

We found a decline in incidence of *S.aureus* and a marked increase in *S.aureus* resistance towards macrolides. All *P.aeruginosa* infected patients used macrolide maintenance therapy. Rate of decline of pulmonary function was comparable in both *P.aeruginosa* infected and non infected patients.

# 5.2 Antimicrobial surveillance and decline in pulmonary function in adult Cystic Fibrosis patients with and without chronic pulmonary infection with *Pseudomonas aeruginosa*

## INTRODUCTION

Cystic fibrosis is the most common hereditary disease in Caucasians caused by a mutation in gene coding for the Cystic Fibrosis Transmembrane conductance Regulating protein (CFTR)[1,2]. One of the symptoms is a reduced bacterial clearance [3]. Repetitive pulmonary infections and inflammation are the main cause of morbidity and a reduced life expectancy in CF patients. In childhood *S.aureus* and *H.influenzae* are the dominant pathogens. Later on, *P.aeruginosa* is becoming the dominant pulmonary pathogen [3-5]. The incidence of *P.aeruginosa* infection increases from 30% in infancy to 80-90% in adults [4,5]. Chronic *P.aeruginosa* infection in cystic fibrosis (CF) patients is associated with a continuous decline in pulmonary function due to infection and inflammation [5,6].

In 1998 macrolide maintenance therapy (MMT) was introduced in our clinic as adjuvant therapy in CF patients with chronic *P.aeruginosa* infection [7]. Also maintenance therapy with other antibiotics (tobramycin and/or colistin per inhalation) and mucolytic therapy with dornase alfa and hypertonic saline were introduced in this decade.

We retrospectively analysed the effects of CF treatment (including maintenance antimicrobial therapy) on changes in pathogens found in sputum isolates in the entire population and we analysed the rate of decline in pulmonary function and compared patients with chronic infection with *P.aeruginosa* to those without. Also the changes in antibiotic susceptibility of isolated *P.aeruginosa* and *S.aureus* strains were analyzed.

## METHODS

Microbiological data of sputum samples collected in the year 2007 were compared to those collected in 1997, the last year before introduction of macrolide maintenance therapy. For *P.aeruginosa* and *S.aureus* susceptibility data of the first isolate of both years were used. Susceptibility testing was done by the disk diffusion method. The break points for 1997 and 2007 were identical and were set by the Dutch Committee 'Guidelines for Susceptibility Determination' [8,9]. Susceptibility for macrolides was tested with erythromycin disks.

A sub analysis was made of patients of whom data of 1997 and in 2007 were available. Of this group, data of patients chronically infected with *P. aeruginosa* (in 1997 and in 2007) were compared to those not infected with *P. aeruginosa*.

Lung function data and patient characteristics were collected from 2000 up to and including 2007. For analysis of lung function the best result of the forced expiratory volume in 1 second as % of predicted ($FEV_1$) per patient per year was used. Adult CF patients with chronic *P.aeruginosa* infection in year 2000 and on macrolide maintenance therapy were compared to CF patients without chronic *P.aeruginosa* infection and without macrolide maintenance therapy. Patients with three or more sputum samples positive for *P.aeruginosa* within one year were defined to be infected chronically. Patients who deceased, newly acquired a chronic infection, received a lung transplantation and or with incomplete data (including lost to follow up and transfer to another hospital) between 2001 and 2007 were excluded. Rate of decline in $FEV_1$ over the years was estimated by linear mixed models (SPSS version 17) [10].

# RESULTS

## Microbiology

In 1997, 154 patients (55% male, age 27.7 years, BMI 20.5, $FEV_1$ 54.0%, all medians) and in 2007, 160 patients (53% male, age 34.1 years, BMI 21.6, $FEV_1$ 57.5%, all medians) were included. Presence of *P.aeruginosa* remained stable (respectively 66.9% and 67.5%), presence of *S.aureus* declined from 52.6% to 32.5% and *H. influenza* from 23.4% to 5.6% (Table 1). Resistance of *P.aeruginosa* for tobramycin increased from 8.8% to 13.1% (p=0.37). Susceptibility for other commonly used antibiotics, including colistin, remained stable (Table 2).

**Table 1:** *Prevalence of pathogens in CF patients in 1997 and 2007 (154 samples 1997; 160 in 2007).*

| Micro-organism | 1997 n=154 n(%) | 2007 n=160 n(%) |
|---|---|---|
| *Achromobacter xylosoxidans* | 5(3.2) | 10(6.2) |
| *Aspergillus fumigatus* | 82(53.2) | 75(46.9) |
| *Atypical Mycobacteria* | 3(1.9) | 1(0.6) |
| *Burkholderia cepacia* | 2(1.3) | 3(1.9) |
| *P. aeruginosa (muc. and non muc.)* | 103(66.9) | 108(67.5) |
| *S. aureus* | 81(52.6) | 52(32.5)* |
| *Stenotrophomonas maltophilia* | 18(11.7) | 21(13.1) |
| *H. influenzae* | 36(23.4) | 9(5.6)* |
| *E. coli* | 11(7.1) | 8(5.0) |
| *Bordetella bronchoseptica* | 0(0) | 4(2.5)** |
| *Serratia marcescens* | 5(3.2) | 5(3.1) |

**p<0,001; **p=0,048 (Chi-squared)*

**Table 2:** *Susceptibility of P.aeruginosa towards commonly used antibiotics.*

| antibiotics | 1997 n=103 | | | 2007 n=108 | | |
|---|---|---|---|---|---|---|
| | S(%) | R(%) | I(%) | S(%) | R(%) | I(%) |
| ceftazidim | 80 | 13 | 8 | 78 | 12 | 10 |
| imipenem | 82 | 18 | 2 | 81 | 18 | 1 |
| tobramycin | 88 | 9 | 3 | 86 | 13 | 1 |
| tazocin | 96 | 3 | 1 | 97 | 3 | 0 |
| colistin | 95 | 5 | 0 | 95 | 5 | 0 |
| ciprofloxacin* | - | - | - | 51 | 35 | 14 |
| aztreonam | 79 | 18 | 3 | 85 | 14 | 1 |

**Ciprofloxacin susceptibility was not tested in 1997*

For 81 patients continuous data between 1997 and 2007 were available. Of this subgroup 71 patients were colonized with *P.aeruginosa* and were all treated with macrolide maintenance therapy since the year 2000. In 1997 in 36 of them a *S.aureus* was isolated of which 10% was resistant to macrolides. In 2007 in 20 patients a *S.aureus* was isolated, of which 85% was resistant to macrolides.

*Atypical mycobacteria* were found in 3 patients in 1997, none of the isolates was multiresistant. In 2007 in 1 patient a multiresistent *Mycobacterium absessus* was found in a patient using azithromycin.

## Pulmonary function

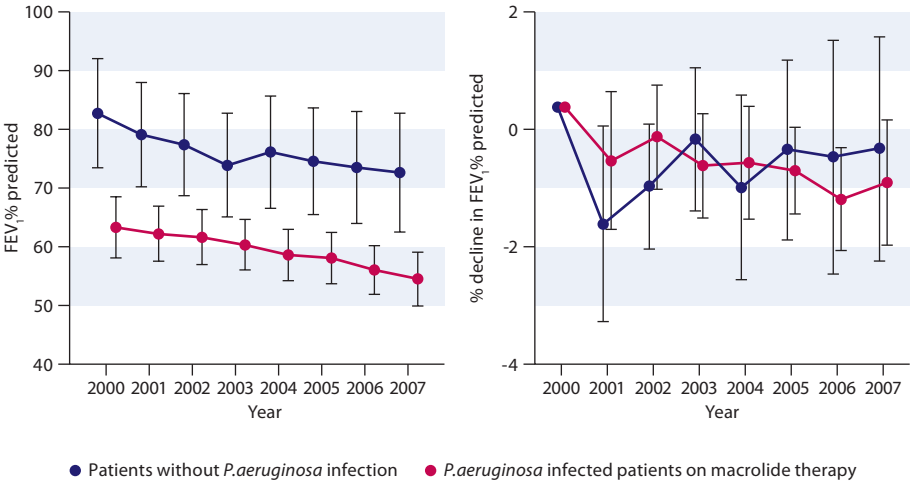
Lung function data (forced expiratory volume in 1 second as % of predicted: $FEV_1$) of 64 CF patients with chronic *P.aeruginosa* infection (m/f: 33/31) were included and compared to data of 23 CF patients without chronic *P.aeruginosa* infection(m/f: 16/7). Median age in 2000 was 28,5 and 30,9 years for patients with and without *P.aeruginosa* infection respectively. Between the year 2001 and 2007 12 patients newly acquired a *P.aeruginosa* infection and were not included. Other excluded patients in the *P.aeruginosa* infected and uninfected group were: patients who deceased 19 and 8; with incomplete data or lost to follow up 12 and 5 and a lung transplantation 11 and 1. Body Mass Index was comparable in both groups throughout the years. Median number of in-hospital days per year was higher in patients with chronic *P.aeruginosa* infection (19 versus 0 days per year, p=0.001). Pathogenic co-infections were reported in table 3. At baseline, median $FEV_1$ was lower in patients with chronic *P.aeruginosa* infection (62.3% versus 79.0%, p<0.001). The rate of decline in $FEV_1$ over the years in *P.aeruginosa* infected and uninfected patients, estimated by linear mixed models, was equal (1.28% versus 1.31% decline per year, p=0.722). Data are depicted in figure 1.

