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Investigations of *Aspergillus* infection in Cystic Fibrosis

A thesis submitted to Trinity College Dublin for the Degree of Doctor of Philosophy

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By

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Summary

Aspergillus fumigatus is a ubiquitous saprophytic fungus that is a pathogen in principally immunocompromised hosts. It is the most common fungal pathogen in Cystic Fibrosis (CF), most often associated with Allergic Bronchopulmonary Aspergillosis (ABPA). However, CF patients are also commonly colonised with *A. fumigatus* without displaying any symptoms of ABPA. There is a wide array of antifungal drugs available to treat *A. fumigatus* infections in CF including the triazoles but drug resistance has been reported in a number of countries. No triazole resistance in clinical *A. fumigatus* isolates has been reported in Ireland to date, with the exception of fluconazole (*A. fumigatus* is inherently resistant to fluconazole). However antifungal susceptibility testing is not routinely performed in Irish diagnostic microbiology laboratories. The epidemiology of *A. fumigatus* and patterns of colonisation in Irish CF patients is unclear. Furthermore the virulence of isolates that persist in the CF airways over time and how they may evolve to adapt to the hostile CF lung is unknown. What impact asymptomatic colonisation has on the CF lung epithelium over time is also not fully understood. We sought to monitor the bioburden of *Aspergillus* pre- and post-itraconazole treatment in a CF patient population who were asymptomatically colonised and to determine the antifungal drug susceptibility of these CF *A. fumigatus* isolates and additionally isolates collected from other CF patients. The epidemiology of *A. fumigatus* isolates from these CF patients was monitored and furthermore the virulence of *A. fumigatus* genotypes from CF patients was investigated. Finally, the ability of the *A. fumigatus* CF isolates to interact with both human bronchial epithelial cells (HBEs) and human bronchial epithelial cells with the cystic fibrosis $delF_{508}$ mutation (CFBEs) was explored.

The bioburden of *A. fumigatus* in an Irish CF cohort (n=13), who were asymptomatically colonised with the fungus was examined by standard culture and qPCR at pre- and post-itraconazole treatment time points. *A. fumigatus* isolates collected from these patients and additional isolates collected from other CF patients from a total of four CF centres in Ireland were identified to the species level by PCR and sequencing of the *ITS* region. The epidemiology of these *A. fumigatus* isolates was investigated using the *A. fumigatus* specific STR*Af* genotyping assay. Antifungal drug susceptibility of all isolates collected was established using the commercial Trek Sensititre susceptibility system. The virulence of different genotypes of *A. fumigatus* was determined using the *Galleria mellonella* insect model. The ability of the *A. fumigatus* CF isolates to open epithelial cell tight junctions in both HBEs and CFBEs was determined using transepithelial resistance (TER). Changes in concentration and distribution in tight junction proteins zonula occludens-1 (ZO-1) and junctional adhesion molecule-A (JAM-A) in response to *A. fumigatus* was determined by western blot analysis and confocal microscopy.

Aspergillus bioburden, measured by CFU counts (CFU/g) and qPCR, was significantly reduced following itraconazole treatment and remained low at 6 and 12 month follow up time points. All isolates collected were confirmed as *A. fumigatus* with a 99-100% identity. Within our CF patients isolates no antifungal drug resistance was observed for a panel of nine antifungal drugs including three echinocandins and four triazoles, with the exception of fluconazole which has been reported to be inactive against *A. fumigatus*. Two patterns of airway colonisation were observed within our CF patients; persistent colonisation (with an indistinguishable genotype in $\geq$2 consecutive samples) and non-persistent colonisation (with distinguishable genotypes in consecutive samples) and furthermore patients sharing an indistinguishable genotype was also observed. Analysis of multiple colonies per sample showed examples of some CF patients being colonised with a unique genotype, while others were colonised with several genotypes. In the *G. mellonella* model different genotypes caused different rates of mortality however no significant difference could be found between, representative persistent and non-persistent colonisers. However virulence of a persistent isolate over time demonstrated that later isolates were more virulent than earlier ones, in the model. *A. fumigatus* conidia and culture supernatants (CSNs) were able to cause significant disruption to tight junctions of HBEs and CFBEs. CSNs of persistent and non-persistent isolates produced different rates of disruption of tight junction integrity of the respiratory epithelium and only early CSNs from the persistent isolate were capable of significantly tightening the tight junction in the first 6 hours post exposure. Later CSNs from the persistent isolate also opened tight junctions more readily. CSNs from longer culturing times caused the greatest disruption of tight junction integrity and breakdown of tight junction proteins ZO-1 and JAM-A, which is in part due to gliotoxin.

These results demonstrate that itraconazole treatment effectively reduces *Aspergillus* burden in the CF airways and resulted in no emergence of triazole resistance. Furthermore no triazole resistance was detected in any of the *A. fumigatus* isolates collected. Genotyping of the isolates from this study revealed patients persistently and non-persistently colonised and additionally CF samples could be found to contain a single genotype or multiple genotypes. These different genotypes caused different rates of mortality in the *G. mellonella* model. However the virulence of a persistent coloniser showed later isolates were more virulent than earlier ones, suggesting an increase of virulence or adaptation over time in the patient. *A. fumigatus* conidia may directly affect the tight junctions of respiratory epithelial cells. Differences in the response of tight junction integrity to CSNs of persistent and non-persistent isolates, illustrates that differences between the persistent and non-persistent colonising isolates exist. Finally, later CSNs revealed the production of gliotoxin by *A. fumigatus* plays a role in the breakdown of tight junction integrity which could have implications for the human lung, particularly the CF lung where isolates may persist over time while remaining viable.

Acknowledgements

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List of Abbreviations

ABPA	Allergic Bronchopulmonary Aspergillosis
ATCC	American type culture collection
AF293	*Aspergillus fumigatus* 293
AFLP	Amplified fragment length polymorhism
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage fluid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
CFBE	Cystic Fibrosis Bronchial Epithelial cells
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony Forming Units
CLD	Chronic Lung Disease
CLSI	Clinical and Laboratory Standards Institute
CN	Control
CNPA	Chronic Necrotizing Pulmonary Aspergillosis
COPD	Chronic Obstructive Pulmonary Disease
CO_2	Carbon dioxide
CSN	culture supernatant
CT	Computed Tomography
Ct	Cycle threshold
ctrA	*Meningococcal* capsular transfer gene A
Cv	Coefficient of variance
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EAPCRI	European *Aspergillus* PCR Initiative
ECV	Epidemiological cut off value
ELISA	Enzyme linked immunosorbent assay
EORTC	European Organisation for the Research and Treatment of Cancer
ETP	Epipolythiodioxopiperazines
EUCAST	European committee on antimicrobial susceptibility testing

FAM	6-carboxyfluorescein
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM	Galactomannan
GvHD	Graft versus Host Disease
HBE	Human Bronchial Epithelial cells
HEX	hexachlorofluorescein
HNEC	Human nasal epithelial cell
Hr	Hour(s)
HRP	Horseradish peroxidase
IA	Invassive Aspergillosis
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISHAM	The international society for human and animal mycology
ITS	Internal Transcribed Spacer
JAM-A	Junctional adhesion molecule A
kDa	Kilodalton
LB	Luria Burtoni
LD	Lethal dose
LFD	Lateral flow device
LOD	Limit of Detection
LOQ	Limit of Quantification
M	Molar
MBL	Mannose-binding lectin
MEA	Malt Extract Agar
MEC	Minimum effective concentration
MEM	Minimal essential medium
MIN	Minute(s)
MgCl	Magnesium Chloride
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MOI	Multiplicity of Infection
MRSA	Methicillin resistant *Staphylococcus aureus*
MW	Molecular Weight

NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	The National Centre for Biotechnology Information
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP-Af	Non-persistent *A. fumigatus*
P-Af	Persistent *A. fumigatus*
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PTX3	Pentraxin 3
PRR	Pathogen Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction
RAPD	Random amplification of polymorphic DNA
R^2	Pearson Coefficient of Determination
RCSI	Royal College of Surgeons in Ireland
RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay buffer
ROI	Reactive oxygen intermediates
RodA	Rodlet A
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
Sec	Second(s)
S-MEM	supplemented minimal essential medium
SOC	super optimal broth with catabolite repression
SSPD	Sequence specific DNA primers
STR	Short tandem repeat
TARC	Thymus and activation-regulated chemokine
TB	Tuberculosis
TBS	Tris buffered saline
TBST	Tris buffered saline with tween
TER	Trans Epithelial resistance
TET	Tetrachlorofluorescin
TLR	Toll-like Receptor
TNF-α	Tumour necrosis factor-α
Th2	T helper 2 cells
UV	Ultraviolet
V	Volts

VDR	Vitamin D receptor
v/v	Volume per volume
w/v	Weight per volume
X-CGD	X-linked chronic granulomatous disease
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
ZO	Zonula occludens