**Table 3:** *Characteristics patients 2000 to 2007, with and without P.aeruginosa infection*

| | P. aeruginosa + | P. aeruginosa - |
|---|---|---|
| Number of patients | 63 | 23 |
| Male (%) | 33 (51.6) | 16 (69.6) |
| Age in 2000, median (SD) | 28.5 (9.7) | 30.9 (8.4) |
| Range | 17.3 - 61.0 | 18.3 - 44.4 |
| In-hospital days, median (SD) | 19 (80.6) | 0 (28.9) * |
| Range | 0 - 429 | 0 - 102 |
| | | |
| ***Co-infection with:*** | n | n |
| Stenotrophomonas maltophilia | 5 | 1 |
| Acinetobacter xylosidans | 4 | 0 |
| Haemophilus influenzae | 2 | 2 |
| E. coli | 3 | 0 |
| Bordetella bronchoseptica | 3 | 0 |
| Serratia marcescens | 2 | 0 |
| Burkholderia cepacia | 1 | 0 |

** Wilcoxon Mann Whitney p = 0,001*

**Table 3:** *Decline in lung function 2000-2007. In blue: patients without P.aeruginosa infection and without macrolide maintenance therapy, in red: P.aeruginosa infected patients on macrolide maintenance therapy. On the left the average predicted $FEV_1$ (± SD). On the right the annual change in $FEV_1$ (± SD).*




## DISCUSSION

In the last decade several new antimicrobial and mucolytic therapies have been introduced in the treatment of patients infected with *P.aeruginosa*: maintenance treatment with azithromycin (systemic), with tobramycin and colistin (local by inhalation). Furthermore inhalation of dornase alfa and hypertonic saline were introduced [11-13]. The degree of decline in lung function can only be related to the combination of therapies in CF.

The microbiological surveillance revealed a significant increase in resistance of *S.aureus* towards macrolides. This effect can be seen in the entire population but is most prominent in the subgroup of patients being treated with azithromycin maintenance therapy. The amount of samples in which *S.aureus* could be isolated, decreased, which could both be related to the use of azithromycin or to aging of the patient population. These data confirm the data found in other CF centres, the level of resistance was comparable to what was found in Dutch CF centers (Utrecht, Rotterdam) and higher than the level found in Denmark where resistance increased from 7% to 52% in patients on macrolide maintenance therapy [14-18].

The incidence of *P.aeruginosa* positive samples remained the same. The level of resistance of *P.aeruginosa* towards tobramycin only slightly increased, which is of importance since tobramycin was used in most patients during exacerbations (systemic) and in some patients per inhalation as maintenance therapy. Resistance towards imipenem, cefalosporins and colistin did not change. This indicates that the current treatment of exacerbations does not lead to the development of uncontrolled resistance in the *P.aeruginosa* positive patient population.

Azithromycin has also been associated with an increased incidence of nontuberculous mycobacterial infections, particularly of multi-drug-resistant species like *Mycobacterium abscessus* [19]. In our population the number of mycobacterial positive samples decreased from 3 in 1997 to 1 in 2007, the last one being a multiresistant *Mycobacterium absessus* determined in a patient using azithromycin which stresses the need for close monitoring of the incidence and susceptibility of mycobacteria.

In our patients infected with *P.aeruginosa* the baseline lung function was lower than in patients without *P.aeruginosa* infection. This is in line with data from other centre's. Also the number of in hospital days, an indication for the number of exacerbations, is higher in the *P.aeruginosa* infected group. In the sub group of patients with data from 1997-2000 all patients with a *P.aeruginosa* infection were treated with macrolide maintenance therapy, and macrolide maintenance therapy was restricted to this group. This implies that the guideline regarding macrolide maintenance therapy was successfully implemented.

Co-infection with other pulmonary pathogens were more often found in *P.aeruginosa* infected patients. The number of deceased patients was comparable in both groups and we expect this not to influence our analysis. Number of transplanted patients was higher in the *P.aeruginosa* infected group. This could influence our analysis since the transplanted patients represent the group with end stage pulmonary function.

Furthermore the male to female number of patients ratio was not equally distributed. In the *P.aeruginosa* positive group the ration m/f was 33/31; in the *P.aeruginosa* free group m/f was 16/7. In general the pulmonary function of female patients tend to decline faster than the pulmonary function in male patients.

The annual rate of decline in pulmonary function expressed as $FEV_1$% predicted was equal in infected and non infected patients (1.28% versus 1.31% decline per year, p=0.722). Parad found in 1999 a rate of decline of 1.4 and 0.9% in uninfected female and male patients and a rate of decline of 2.2 and 1.7 % in infected male and female patients [6]. In a large US-Canadian epidemiologic study (n=4866) in children and adolescents *P.aeruginosa* infection was identified as an independent factor related to decline in pulmonary function in 2 out of 3 age groups. In contrast to the children, in the adolescent group (age 13-17 years) the difference was no longer statistically significant, pointing at lesser difference between infected and not infected patients [20].

The data of our limited number of patients, but also larger epidemiologic studies indicate that current treatment directed towards *P.aeruginosa* in combination with anti inflammatory therapy and mucolytic therapy largely overcomes the difference in yearly decline in lung function between *P.aeruginosa* infected and non infected patients to a rate which could not be observed within the ranges of statistical significance.

Keeping the limitations of retrospective analysis of patient data in mind we conclude that current treatment of adult CF patients infected with *P.aeruginosa* no difference in the rate of lung function decline with uninfected patients could be observed over 7 years. Antimicrobial therapy directed towards *P.aeruginosa* combined with anti-inflammatory and mucolytic therapy is the most probable explanation. The development of resistance in *S.aureus* towards macrolides was confirmed. The use of anti *P.aeruginosa* antibiotics did not lead to uncontrolled development of resistance in 10 years time.

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# Chapter 6

# **Azithromycin maintenance therapy in patients with cystic fibrosis:** a dose advice based on a review of pharmacokinetics, efficacy and side effects

A review about azithromycin dosing in maintenance therapy

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# 6 Azithromycin maintenance therapy in patients with cystic fibrosis: a dose advice based on a review of pharmacokinetics, efficacy and side effects


## ABSTRACT

Azithromycin maintenance therapy results in improvement of respiratory function in patients with Cystic fibrosis (CF). In azithromycin maintenance therapy, several dosing schemes are applied. In this review we combine current knowledge about azithromycin pharmacokinetics with the dosing schedules used in clinical trials in order to come to a dosing advise which could be generally applicable. We used data from a recently updated Cochrane meta analysis (2011), the reports of clinical trials and pharmacokinetic studies. Based on these data, it was concluded that a dose level of 22-30 mg/kg/week is the lowest dose level with proven efficacy. Due to the extended half-life in patients with CF, the weekly dose of azithromycin can be divided in 1 to 7 dosing moments, depending on patient preference and gastro-intestinal tolerance. No important side-effects or interactions with other CF-related drugs have been documented so far.

# 6 Azithromycin maintenance therapy in patients with cystic fibrosis: a dose advice based on a review of pharmacokinetics, efficacy and side effects

## INTRODUCTION

Maintenance therapy with the macrolide antibiotic azithromycin has been introduced 10 years ago and is currently widely used in the treatment of patients with Cystic Fibrosis (CF) [1]. Several randomized controlled trials showed that azithromycin can increase lung function, decrease the number of pulmonary exacerbations, the number of hospital days and the use of other antibiotics [1-8]. Beneficial effects on lung function are most prominent in patients who are chronically infected with *P.aeruginosa* [9]. In *P.aeruginosa* uninfected patients a positive effect on lung function could not be found, although other positive effects like a reduction in number of exacerbations, were found [10]. The beneficial effect highly varies between patients and could, apart from chronic infection with *P.aeruginosa*, not be related to other specific patient characteristics [9]. In a recently updated review on behalf of the Cochrane Collaboration it was concluded that a consistent improvement in $FEV_1$ over 6 months was demonstrated. A mean difference of 3.97% (95% conf interval 1.74%-6.19%) was calculated using combined data of 4 studies (n=549 patients). The likeliness to be free of exacerbations during 6 months was 1.96 times higher in the patients treated with azithromycin [11]. The consistent improvement in respiratory function after 6 months of therapy has not been demonstrated for extended periods of time. Only 1 study (Clement) presented data over 1 year, with a tendency towards equivalence between azithromycin and placebo [5,11].

The mode of action of azithromycin in patients with CF is likely to be a combination of antimicrobial and anti-inflammatory effects. Although azithromycin lacks a direct antimicrobial effect against *P.aeruginosa*, indirect effects have been found like a sub MIC inhibitory effect on the production of alginate, virulence factors, quorum sensing signals and *P.aeruginosa* motility [12-17]. In addition, direct anti-inflammatory effects have been found like decreased production of pro-inflammatory cytokines by monocytes and epithelial cells [18-20].

Sub MIC antimicrobial and anti-inflammatory effects have been found at concentrations of 2-10 mg/L [14,20], but no proof is available that these concentrations are directly related to clinical activity. For the anti-infectious effect sputum levels of azithromycin are expected to be relevant, while anti-inflammatory effects might be exerted systemic in blood and plasma.

Azithromycin is a so called 'lysosomotrophic' drug [21,22]. After resorption, at physiologic pH, azithromycin is able to pass cellular membranes. Due to its dibasic chemical nature, azithromycin is trapped in the acidic inner environment of lysosomal compartments of, for example, white blood cells. As a consequence, polymorphonuclear neutrophils (PMNs) contain high concentrations of azithromycin [21,22].

In healthy volunteers 37% of oral administered azithromycin was absorbed within 2-3 hours [23,24]. $T_{½}$ in plasma was between 11-14 h the first 24 h post dose and increased to 57 h 1-6 days after dosing [23]. In tissue concentrations exceeding serum a 10-100 fold were found [23]. Excellent tissue penetration and the long elimination half-life make that in general an infection with a susceptible pathogen can be treated with a once daily dose during 3 days [24].

In clinical trials in cystic fibrosis maintenance therapy, a variety of dosing schemes was used. In this paper we review the dosing levels and outcomes of these clinical trials and combine current knowledge about the pharmacokinetics of azithromycin in relation to the effects, side effects and possible drug interactions with other CF related medications. From these data we propose a dosing advice which could be used uniformly in clinical practice.

## METHODS

We searched the Pubmed database up to December 2010. To review the pharmacokinetics of azithromycin we used mesh terms 'azithromycin', 'cystic fibrosis' and 'pharmacokinetic'. We included primary reports which used a validated assay of azithromycin in blood, serum/plasma and/or sputum in CF patients. Efficacy of azithromycin was reviewed in the most recent version of the Cochrane systematic review (2011) and meta analysis on efficacy of azithromycin in CF [11]. For analysis of a relationship between dose level and efficacy we used reports of clinical trials which were incorporated in the Cochrane review (2011). We selected reports with a low risk of bias according to the assessment in the Cochrane review. In order to compare different dosing regimens we calculated the average dose per kg bodyweight per week using the intended dose level (e.g. 10 mg/kg bw/d) or in case of a fixed dose, the dose divided by the average bodyweight of the patients included. In case of a fixed dose per weight category we calculated the weighted average correcting for unequal distribution between the categories, in case the average dose was not included in the report. The correlation coefficient between average dose/kg bw/week and effect parameters was calculated (Pearson, two tailed probability). We used the data at six months of therapy or the data at the nearest time point reported of the relative change in $FEV_1$, hospitalization rate, days with intravenous antimicrobial therapy and number of subjects dropped out of the study as effect parameters.

To review the interaction of azithromycin with other CF-related drugs we used the mesh terms 'azithromycin' and 'interaction' or 'QT time' and selected reports, including case reports and reports about in-vitro experiments, about drug-drug interactions which could be applicable in cystic fibrosis treatment. Finally we searched with the mesh terms 'azithromycin', 'pediatric dosage' and 'pharmacokinetics' and selected reports which could add information towards the primary searches.

## RESULTS

### Collected reports

The search of pharmacokinetics in cystic fibrosis patients revealed 12 reports. Excluded were 4 reviews, 1 report about analytical aspects of azithromycin and 2 reports about experiments in mice and cell lines. We used 4 reports of pharmacokinetic studies and 1 report of a clinical trial which reported pharmacokinetic data as well. Efficacy data were used from 8 reports of clinical trials, which were assessed in the Cochrane review [11].

The search of interactions showed 122 reports. Of these, 13 reports were selected being of importance for cystic fibrosis treatment. We used 3 out of 5 case reports on azithromycin and prolongation of QT time.

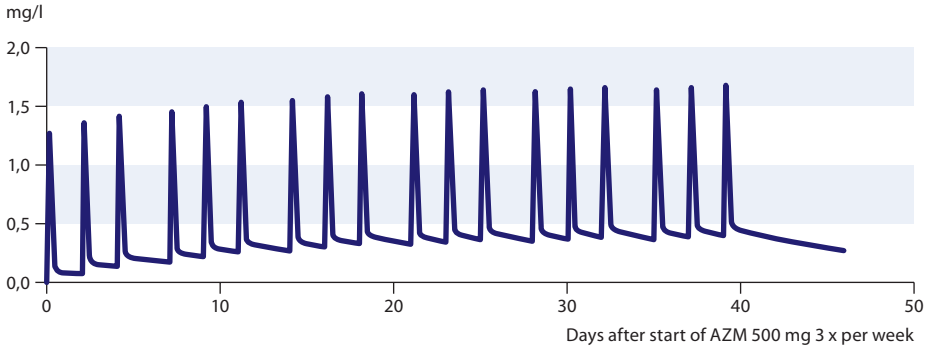
The final search using the mesh terms 'azithromycin' and 'pediatric dosage' and 'pharmacokinetic' revealed 8 reports, all about non CF patients. We used 3 reports because of their additive information about azithromycin dose relationship to body weight in pediatric patients.

### Azithromycin pharmacokinetics during maintenance therapy in cystic fibrosis

After a single oral administration azithromycin is absorbed within 3 hours from the gastro intestinal tract and is distributed in plasma with a distribution $t_{½}$ of 5-10 minutes. These data do not differ from kinetic data in non CF volunteers [23-25]. Azithromycin is excreted in bronchial secretion and in saliva [26,27]. In patients with CF, bronchial concentrations reached far higher levels than those in blood, plasma and saliva [26-30].

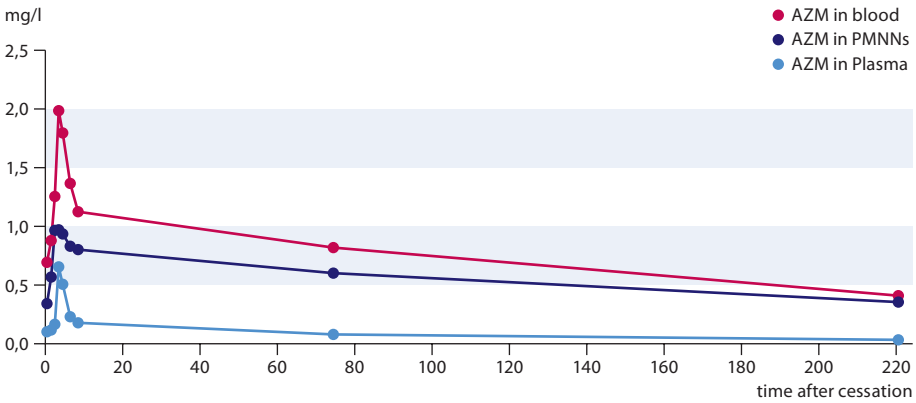
To illustrate azithromycin kinetics in a cystic fibrosis patient, the concentration in blood of 500 mg 3 times per week (monday-wednesday-friday) was simulated in figure 1. Pharmacokinetic parameters to make this simulation were taken from Wilms et al [27]. After 30 days a stable trough level in blood was reached. After every dose a peak was reached within hours and was distributed quickly.

**Figure 1:** *Simulated blood azithromycin concentration to time profile, dosing 500 mg 3 times per week(Monday-Wednesday-Friday) in an adult CF patient (70 kg).*



In figure 2 we depicted the decline of azithromycin levels after cessation of chronic dosing as was measured in a representative subject from the same report [25]. In this graph we show the concentration in blood, PMNs and plasma separately to demonstrate that about 70% of azithromycin in blood was incorporated in PMNs. After cessation a terminal $t_{½}$ of 180 h (sd 68 h) was found [27]. Especially the terminal $t_{½}$ is longer than reported in non CF subjects were a terminal $t_{½}$ of 57 h was found 1-6 days after dosing [23].

**Figure 2:** *Azithromycin concentration to time profile after a dose of 500 mg of azithromycin. The concentration in blood and the fraction in plasma and PMNs. The concentration in PMNs is expressed as azithromycin in PMNs per 1 L blood (figure from Wilms et al [27]).*




Cipolli and co-workers studied the short term pharmacokinetics in adult patients with CF using azithromycin 500 mg/day and 1000 mg/day during 5 consecutive days. At day 5, in bronchial secretions azithromycin was found in a concentration (4.06 ± 1.9 mg/L and 7.8 ± 1.55 mg/L respectively) 30-40 times higher than in serum. The concentration in bronchial secretions was doubled after doubling the oral dose, suggesting a linear relationship between oral dose and concentration in bronchial secretions [28].

Wilms and co-workers showed that concentrations in bronchial secretion further increased after prolonged administration. In adult patients with CF who had received 500 mg/d for more than 35 days on average 32 mg/l ± 16 mg/l was found in bronchial secretions, pointing to gradual accumulation of azithromycin in the lung [27].