Chapter 1

Introduction

Chapter 1

Introduction

1.0 *Aspergillus*

Aspergillus is a ubiquitous saprophytic filamentous fungus that survives in a broad range of environmental conditions and is known to be an opportunistic pathogen. *Aspergillus* was first described in 1729 by Pier Antonio Micheli, an Italian priest and biologist [1]. The genus was named *Aspergillus* because of its structural resemblance to an aspergillum (used to sprinkle holy water by Roman Catholic priests) [1]. As a result of this, the presence of the aspergillum spore bearing structure is a defining characteristic of the genus *Aspergillus* [1]. The *Aspergillus* genus comprises of over two hundred species and variants, however only forty species are known to be pathogenic to humans [1] and of those *A. fumigatus* has been reported to be the most virulent of all the *Aspergillus* species [2, 3].

1.0.1 *Aspergillus fumigatus*

A. fumigatus plays an important role in the recycling of nitrogen and carbon in the environment and produces large numbers of small airborne asexual spores called conidia. It is estimated that an individual inhales several hundred conidia on a daily basis [4]. Conidia are 2-3μm in diameter and are therefore small enough to reach the alveoli of the lungs. In immune-competent individuals these conidia are usually cleared effectively by pulmonary innate immune responses [5]. However, in immunocompromised individuals or those with Chronic Lung Diseases (CLD) such as cystic fibrosis (CF), *A. fumigatus* can cause a spectrum of diseases.

1.0.2 Appearance, morphology and identification

Aspergillus has a powdery texture/appearance and *A. fumigatus* colonies are characterised by a blue-green to grey colour (Figure 1.1). *A. fumigatus* has a wide range of thermotolerance, with the ability to grow at temperatures between 20 and 50°C. Therefore the ability to grow at 48 to 50°C aids in its identification [6, 7]. Microscopically all *Aspergillus spp.* demonstrate septate and hyaline hyphae (Figure 1.2), with conidiophores that emerge from the basal foot cell of supporting hyphae and ending in a vesicle at the apex (Figure 1.2). These conidiophores can differ in morphology and colour between *Aspergillus spp.* and thus can aid in species identification. At the apex of the conidiophores the vesicle is covered with phialides from which chains of conidia emerge (Figure 1.2). Vesicles may be entirely covered with phialides (radiate) (Figure 1.2B) or partially covered at the upper surface (columnar) (Figure 1.2A) and this can aid species identification. Furthermore phialides may be attached to the vesicle directly (uniseriate) (Figure 1.2A and 1.2B) or be attached via a supporting cell called metula (biseriate) (Figure 1.2C). The

morphology of the conidiophores taking into account, shape, colour, whether vesicles are radiate / columnar and whether phialides are uniseriate / biseriate can help identify *Aspergillus* to the species level microscopically. *A. fumigatus* has short smooth colourless/ greenish conidiophores with a round columnar vesicle and uniseriate phialides (Figure 1.2A) [4, 8, 9].

In order to fully understand the clinical impact of *A. fumigatus*, it is vital to correctly identify the *fumigatus* species as this is known to be the most virulent [2, 3]. For a long time identification relied on macroscopic and microscopic characteristics. However, more recently identification and quantification of *A. fumigatus* has utilised a combination of morphological characteristics and/or molecular methods, namely PCR. Furthermore while macroscopic and microscopic characteristics can aid in species identification of *Aspergillus, A. fumigatus* can be difficult to distinguish morphologically from some other species of *Aspergillus* [9]. In particular, the recently described *A. lentulus* has a very similar appearance to *A. fumigatus*, with the exception of the poor sporulation associated with *A. lentulus* and therefore identification by molecular methods is more reliable [9, 10]. There are a number of target genes used for molecular identification of *Aspergillus* to the species level including the internal transcribed spacer (*ITS*) [11-13], *β-tubulin* [14, 15] and *calmodulin* [15] [16] regions.