Baumann and co-workers performed a study in children and young adults comparing a dose level of 250 mg/d in a group of patients aged 24 ± 8 years and 250 mg twice weekly in patients aged 14 ± 2 years of age. Mean sputum concentrations were 9.5 mg/l (interquartile ranges 1.4-33.4 mg/L) in the daily dosing group and 0.5 mg/L (interquartile ranges 0.2-1.4 mg/L) in the twice weekly dosing group. After 4 weeks a stable azithromycin concentration in sputum was reached. The results do not show a linear relationship between dose and sputum level. In this study, the patients were considerable younger in the twice daily group compared to the daily dosing group and had a better lung function (expressed as $FEV_1$) both of which could influence azithromycin pharmacokinetics and its sputum accumulation [29].

In another pharmacokinetic study a maintenance dose of 500 mg/day was compared with 1000 mg once weekly in adult CF patients. At 500 mg/day a concentration in bronchial secretion comparable to an earlier study was found ( 26.6 mg/L ± 16 mg/L). At 1000 mg once weekly the average concentration in bronchial secretions collected between 144 and 168 hour after the dose was still considerable (9.6 mg/L ± 7.1 mg/L) demonstrating a very slow pulmonary clearance. The concentration of azithromycin in bronchial secretions varied widely between patients. In an individual patient however, the concentration remained remarkably stable with little differences between the concentrations measured after 1, 2 and 3 months treatment. Reduction of the cumulative weekly dose with a factor 3.5 (7 x 500 mg versus 1 x 1000 mg per week) resulted in a reduction in exposure (calculated as area under the concentration-time curve) with a factor 2.5 ± 0.8 in plasma, 2.8 ± 0.9 in blood and 2.2 ± 1.1 in PMNs. In bronchial secretion the concentration was reduced with a factor of 3.0 ± 1.5. These data showed, again, an almost linear dose – concentration relationship in the studied tissues [30].

Steinkamp and co-workers performed an 8 weeks pharmacokinetic study as part of a randomized clinical trial of 17 to 25 mg azithromycin per kg body weight per week [8]. They found concentrations of 14.4 ±10.1 ug/g (1 ug/g ≈ 1 mg/L) in bronchial secretions, which is in the same range as in the Wilms study where adults were given 1000 mg per week [30].

Pharmacokinetics of azithromycin 10 mg/kg bodyweight was determined in a wide age range of (non cystic fibrosis) children (0.5-16 years, divided in 4 age groups) at short term use (3 days). Mean serum concentration-time data were comparable for the 4 age groups. An elimination half-life ($t_{1/2}$) of 65.2 hours (n = 25) was found. The $AUC_{0\text{-}72\ hour}$ and $C_{max}$ were not associated with age. Concluded was that a comparable $C_{max}$, AUC and $t_{½}$ was found after a dose of 10 mg/kg bodyweight in children from 0.5-16 years of age [31]. Other pharmacokinetic reports show a similar AUC of the oral suspension (10 mg/kg bw/d followed by 5 mg/kg bw/d, 4 days) compared to adult data and an increase in tissue (tonsil) penetration when the dose was increased from 10 mg/kg/ bw/d to 20 mg/kg bw/d during 3 days [32,33]. Pharmacokinetic data and concentrations of azithromycin in brochial secretion have not been determined in pediatric CF patients at chronic use.

From these reports we conclude that in CF patients azithromycin maintenance therapy leads to concentrations in bronchial secretion approximately linearly related to the oral dose and irrespective of the azithromycin dosing frequency and interval. Accumulation in bronchial secretions still occurs after 5 days of treatment, reaching stable values in about 1 month of treatment. A wide inter-individual variation in bronchial concentration occurs but the intra-individual variation in concentrations is rather small [29,30]. Up to now no straightforward relationship between pulmonary concentration of azithromycin and individual efficacy has been described. We advocate to include this in future efficacy studies.

## Dose level, effects and side-effects of azithromycin

Of the 8 reports of clinical trials, 6 reported efficacy data up to at least 6 months of therapy, 2 reported data up to 2 and 3 months. Thereof 7 reports were evaluated in the Cochrane review [11] to be reported without or with a low risk of bias (Equi [3], Clement [5], Saiman 2003 [4], Saiman 2010 [10], McCormack [6], Wolter [2] and Steinkamp [8]). Apart from the McCormack study, all studies compared azithromycin to placebo. In the Steinkamp study the absolute change in $FEV_1$ from baseline was reported, not the relative change. We recalculated the dose levels used to the dose per kg body weight per week. The average dose varied between 21 and 65 mg per kg bodyweight per week. Data are summarized in table 1.

**Table 1:** *Table 1: Dosing regimen and outcome parameters of clinical trials towards the efficacy of azithromycin maintenance therapy in CF. Data at 6 months or the nearest time point available were used.*

| Reference | Dose | Frequency | Compared to # | Duration of study | n active | Av weight | Dose range | Av dose | Mean Δ $FEV_1$ | SE Δ $FEV_1$ | Δ Days in hospital | Δ Days with IV antibiotics | P aeruginosa + | Drop out AZM treatment | Absence of risk of bias (Cochrane) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg | per week | | months | | kg | mg/kg/w | mg/kg/w | % | | % | % | % | % | |
| Steinkamp [8] | | 1 | p | 2 | 21 | | 17-25 | 21.2 | 2.3* | | | | 100 | 19.0 | ± |
| McCormack weekly [6] | 1200 | 1 | b | 6 | 103 | 54 | 17-34 | 22.2 | 2.4 | 1.0 | -39 | | 68 | 21.4 | + |
| Saiman 2003 [4] | 250-500 | 3 | p | 6 | 87 | 54 | | 25.4 | 6.2 | 1.8 | -47 | -39 | 90 | 2.3 | + |
| Clement [5] | 250-500 | 3 | p | 12 | 40 | | 19-34 | 28.0 | -2.8 | 3.7 | | -50 | 25 | 12.5 | + |
| Saiman 2010 [10] | 250-500 | 3 | p | 6 | 131 | 37 | 21-42 | 28.3 | -0.5 | 11.7 | -10 | -27 | 0,0 | 1.5 | + |
| Wolter [2] | 250 | 7 | p | 3 | 30 | 57 | | 30.7 | 3.9** | 2.4 | -60 | -72 | 100 | 20.0 | + |
| McCormack daily [6] | 250 | 7 | b | 6 | 105 | 56 | 24-44 | 31.3 | 3.1 | 0.8 | -58 | | 66 | 14.3 | + |
| Kabra [7] | 5 mg/kg | 7 | b | 6 | 24 | 17 | | 35.0 | -4.0 | 3.5 | | | 33 | 16.7 | - |
| Equi [3] | 250-500 | 7 | x | 6 | 41 | | 44-87 | 65.0 | 3.9 | 4.8 | | | 50 | 4.9 | + |
| Kabra [7] | 15 mg/kg | 7 | b | 6 | 23 | 16 | | 105.0 | 0.0 | 15.0 | | | 50 | 21.7 | - |

*# Data from 6 randomized placebo (p) controlled clinical trials, 1 randomized placebo controlled cross over trial (x) and 2 randomized trials comparing two dosing regimens to baseline (b). *Data at 2 months. **Data at 3 months.*

In the study performed by Kabra [7] the highest dose level in children was used, comparing 35 and 105 mg/kg bw/week. This study was not included in the analysis because it was assessed to contain a risk of bias on several issues [11]. The safety data, however, remain of interest [7].

In the other 7 studies (Equi [3], Clement [5], Saiman 2003 [4], Saiman 2010 [10], McCormack [6], Wolter [2] and Steinkamp [8]) no correlation between dose and primairy effect parameter $FEV_1$ could be found (correlation coefficient r= 0.19; P>0.2), Secondary parameters like the number of patients who dropped out of the study, neither showed a correlation with the dose level. Data about decline in hospitalization rate and days with intravenous antibiotic treatment were available in 4 and 5 reports and again showed no correlation with the dose level. Only the percentage of *P. aeruginosa* infected patients showed a correlation (r=0.73; P<0.05) with the outcome parameter changes in $FEV_1$.

A limitation to our approach was that we only used reported data. Using the raw individual patient data would have been a more preferable method.

In the trial reported by Equi and co-workers, the dose-level varied between 44 and 87 mg/kg bw/week for the individual participants, within this protocol no relation between dose and outcome could be made [3].

Combining these observations with the linearity between dose and sputum concentration, the stable expression in sputum and the length of the $t_{½}$ and the linear relation between dose and bodyweight in short term use we propose to use the lowest dose level with a documented favorable outcome with a dose related to bodyweight. The study of Steinkamp [8] used the lowest dose reported (17-25 mg/kg bw/week, average 21 mg/kg bw/week). However, the treatment duration in this study was only 2 months, only a limited number of patients participated (n=38), the absolute and not the relative change in $FEV_1$ was reported and the report was evaluated to contain some issues related to a risk of bias [11]. We therefore conclude the lowest dose level with proven efficacy was used in the McCormack [6] study, which reported data over 6 month of therapy, without an apparent risk of bias [11]. A dose level between 22 (the average dose in the once weekly group) and 30 mg/kg bodyweight/week, irrespective of its dosing scheme, could be advised as being the lowest dose level with proven efficacy. Using different dosing categories related to body weight, we propose a scheme with 5 dose steps leading to a dose level between 20 and 33 mg/kg bodyweight/week as shown in table 2.

**Table 2:** *Proposed azithromycin dosing schedule in patients with cystic fibrosis.*

| Body weight | Weekly dose | Alternative | or | Dose range in mg/kg/week |
|---|---|---|---|---|
| < 15 kg | | 10 mg/kg 3x per week suspension 40 mg/ml | | 30 |
| 15-25 | 500 mg | 250 mg 2x per week | | 20-33 |
| 25-35 | 750 mg | 250 mg 3x per week | | 21-30 |
| 35-45 | 1000 mg | 250 mg 4 x per week | 500 mg 2 x per week | 22-29 |
| 45-55 | 1250 mg | 250 mg 5 x per week | | 23-28 |
| >55kg | 1500 mg | 500 mg 3 x per week | | < 27 |

For neonates, no efficacy data are available at present, but if azithromycin therapy is to be studied in a clinical trial a dose of 30 mg/kg bodyweight/week could be given in 3 dosing moments using the azithromycin suspension.

We advocate to include pharmacokinetic measurements of azithromycin in blood and sputum in future studies to further explore the relationship between systemic and local (pulmonary) exposure and efficacy.

In all studies azithromycin was well tolerated. Gastro intestinal side effects (nausea and diarrhea) were reported with a higher incidence in the azithromycin treated patients [34]. An odds ratio for nausea of 1.69 (95% CI 0.99-2.87) was calculated in the Cochrane meta analysis from the combined data of 2 studies (Saiman 2003 and 2010) [11]. In two studies audiometry was incorporated in the protocol [3,4]. Although azithromycin has been related to ototoxicity in an earlier report [35] and some changes were noted, no differences between the azithromycin and the placebo group were observed [3,4,35]. An increased incidence of wheezing was found in the Saiman 2003 study but was not reported in later studies [4].

Comparing daily and weekly administration in a randomized study (McCormack) resulted in a favorable safety profile at a daily dosing regimen with less gastro intestinal side effects and less drop outs compared to once weekly dosing [6].

In the study with the highest dose level in children, comparing 35 and 105 mg/kg bw/week (Kabra) no specific side effects were reported and the number of drop outs was comparable at both levels [7].

An important side effect of macrolide maintenance therapy is the introduction of resistance towards macrolides in micro-organisms that are normally sensitive. This accounts especially for *Staphylococcus aureus*. The level of resistance increased from 10% to 83% within the first year of azithromycin therapy [36,37]. Azithromycin has also been associated with an increased incidence of nontuberculous mycobacterial infections, particularly of multi-drug-resistant species like *Mycobacterium abscessus* [38]. Nontuberculous mycobacterial infections are rare but the incidence and resistance have to be monitored closely. These disadvantages have to be weighed against the positive effects.

Apart from antimicrobial resistance and safety data up to 6 months in clinical trial reports no other long term safety data are reported so far.

## Interaction with other CF pharmacotherapy

Only limited data are available about drug-drug interactions of short-term use of azithromycin with other drugs used in treatment of CF. Even less information is available after chronic use of azithromycin. Whereas the other macrolide antibiotics erythromycin and clarithromycin have a strong inhibitory effect on the metabolising liver enzyme cytochrome P450-3A4 and the membrane pump P glycoprotein (PgP), azithromycin has little or no interference with both systems [39,40].

Bioavailability of azithromycin in CF patients using pancreatic enzymes was comparable to healthy volunteers not using pancreatic enzymes [25].

Some reports about possible drug-drug interactions are noticeable: a case of a potentially harmful elevation of disopyramide levels has been reported, probably based on inhibition of disopyramide dealkylation [41]. Furthermore there are incidental reports about prolongation of QTc time that make that we should be careful when azithromycin is used in combination with other QTc time prolonging drugs, like cisapride, haloperidol, verapamil and B blockers [42-44].

A specific interaction in CF therapy is the potential interaction of azithromycin with dornase alfa. Ripoll and co-workers found a concentration dependent inhibition of hydrolysing capacity of dornase by macrolides (azithromycin, roxitromycin and erytromycin) in an in-vitro experiment [45]. This noticeable effect however, was found at concentrations of DNA far below those found in CF bronchial excretions and can therefore not directly be extrapolated to clinical practice [45,46]. In two of the randomised clinical studies towards the efficacy of azithromycin, the use of dornase alfa was not excluded. Equi and co-workers found a decreased $FEV_1$ in patients on azithromycin who had been using dornase alfa before (since > 2 months) inclusion [3]. This could indicate inhibition of dornase alfa by azithromycin. The subgroup however, was too small to draw a statistically significant conclusion. Saiman and co-workers showed that CF patients using azithromycin and dornase alfa concomitantly, had similar benefits from azithromycin as CF patients that did not use dornase alfa [4]. In this study also patients who were treated with dornase alfa further showed an improvement of lung function when azithromycin was added. As both the clinical effect of dornase alpha and azithromycin is reflected in lung function, this may argue against a negative effect of azithromycin on dornase alfa. These data do, in our opinion, not restrict the concurrent use of dornase alfa and azithromycin.

Conflicting data about interactions with statins are available. No pharmacokinetic interaction was found with atorvastatin (a cytochrome P450-3A4 metabolised statin), but a systematic screening of the WHO drug interaction database by Strandell using a disproportionality analysis, found an elevated (2-3 fold) incidence of rhabdomyolysis after addition of azithromycin to statins (simvastatin, lovastatin, atorvastatin, pravastatin, rosuvastatin) [47,48]. No difference was found between the cytochrome P450-3A4 metabolised statins on the one hand and pravastatin and rosuvastatin, which are metabolized by non-cytochrome P450-3A4 pathways on the other [48]. This suggests an alternative cause or relationship than cytochrome P450-3A4 inhibition. In co prescribing azithromycin and a statin one should be aware of the 2-3 fold increased risk of rhabdomyolysis.

For lung transplant patients case reports about elevation of cyclosporine and tacrolimus serum levels during intravenous azithromycin therapy are noticeable [49,50]. Although a definite interaction with azithromycin could not be assessed, tacrolimus and cyclosporin levels should be closely monitored when azithromycin therapy is initiated or discontinued.

The serum levels of the antihistamine desloratadin slightly increased after combining with azithromycin. The increase however was evaluated as not clinically relevant [51].

Up to date no other major drug-drug interactions have been reported between azithromycin and medication used by CF patients concomitantly.

## CONCLUSION

Maintenance therapy with the macrolide antibiotic azithromycin has been introduced 10 years ago and is currently widely used in the treatment of patients with Cystic Fibrosis. Azithromycin can improve lung function, decrease the number of pulmonary exacerbations, the number of hospital days and the use of other antibiotics. A recent update of the Cochrane systematic review (2011) has demonstrated evidence from meta-analysis to support a small but consistent improvement in respiratory function over six months. Data about a longer treatment period are less clear and show a tendency towards equivalence between azithromycin and placebo treatment. A relationship between dose level and efficacy could not be found. Based on the current literature a dose level of 22-30 mg/kg/week is the lowest dose level with proven efficacy over a treatment period of 6 months. Due to its extended half-life in patients with CF, this weekly dose can be divided in 1 to 7 dosing moments, depending on patient preference and gastro-intestinal tolerance. Azithromycin has a mild side effect profile and a only a limited number of drug interactions with other CF-related drugs has been documented so far.

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# Chapter 7

# Summary, General Discussion and Perspectives

# 7 Summary, General Discussion and Perspectives

## AZITHROMYCIN MAINTENANCE THERAPY

The widespread acceptance and clinical use of azithromycin maintenance therapy in patients with cystic fibrosis (CF) started a decade ago, after the first publications of Jaffe in 1998 and the first randomized clinical trial in 2002 [1,2]. Up to then azithromycin had been used merely for antibiotic treatment of short duration. Its pharmacokinetics and distribution are characterized by accumulation in lysosome containing cells (mainly polymorphonuclear neutrophils) and an excellent tissue penetration, resulting in prolonged exposure in tissues [3-5]. Because of these characteristics a limited dosing time (in general 3 or 5 days) proved to be sufficient for the treatment of infections with susceptible micro-organisms [6].

In cystic fibrosis treatment, chronic use or 'maintenance therapy' with azithromycin proved to be effective in randomized clinical trials and was well tolerated. The effect was most prominently seen in patients infected with *P. aeruginosa*, a micro organism able to colonize the CF patients' lung [7-9]. This was remarkable, since *P. aeruginosa* is normally not susceptible for antibiotic treatment with azithromycin. However, despite the unclear exact mechanisms of action in CF, chronic use of azithromycin causes an increase in (or a lesser rate of decline of) pulmonary function, a reduction in number of days in hospital, a reduction in number of exacerbations and a reduction in other antibiotic use in CF patients [10-17]. Consequently, azithromycin maintenance therapy became standard of care in patients infected with *P.aeruginosa* [18].