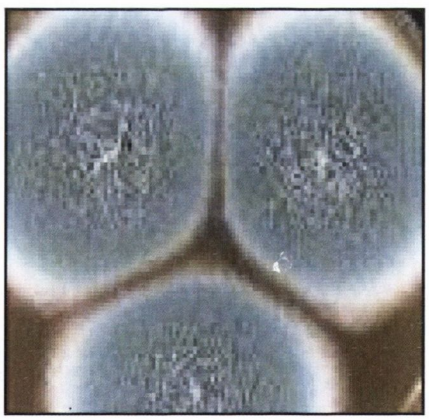


Figure 1.1: *A. fumigatus* macroscopic characteristics

A. fumigatus grown on malt extract agar displaying blue-green to grey colour colonies.

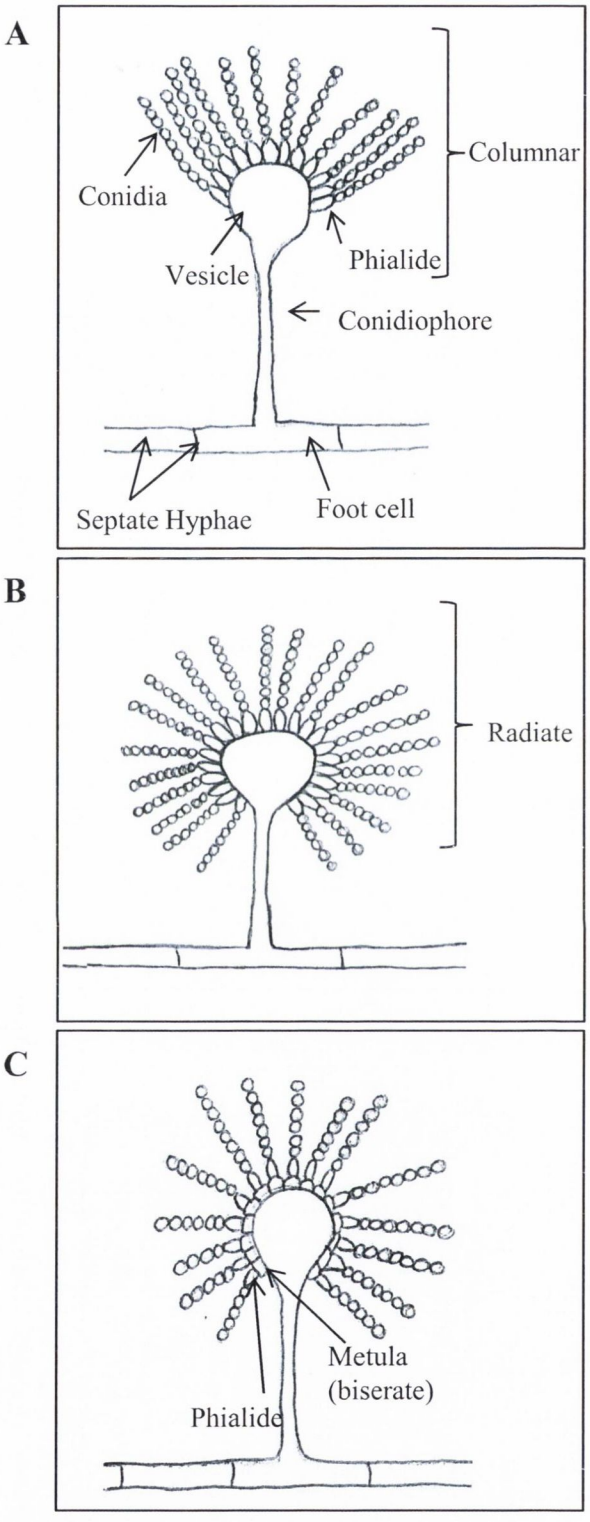


Figure 1.2: Microscopic morphology of *Aspergillus* conidophore

A) *Aspergillus* conidiophores demonstrating columnar head. **B)** *Aspergillus* conidiophores demonstrating radiate head. **C)** *Aspergillus* conidiophores demonstrating phialides attached via metula cells.

1.0.3 *A. fumigatus* conidia

Conidia of *A. fumigatus* are 2-3μm in diameter and their cell wall provides the first line of defence for the organism against its environment. The cell wall of conidia is composed of polysaccharides and proteins which provide a tough exterior [4, 17-19]. The polysaccharides include β-D-glucans (β 1,3-glucans, β 1,3/1,4-glucans, β 1,6-glucans), α 1,3-glucans, chitins and galactomannans (Figure 1.3) [17, 19, 20], with mannoproteins spread on the outer surface of the conidia [20]. Most *A. fumigatus* conidia are hydrophobic and this is due to the presence of a rodlet layer on the surface of the cell wall (Figure 1.3). While the cell wall provides structure and defence for *A. fumigatus*, it also provides targets for recognition by the immune system, for diagnosis and for antifungal therapy. β 1,3-glucans help maintain cell wall integrity, immunomodulation and are required for cellular adhesion. Additionally β 1,3-glucans provides a useful diagnostic marker for *A. fumigatus* and a target for echinocandin antifungal treatment [20]. Galactomannan plays an important role in cell wall integrity and adhesion while also providing a diagnostic marker of *Aspergillus* infection [20].

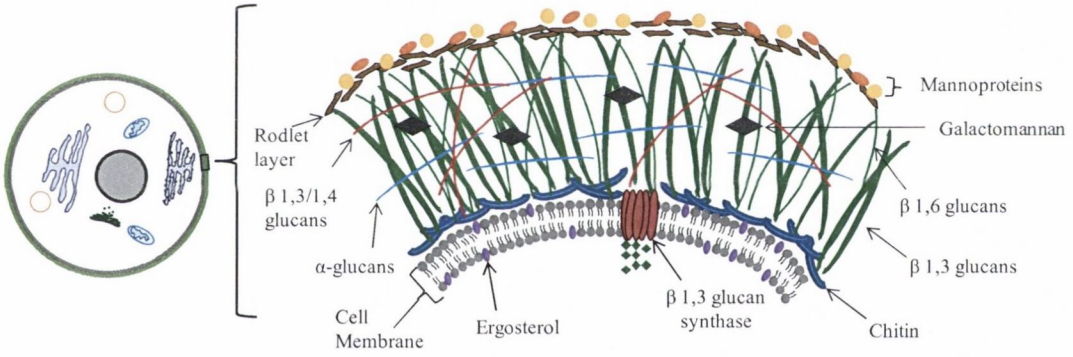


Figure 1.3: *A. fumigatus* conidium cell wall

Cell wall of conidia illustrating the constituent polysaccharides and proteins .

A. fumigatus can grow over a broad range of temperatures and its ability to grow at 37°C contributes to its pathogenic ability, being capable of germinating and growing hyphae within the airways. The growth of *A. fumigatus* begins with the swelling of conidia by up to eight times their size and shedding of their hydrophobic layer in the process [21]. Hyphae bud from the swollen conidia and grow out branching at 45° angles. Hyphae are 7-10μm in size [22] and are associated with invasive aspergillosis. As hyphae grow they release toxins and proteases which can contribute to disease and immune response while also providing diagnostic markers. From hyphae conidiophores emerge and grow from a supporting hyphal foot cell. Conidiophores grow into a conidiophore covered in phialides at the apex (Figure 1.2). From these phialides chains of conidia emerge (Figure 1.2), where they are dispersed in the air and begin the growth cycle again.

1.1 Types of Aspergillosis

A. fumigatus can cause a spectrum of diseases in the form of aspergillosis. Aspergillosis includes all types of *Aspergillus* infection from invasive disease to allergic responses [4]. Aspergillosis may affect many organs but most often begins in the lungs through inhalation of conidia. However cutaneous routes have also been described and following entry *Aspergillus* may then spread to other sites [4, 20, 23]. Most often *A. fumigatus* gains entry through the lungs and the immune status of the patient determines how the infection progresses [4, 18, 20]. While there are a variety of subcategories or types of aspergillosis, *A. fumigatus* is primarily responsible for three disease states; allergic bronchopulmonary aspergillosis (ABPA), invasive aspergillosis (IA) and aspergilloma [4, 5, 18, 20, 22-26].

1.1.1 Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA is due to an allergic response to *Aspergillus*. APBA is generally associated with asthma and cystic fibrosis (CF) patients [21-23, 27, 28] and is also becoming increasingly recognised in chronic obstructive pulmonary disease (COPD) patients [29]. The conidia surface is made of a complex structure mainly composed of polysaccharides (Figure 1.3), with the majority of allergens being released from the cell wall during growth [30]. A number of *A. fumigatus* allergens have been detected to date, namely Asp f1 to Asp f23 [31] [32] [33] [34]. These allergens all appear to promote hypersensitivity and an exaggerated neutrophil and eosinophil response. *A. fumigatus* growth in the trachea and bronchi can evoke both type 1 (formation of IgE) and type 2 hypersensitivity response (formation of IgG) in the host [35-37]. The type 1 response to *A. fumigatus* causes mast cell degranulation with broncho constriction and increased capillary permeability. The type 2 response together with deposits of inflammatory cells within the airway mucous membrane causes necrosis [38]. ABPA is characterised by hypersensitivity that causes an exaggerated allergic Th2-mediated immune response (Figure 1.4) with symptoms that include; wheezing, coughing, shortness of breath, central bronchiectasis, precipitating (IgG) antibodies to *Aspergillus*, elevated *Aspergillus*-specific IgE and elevated total IgE. However some patients may be colonised without displaying symptoms of ABPA and the factors that trigger conversion from asymptomatic colonisation to ABPA are not known (Figure 1.4). There are two treatment aspects for ABPA, one is to control inflammation and hypersensitivity reactions by using oral corticosteroids and the other is to control fungal growth by antifungal drugs.