The aim of this thesis was to describe the pharmacokinetics of azithromycin in cystic fibrosis patients during maintenance therapy, to use this information to evaluate the current dosing regimens and to combine this information to a dosing advise. A further aim was to evaluate long term azithromycin use in cystic fibrosis patients and to investigate potentials for drug-drug interactions.

The first step in this thesis was the development and validation of a method of quantitation of azithromycin in matrices of interest. We were specifically interested in azithromycin kinetic behavior in plasma and blood, but also in polymorphonuclaer neutrophils (PMNs) and in pulmonary excretion (sputum). In **chapter 2** we describe an analytical method including the separation of PMNs and the treatment of CF sputum in a way that azithromycin could be quantified.

Azithromycin lacks UV absorbing properties and we used an in line derivatisation reaction with 9-fluorenylmethyloxycarbonyl-chloride (FOML-Cl) to enable fluorescence detection. We optimized a high performance liquid chromatographic method towards determination of azithromycin. Recovery from blood and PMNs isolated by a gravity separation procedure was also assessed. A lower limit of quantitation of 0.042 ± 0.017 mg/l in plasma, 0.119 ± 0.065 mg/l in blood and 0.072 ± 0.036 mg/l in water was achieved. This method proved sensitive enough for the pharmacokinetic studies.

Nowadays, and for further research, liquid chromatography combined with mass spectrometry would be the method of choice. Pretreatment is less laborious and a further reduction in the lower limit of quantitation will be feasible, even with small sample volumes. In recent a report a limit of detection of 2 microg/L and limit of quantitation of 5 microg/L were achieved using liquid chromatochraphy coupled to electrospray mass spectrometry [19].

In **chapter 3.1** we describe the pharmacokinetics of long term administration of azithromycin in CF patients and the pharmacokinetic consequences of a change in dosing regimen from daily dosing to once weekly dosing is reported in chapter 3.2.

In chapter 3.1 we report the pharmacokinetic parameters of 500 mg per day in adult CF patients. Azithromycin concentrations were quantified in plasma, blood, isolated PMNs and sputum of 8 adult CF patients. The azithromycin distribution $t_{1/2}$ was 0.1 hours in plasma. The (mean ± standard deviation) elimination $t_{1/2}$ was 102 (± 20) hours in plasma, 180 (± 68) hours in blood and 289 (± 166) hours in PMNs. On average, the concentration in PMNs was 2100 times the concentration in plasma 24 h after dosing azithromycin and 70% of azithromycin in blood was incorporated in or bound to PMNs.

In sputum the concentration of azithromycin ranged from 12 to 53 mg/L and was still detectable at concentrations in the range of 4 to 27 mg/L 10 days after the last dose.

In earlier reports a $t_{1/2}$ of 57 h in plasma was found 1-6 days after dosing in healthy volunteers. After chronic use in CF the $t_{1/2}$ in plasma is doubled. In bringing up an explanation we have to take into account that 1-6 days after dosing only limited amounts are found in plasma. More than 70% of azithromycin in blood will be found in PMNs. Presumably a reservoir of azithromycin in PMNs, tissue and bronchial excretion releases azithromycin to some extent, resulting in a prolongation of $t_{1/2}$ in plasma. $T_{1/2}$ in blood or sputum could not be compared since data in healthy volunteers were not available.

The long $t_{1/2}$ in blood and PMNs and the slow decrease in sputum levels indicate a less frequent dosing schedule (for instance once weekly dosing) could be studied in future clinical trials. The elevated concentration of azithromycin in PMNs and the pulmonary retention of PMNs support the hypothesis that PMNs transport azithromycin towards the lung and towards pulmonary excretion (sputum).

A question which needs further research is whether or to what percentage azithromycin in sputum is freely available or still incorporated in lysosomal vesicles of (viable or non-viable disintegrating) PMNs. To be able to relate the concentration in pulmonary excretion to efficacy and microbial susceptibility data, a reproducible measurement of the free (unbound) fraction in pulmonary excretions is warranted.

In **chapter 3.2** we examined pharmacokinetics, systemic exposure and sputum penetration of azithromycin in CF patients after a change from daily dosing of azithromycin to a once weekly dosing scheme. Eight adult CF patients using AZM 500 mg per day, changed to a once weekly dose of 1000 mg during 3 months. The cumulative weekly dose was reduced with a factor 3.5 (7 x 500 mg vs 1 x 1000 mg weekly).This dose level was comparable to 1200 mg once per week which demonstrated a comparable level of efficacy to daily dosing. Once per month, sputum and blood samples were collected. Azithromycin was quantified in blood, plasma, isolated PMNs and sputum.

At 1000 mg once weekly reduced but still substantial concentrations were achieved in PMNs and in sputum. A reduction for AZM exposure (expressed as Area Under the Curve) by a factor (±SD) of 2.5(±0.8) in plasma, 2.8(±0.9) in blood and 2.3(±1.1) in PMNs was found. At weekly dosing the average sputum concentration was 3.0 (±1.5) times lower than at daily dosing. These differences are statistically significant in plasma and PMNs but - regarding the large interindividual variation in AUC - clinically of little relevance.

In this pharmacokinetic study a wide level of variation between the subjects was found but the monthly determined concentrations in sputum within one subject were comparable with only a small level of within subject variation.

As the percentage of azithromycin found in PMNs showed little variation, quantification in isolated PMNs does not seem to add information about the exposure level in kinetic studies or in a therapeutic drug monitoring program. In further kinetic studies quantification in blood and sputum should be sufficient to be able to compare exposure levels.

Simultaneously with our report the results of a randomised trial comparing 250 mg daily to 1200 mg once weekly were published. In this particular clinical trial the dose per week was reduced from 1750 mg to 1200 mg. The outcome of the once weekly dosing scheme was comparable (non inferior) to the daily dosing scheme proving the efficacy of once weekly dosing at a reduced dose level [20]. Both pharmacokinetic data and the results of this clinical trial prove a once weekly dosing scheme is feasible without loss of clinical efficacy.

Introduction of azithromycin maintenance therapy in a patient group already using a wide variation of drugs introduces a risk of drug-drug interactions. In **chapter 4** we focussed on the possibility of an interaction between azithromycin and the DNA hydrolysing capacity of dornase alfa. Dornase alfa is an enzyme used locally (by inhalation) to reduce the increased viscosity caused by elevated concentration of waste DNA in sputum. Azithromycin and dornase alfa are used concomitantly in patients with cystic fibrosis and are both found in substantial concentrations in sputum. In an earlier study, inhibition of the DNA hydrolysing capacity of dornase alfa by azithromycin was reported [21]. In this study however, concentrations of DNA and dornase far below those found in CF sputum were tested. We investigated if inhibition occurred at clinically representative sputum-levels of azithromycin, dornase alfa and DNA. In an in vitro experimental setting, azithromycin 50 mg/L did not affect the hydrolysing capacity of dornase alfa. Azithromycin 100 mg/L, which is above the concentration usually found in sputum, showed a minor inhibitory effect. We concluded that in-vitro azithromycin did not inhibit the DNA hydrolysing capacity of dornase alfa at clinically representative sputum concentrations. These data combined with the data found in clinical trials do, in our opinion, not restrict the concurrent use of dornase alfa and azithromycin.

Other local interactions have not been reported or studied yet. Azithromycin for injection however, proved incompatible with some of the antibiotics which are inhaled in CF treatment, like tobramycin and aztreonam (tested in a Y-side simulated injection, azithromycin concentration 2000 mg/L) due to precipitation [24]. Whether this would lead to a clinical interaction in CF treatment, is dependent on factors like the unbound concentration of both drugs and local circumstances (pH, concentration, sputum constituents). Since the concentration in sputum is about a 40 times lower, the risk will be limited but we should be aware of the possibility that incompatibility could lead to a clinical interaction when locally high concentrations of drugs are present.

In **chapter 5** we retrospectively studied long term effects of azithromycin therapy in children and in adult patients. In **chapter 5.1** we evaluate the long term effect of azithromycin on pulmonary function in children infected with *P. aeruginosa* and compare these results to a non-pseudomonas colonized cohort (not using azithromycin). We found an amelioration in pulmonary function in *P.aeruginosa* infected patients on azithromycin in the first year after start of therapy. This could not be found in the following 6 years were a decline in pulmonary function was found. The rate of decline however was comparable in the two groups.

In **chapter 5.2** we studied the changes in pulmonary function in adult cystic fibrosis patients and also made a comparison between *P.aeruginosa* infected and non-infected patients. Although the baseline pulmonary function ($FEV_1$) was lower in the infected group of patients, the annual rate of decline was, again, comparable in both groups. Up to now, most reports show a difference in yearly decline of pulmonary function in advantage of the uninfected patients.

The finding in both children and adult patients of an equal rate of decline of pulmonary function in both *P. aeruginosa* infected and not infected patients cannot be contributed to the introduction of macrolide therapy alone, since in the timeframe we analyzed, also other anti *P. aeruginosa* therapies, like inhaled tobramycin, and mucolytic treatment (dornase alfa, hypertonic saline) have been introduced.

Furthermore we analysed changes in prevalence of pathogens found in sputum isolates in the adult patients and we monitored changes in susceptibility of isolated *P.aeruginosa* and *S.aureus* strains before and after introduction of azithromycin maintenance therapy.

Intensive anti *P. aeruginosa* antimicrobial treatment did not lead to uncontrolled increase in resistance of *P. aeruginosa* towards the antibiotics that were used. Comparing 1997 (the last year before introduction of azithromycin maintenance therapy) and 2007 we did observed a decrease in incidence and an increase in macrolide resistance of *S. aureus*. This disadvantage of azithromycin maintenance therapy has been documented before in cystic fibrosis centers in Rotterdam and Utrecht [26,27].

The long term contribution of azithromycin therapy alone however, could not be derived from these retrospective analysis. In clinical trials a maximum difference between treatment and placebo was seen at 6 months treatment (Cochrane) [25]. The published randomized clinical trials comparing azithromycin to placebo all lasted between 2 and 12 months. The one single trial lasting 12 months, performed by Clement et al, showed that after 6 months of therapy the maximal difference in pulmonary function ($FEV_1$) between azithromycin and controls was found: a mean difference of 5.58 (-1.98-13.58). At 1 year however the difference in $FEV_1$ was -2.8 (-10.04-4.44) pointing at a gradual decline in efficacy towards the primary outcome parameter $FEV_1$ [23,25]. The long term data in chapter 5.1 and 5.2 both indicate that an initial gain in pulmonary function was not consolidated beyond the first year of therapy.

A longer period of time than the 12 months randomized trial will be necessary to determine the contribution of azithromycin to the preservation of pulmonary function, number of exacerbations, hospitalization rate and use of additive antibiotics, but if this can be studied in a prospective randomized way is questionable. Currently, most CF patients with a *P.aeruginosa* pulmonary infection are treated with macrolide maintenance therapy. An alternative method to study the contribution of long term azithromycin therapy could be a prospective randomized (placebo controlled) trial in which continuation

of azithromycin is compared to discontinuation (stopping). In such a trial the kinetic parameters (long $t_{½}$ in blood and in sputum) have to be taken into account. Furthermore only patients on azithromycin therapy for more than 1 year should be included. Besides changes in pulmonary function also the frequency of exacerbations, days on intravenous antibiotic treatment and the influence on pathogens in sputum cultures and microbial resistance should be used as outcome measures.

In **chapter 6** we review current knowledge about azithromycin pharmacokinetics with the dosing schedules used in clinical trials in order to come to a dosing advise. Based on these data we concluded that a dose level of on average 22 mg/kg/week is the lowest dose level with proven efficacy. Due to the extended half-life in patients with CF, and the almost linear relationship between dose and exposure (expressed as AUC) and the non inferiority of once weekly dosing to daily dosing we conclude that the azithromycin dose can be divided in 1 to 7 administrations per week, depending on patient preference and gastro-intestinal tolerance. We propose a dosing scheme with 4 weight groups and a dose level between 20 and 33 mg/kg/week, which could be generally applicable.

No important side-effects or interactions with other CF-related drugs have been documented so far. Once weekly azithromycin was well tolerated but more gastro intestinal side effects were noticed compared to once daily dosing.

No relationship has been studied yet between azithromycin exposure in blood or sputum and clinical efficacy. In none of the clinical trials performed by other groups to prove clinical efficacy, quantitation of azithromycin was incorporated. This is remarkable since azithromycin was known for its extended $t_{½}$, and a change in dose regimen from short term (3-5 days) to maintenance therapy should have been combined with measurements of azithromycin concentrations in blood and sputum or tissue straight from the beginning. Especially since pharmacokinetics of drugs in cystic fibrosis patients are not always comparable to the general patient population. Further research should be directed towards determination of pharmacokinetic parameters at maintenance therapy in younger CF patients, especially if azithromycin therapy is going to be tested in neonates.
Further research towards a possible relationship between sputum or tissue concentration and efficacy is warranted. If such a relationship exists, patients in whom azithromycin lacks efficacy could possibly benefit from a higher dose level.

An even lower dose level which could be combined with a 'loading dose', should also be the scope of further investigations since the 'minimal effective dose level' with proven efficacy has probably not been reached yet.

The excellent tolerability of azithromycin however, did not stress the necessity of preceding pharmacokinetic, dynamic and toxicological research.

The combination of chronic or repetitive pulmonary infection with sputum production and inflammation can not only be found in cystic fibrosis and diffuse panbronchiolitis but also in chronic obstructive pulmonary disease (COPD) and in patients with non-CF bronchiectasis [28,29]. Although the number of prospective studies is less than in CF, for all of these indications studies reported positive effects on pulmonary function, sputum production and reduction of number of exacerbations. A recently reported randomized trial of daily azithromycin in 1142 patients with severe (GOLD 2-4) COPD resulted in a 27% reduction in the risk of exacerbation of pulmonary infection in one year. In the patients on azithromycin, resistance of pathogens increased from 42% to 81%.

This leads us to the question whether macrolide maintenance therapy has to become standard of care in these groups of patients. The development of resistance of *S. aureus* towards macrolides is fast and documented in several reports. Resistance of other susceptible pathogens including Mycobacterial pathogens is less clear and should be monitored closely.

Balancing development of antimicrobial resistance with therapeutic benefits, azithromycin maintenance therapy would be a valuable addition to the regular therapy for patients at risk for exacerbations and with a restriction in pulmonary function, especially when combined with exaggerate production of sputum. Besides in patients with Cystic Fibrosis infected with *P.aeruginosa* also in patients with severe COPD and in patients with non-CF brochiectasis azithromycin could be used with a positive benefit to risk balance. Although azithromycin did not increase pulmonary function in CF patients not infected with *P.aeruginosa*, azithromycin did reduce the incidence of exacerbations [9] and could therefore also be started in this group of CF patients in case of frequent exacerbations and excessive sputum production.

In developing dosing regimens the long $t_{1/2}$ and accumulation in sputum have to be taken into account leading to a dosing regimen as low as proven effective. Further questions which need to be addressed in future studies: do we need to continue when a patient is stabilized? Or can we apply other strategies like on–off therapy in which macrolide therapy is used when the exacerbation rate or increase in sputum production indicate the start of maintenance therapy?

Based on the pharmacokinetic data and on the reported clinical trial results we propose a dosing scheme with 4 weight groups and a dose level between 20 and 33 mg/kg/week, which can be generally applicable in pediatric and adult CF patients.

The comparable rate of decline in *P. aeruginosa* infected and uninfected patients is a valuable achievement of current CF treatment including azithromycin maintenance therapy but cannot be contributed to azithromycin alone.

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# Chapter 8

8

# Samenvatting

Dit proefschrift gaat over de toepassing van het antibioticum azithromycine bij patienten met cystic fibrose (CF, taaislijmziekte).

CF is een autosomaal recessief erfelijke aandoening veroorzaakt door een mutatie in het gen dat codeert voor het cystic fibrosis transmembrane regulator (CFTR) eiwit. Bij ongeveer 1 op de 32 Nederlanders komt het gen(heterozygoot) voor en 1 op de 4750 pasgeboren heeft cystic fibrose.

De mutatie leidt tot een verstoorde regulatie van zout en watertransport over de celmembraan. Als gevolg hiervan ontstaan afwijkingen in het functioneren van de luchtwegen, het maagdarmstelsel en het voortplantingssyteem. Infecties van de luchtwegen zijn de belangrijkste oorzaak voor ziekte en een verlaging van de levenverwachting bij patienten met CF. Longinfecties worden veroorzaakt door een verminderde natuurlijke verdediging van de luchtwegen tegen binnenkomende bacterien en een sterke, chronische ontstekingsreactie hierop.

Longinfecties bij CF patienten worden in eerste instantie veelal veroorzaakt door *Staphylococcus aureus* en *Haemophilus influenzae*. Als de patienten ouder worden, wordt de infectie vaak veroorzaakt door *Pseudomonas aeruginos*a, een bacterie die zich goed kan aanpassen aan de omstandigheden in de long van de CF patient en daardoor ook een chronische infectie kan veroorzaken.

Het als onderhoudsbehandeling gebruiken van azithromycine bij CF patienten startte na een eerste publicatie van Jaffe hierover in 1998 en werd breed geïmplementeerd in CF behandelcentra na publicatie van de eerste gerandomiseerde klinisch studie in 2002. Tot die tijd werd azithromycine voornamelijk kortdurend, als kuur, gebruikt. Door chronisch azithromycine gebruik werd de longfunctie verbeterd en ontstonden er minder exacerbaties (opvlammingen) van longinfecties. Deze effecten werden vooral gezien bij patiënten die reeds een chronische infectie met *Pseudomonas aeruginosa* hebben en in de praktijk wordt azithromycine voornamelijk in deze groep patiënten toegepast.