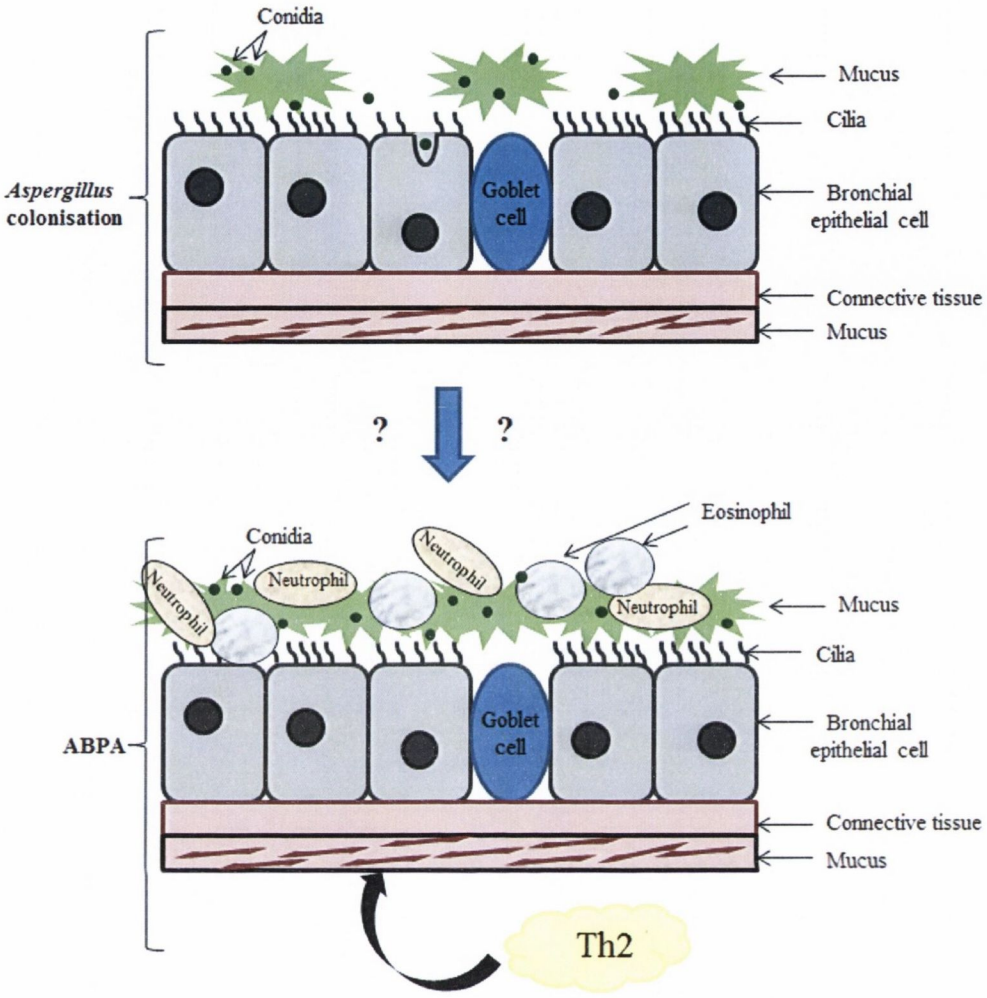


Figure 1.4: *A. fumigatus* conidia and the Respiratory Epithelium

The airways are composed of ciliated bronchial epithelial cells and goblet cells. These cells are tightly bound together and are attached to connective tissue which is surrounded by smooth muscle cells [39]. Inhaled *A. fumigatus* conidia come into contact with the respiratory epithelium on a daily basis. Following inhalation *A. fumigatus* conidia may colonise the epithelium or be cleared effectively by the immune system or by becoming phagocytosed by epithelial cells. In ABPA a Th2-mediated immune response to conidia causes increased mucus production and recruitment of eosinophils and neutrophils from the bloodstream into the airways. This results in inflammation and damage to the airways. It is unclear what triggers the onset of APBA following *A. fumigatus* colonisation