Azithromycine is een macrolide antibioticum waarvan de kinetiek in het lichaam wordt gekenmerkt door een snelle opname in witte bloedcellen en in weefsel. Hierdoor heeft het een lange verblijfsduur in het lichaam en kan het normaal gesproken gedurende een korte periode, 3-5 dagen worden gedoseerd bij infecties met een gevoelig microorganisme.

Het doel van dit proefschrift was het meten en beschrijven van de kinetiek van azithromycine tijdens chronisch gebruik bij patiënten met CF. Deze informatie is van belang om te kunnen bepalen of de gebruikte doseerschema's wel de optimaal zijn. Daarnaast werd het langdurig gebruik van azithromycine geëvalueerd en werd onderzoek gedaan naar mogelijke interacties met andere geneesmiddelen die CF patiënten gebruiken.

De eerste stap bestond uit de ontwikkeling van een analyse methode waarmee azithromycine kan worden gemeten in het bloed, plasma, witte bloedcellen en in sputum. Hierbij werd een methode gebruikt waarbij azithromycine met behulp van een hulpstof wordt omgezet in een fluorescerende verbinding die gevoelig genoeg kon worden geanalyseerd. De methode staat beschreven in hoofdstuk 2.

# Samenvatting

In hoofdstuk 3 werd de farmacokinetiek van azithromycine beschreven. In hoofdstuk 3.1 zijn de farmacokinetische parameters van een dosering van 500 mg per dag gemeten in bloed, witte bloedcellen (polymorfonucleare neutrofielen), plasma en sputum. De concentratie in witte bloedcellen was opvallend hoog, ongeveer 70% van de azithromycine in het bloed bevindt zich in de witte bloedcel. Verder bleek de concentratie in het sputum hoog, dit is van belang omdat dit een afspiegeling van de concentratie in de long is en azithromycine daar een deel van zijn werkzaamheid uitoefent. De halfwaardetijd (tijd waarin de concentratie met 50% vermindert) in bloed bedroeg 180 uur, hetgeen langer is dan de halfwaardetijd bij kortdurend gebruik in niet CF patienten.

Vanwege de lange halfwaardetijd en de hoge concentratie in het sputum is in een vervolgonderzoek de kinetiek gemeten bij een dosering van 1000 mg 1 maal per week. Het verlagen van de dosering met een factor 3,5 (500 mg per dag versus 1000 mg 1 maal per week) leidde tot een verlaging van de blootstelling aan azithromycine met een factor van 2,5 (±0,8) in plasma, 2,8 (±0,9) in bloed, 2,3 (±1,1) in witte bloedcellen en 3,0 (±1,5) in sputum. Geconcludeerd werd dat de verlaging in blootstelling ongeveer lineair evenredig was met de dosering.

Gelijktijdig werd een Australisch onderzoek gepubliceerd waarin de effectiviteit van een 1 maal per week dosering werd vergeleken met een dagelijkse dosis azithromycine. Uit dat onderzoek kwam naar voren dat beide doseringen even effectief bleken. Op grond van de effectiviteitstudie en onze kinetiek gegevens kan worden geconcludeerd dat een dagelijks dosering niet nodig is en dat azithromycine ook 1 maal per week kan worden gegeven. Hierbij moet wel worden opgemerkt dat een dosering van 1 maal per week 1200 mg, die in de studie werd gebruikt, meer misselijkheid door de patiënten werd aangegeven.

De introductie van een chronische behandeling bij een groep patiënten die al veel geneesmiddelen gebruiken kan leiden tot ongewenste geneesmiddel interacties. In hoofdstuk 4 hebben we een interactie verder onderzocht, namelijk die tussen dornase alfa en azithromycine. Dornase alfa is een enzym dat wordt geïnhaleerd en dat in staat is om rest DNA strengen in het sputum van CF patienten af te breken. Rest DNA kan afkomstig zijn van witte bloedcellen die door de chronische longontsteking in de long aanwezig zijn. Rest DNA verhoogt de viscositeit van het sputum. In een eerdere publicatie werd weergegeven dat azithromycine de werking van het enzym dornase alfa zou kunnen verminderen. In het gepubliceerde experiment werd echter een lage concentratie dornase alfa en DNA gebruikt. Wij hebben een soortgelijk experiment uitgevoerd met concentraties die in de range zijn van wat in CF patiënten wordt gemeten. Hierbij trad geen vermindering van het effect van dornase alfa op. Op basis van deze resultaten en op basis van wat in studies is gemeten bij gelijktijdig gebruik, achten wij het gelijktijdig gebruik verantwoord.

In hoofdstuk 5 hebben we retrospectief de resultaten van de behandelde patiënten over een lang tijdsinterval geanalyseerd. In hoofdstuk 5.1 is de ontwikkeling van de longfunctie bij kinderen met CF bestudeerd. Hierbij werd gezien dat de longfunctie in het eerste jaar na start van azitromycine verbeterde maar in de daaropvolgende jaren weer achteruitging.

# Samenvatting

De mate van achteruitgang per jaar is vergeleken tussen twee groepen patienten: zij die een chronische infectie met de bacterie *Pseudomonas aeruginos*a hebben én azithromycine gebruiken en een groep patiënten die geen *Pseudomonas aeruginosa* infectie hebben en geen azithromycine gebruiken. In beide groepen bleek de achteruitgang vergelijkbaar. Dit is opmerkelijk omdat in het verleden patiënten met een *Pseudomonas aeruginosa* infectie een snellere achteruitgang vertoonden.

In hoofdstuk 5.2 hebben we een soortgelijke vergelijking gemaakt bij volwassen CF patiënten. Ook hier bleek de achteruitgang van de longfunctie per jaar gelijk in een groep patiënten met een *Pseudomonas aeruginosa* infectie en met azithromycine in vergelijking met een groep die geen *Pseudomonas aeruginosa* infectie had en geen azithromycine gebruikte. Verder is hier gekeken of de bacterien die in het sputum werden gekweekt in 10 jaar na introductie van azithromycine veranderde. De bacterie *Staphylococcus aureus* werd minder vaak gekweekt, maar van deze bacterie bleek >80% resistent (ongevoelig) voor azithromycine terwijl dit voor de introductie van azithromycine 15% bedroeg.

Het gunstige resultaat dat patiënten met een *Pseudomonas aeruginosa* longinfectie niet een snellere achteruitgang in longfunctie ondervinden dan de andere patiënten kan niet alleen aan azithromycine worden toegeschreven. In het tijdbestek van de analyse hebben ook andere therapieën hun intrede gedaan zoals dornase alfa, tobramycine inhalatie en inhalatie van een hypertone zoutoplossing.

In hoofdstuk 6 is de huidige beschikbare informatie en kennis over het doseren van azithromycine in een overzichtsartikel verwerkt. Op basis van ons eigen onderzoek en andere beschikbare literatuur is een voorstel voor een doseertabel met verschillend gewichtsgroepen patiënten opgesteld waarbij de dosering tussen 20 en 33 mg/kg/week valt. Het aantal doseringen per week in het schema kan variëren en aangepast worden aan de wensen van de individuele patiënt.

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# Curriculum Vitae

Erik Bert Wilms werd geboren in Zuidwolde op 5 april 1963. Na het behalen van het VWO diploma aan het Gemeentelijk Scholengemeenschap Emmen startte hij in 1981 met de studie farmacie aan de Rijks Universiteit Groningen. Tijdens zijn bijvakonderzoek deed hij farmacokinetisch onderzoek aan de Universiteit van Montpellier (Frankrijk). In 1987 behaalde hij zijn doctoraal examen en een jaar later het apothekersexamen.

Aansluitend heeft hij gewerkt in the William Harvey Hospital in Ashford (Kent, UK) als klinisch apotheker en daarna gedurende 1,5 jaar als dienstplichtig militair apotheker bij het Nationaal Vergiftigingen Informatiecentrum (NVIC).

De opleiding tot ziekenhuisapotheker heeft Erik gevolgd in het Erasmus MC (opleider dr. JW Meilink en drs. BH Graatsma). Aansluitend hieraan is hij 5 jaar werkzaam geweest in het oncologisch centrum Daniel den Hoedkliniek (Erasmus MC). In 2000 maakte hij de overstap naar de Apotheek Haagse Ziekenhuizen(AHZ) met tot 2012 de aandachtgebieden klinische dienstverlening Medisch Centrum Haaglanden en ICT.

In 2005 heeft hij de aantekening Klinisch Farmacoloog (NVKF&B) behaald en het onderzoek wat in dit proefschrift is beschreven, is daaruit voortgekomen. Het onderzoek heeft hij uitgevoerd in het klinisch farmaceutisch en toxicologisch laboratorium van de AHZ en in het HagaZiekenhuis, waar hij sinds februari 2012 werkzaam is.

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