1.1.2 Invasive Aspergillosis (IA)

Invasive aspergillosis is a serious form of aspergillosis most often associated with patients with a compromised immune system, particularly neutropenic patients and has a 90% mortality rate [40]. IA generally begins with colonisation of the lung with *Aspergillus* conidia, which germinate and penetrate the respiratory epithelial barrier and spread to other organs or sites of the body through angioinvasion and haematogenous dissemination. Risk factors associated with IA include, chemotherapy induced neutropenia, solid organ/bone marrow transplant, graft versus host disease, AIDS, primary immunodeficiency or any patients undergoing immunosuppressive therapy [4, 20, 25, 41, 42]. The incidence of IA varies between the patient's underlying condition and geographical location; in Europe the incidence of IA is estimated to occur in 38% of acute myelogenous leukaemia, 70-85% of other immunocompromised patient and 50-60% of organ transplant patients however it is more common in leukaemic patients [43-45]. IA is recognised as the main fungal infection in patients with cancer however the incidence may be underestimated due to low sensitivity of diagnostic tests [4]. Symptoms may include fever, cough, poor response to antibiotics and fatigue [4, 25]. Patients with IA have poor prognosis and diagnosis can be problematic. Radiological abnormalities, microscopy and culture from sputum, serological and molecular diagnostic tests are often used for diagnosis. For patients with cancer IA may be diagnosed as possible, probable or proven according to the European Organisation for the Research and Treatment of Cancer (EORTC) and Mycoses Study Group guidelines [46]. Possible IA includes the following criteria; a host risk factor for IA and a clinical or radiological criterion at a visceral site. Probable IA requires; host risk factors, a major clinical/radiological criterion in a visceral site (for example: pulmonary infiltrate with or without a halo sign, isolated nodule, air crescent sign or cavity) and a direct or indirect microbiological criterion (antigen or mycological) [40]. Finally proven IA requires the following diagnostic criteria; associated host risk factors, histopathological evidence of tissue invasion by filamentous fungi and the isolation of *Aspergillus* species from a sample from a normally sterile site [40]. Early diagnosis and treatment have been correlated to improved outcome [4, 40] and research into new or improved diagnostic tests continues.

1.1.3 Aspergilloma

Aspergilloma also known as a fungal ball, is a hyphal mass of *Aspergillus* found inside scarred lungs or within pre-existing body cavities such as cavities produced as a consequence of tuberculosis (TB) and is probably the least well studied form of aspergillosis. Cavities in the lungs of patients with TB, lung cancer, coccidioidomycosis and histoplasmosis are susceptible to the development of IA [4, 20, 47-50]. Patients with aspergilloma are often asymptomatic, however some patients may present with haemoptysis [4, 47]. Asymptomatic patients are often diagnosed by radiological appearance, sputum culture or serological tests carried out in relation to another

medical issue. Aspergilloma often do not require treatment however it may be provided by antifungal therapy or by surgical excision where required.

1.2 *A. fumigatus* Virulence

Secreted fungal enzymes, proteins and toxins may promote *A. fumigatus* virulence [4, 18, 20]. *A. fumigatus* synthesizes a number metabolites and toxins that are secreted at different stages of growth or in response to stress [34]. Examples of *A. fumigatus* metabolites include ribotoxin, superoxide dismutases, catalases, proteases, phospholipases, hemolysin, fumagillin, sphingofungins, fumitremorgin, verruculogen, fumigaclavine c, helvolic acid and gliotoxin and these can have negative effects on the host and immune response [4, 20, 34]. For example, helvolic acid belongs to a group of secondary metabolites know as fusidanes which act as natural steroid antibiotics. Helvolic acid has been shown to reduce the oxidative burst of macrophages and at elevated concentrations can inhibit the ciliary beat function of epithelial cells which may aid survival of *A. fumigatus* in the host [51, 52]. Fumagillin is a complex biomolecule and acts in the inhibition of endothelial cell proliferation. Fumagillin is reported to be an antimicrobial and inhibitor of angiogenesis due to its anti-tumour antibiotic characteristics [34]. Fallon *et al* demonstrated that exposure of polymorphonuclear neutrophils to fumagillin resulted in the inhibition of the formation of the NADPH oxidase complex and reduced degranulation. These authors suggest that fumagillin might also impact upon neutrophil migration [53].

1.2.1 Gliotoxin

Mycotoxins that bypass the immune system particularly in at risk patients represent an important virulence factor of *A. fumigatus* [54]. The most abundant and also most studied mycotoxin is gliotoxin [4, 55]. Gliotoxin is a hydrophobic low molecular weight toxin and a member of the family of toxins called epipolythiodioxopiperazines (ETP), which are characterised by a quinoid moiety and disulfide bridge across a piperazine ring (Figure 1.5) [4, 55]. The di-sulphide bridge has been shown to be crucial for the damaging activity of gliotoxin [56]. Gliotoxin has been found in the sera of patients with IA [57, 58] and in other patients with *Aspergillus* colonisation only [59]. Gliotoxin is released from the hyphae of *A. fumigatus* and causes many damaging effects on the host and furthermore *A. fumigatus* strains that do not produce gliotoxin are less virulent [60]. Gliotoxin inhibits NADPH oxidase activity which is associated with the neutrophil oxidative burst [61]. Gliotoxin prevents B and T cell activation and has been shown to induce apoptosis in macrophages [62] which may be as a result of the inhibition of the transcription factor NFκB [63]. Additionally, gliotoxin causes negative effects on the respiratory epithelium [59, 64]. Coughlan *et al* showed that gliotoxin caused a down-regulation of the vitamin D receptor (VDR) which reduced Th2 cytokine production and this was observed in both macrophages and respiratory

epithelial cells [59]. The authors reported VDR expression was recovered after a reduction in *A. fumigatus* bioburden as a result of itraconazole treatment [59]. Amitani *et al* demonstrated that gliotoxin caused epithelial damage and slowed ciliary beat frequency in respiratory epithelial cells [64].

There are several genes involved in gliotoxin production by *A. fumigatus* located in the gliotoxin gene cluster and deletions in these genes including *gliZ* [65] , *gliP* [66], *gliG* [67], *gliK* [68] and *gliT* [65, 69-71] have been shown to eliminate gliotoxin production. In addition to its role in gliotoxin production, *gliT* also functions in mediating *A. fumigatus* self-resistance against gliotoxin [65, 69-71]. It has been suggested that this self-resistance is dependent on *gliT* maintaining gliotoxin's di-sulphide bridge structure and the di-sulphide bridge has been suggested to be a requirement for gliotoxin production in *A. fumigatus* [70]. Interestingly exposure of *A. fumigatus* to exogenous gliotoxin resulted in significant changes in *A. fumigatus* protein expression including up regulation of the allergen Asp f3 [72]. However, virulence tests in mice have shown that deletion of the gene *gli*P or *gli*Z which block gliotoxin production did not affect virulence suggesting that gliotoxin might not be important for virulence in every host [73]. Gliotoxin has been reported to be an important factor for virulence, particularly in the immunosupressed host [74]. In support of this, Orciuolo *et al* reported that methylprednisolone and the production of gliotoxin from *A. fumigatus* lead to an inflammatory reaction, along with impairment of T cell function and development of IA, and consequently an increase in morbidity [75]. The full impact of gliotoxin on the host is still not fully understood and its full arsenal of negative effects on the host, are a hot topic of research.

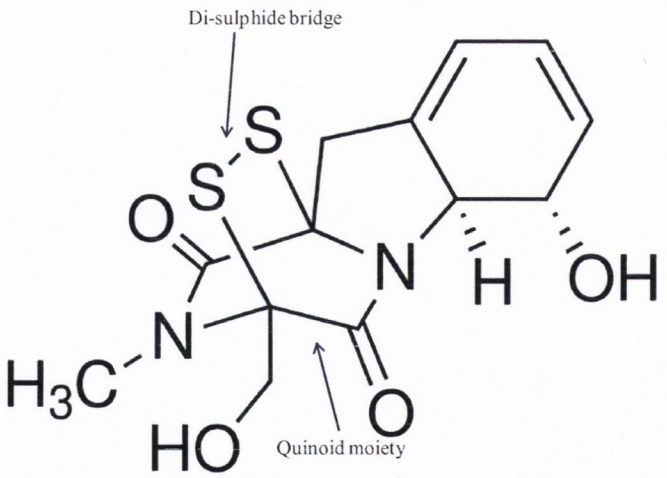


Figure 1.5: Structure of gliotoxin

Chemical structure of gliotoxin, illustrating a quinoid moiety and disulfide bridge across a piperazine ring.

1.2.2 Secreted Fungal Proteases

Secreted fungal proteases may promote *A. fumigatus* virulence resulting in invasive hyphal growth in the lung [76]. *A. fumigatus* secretes a range of proteases such as serine protease (ALP1) [77, 78] and an aspartic protease (PEP1) [79] and other extracellular hydrolases such as lipases, phosphatases and glycosyl hydrolases that are able to degrade macromolecules which are used as nutrients for growth [80]. A number of studies have suggested that the extracellular proteases play a major role in the pathogenicity of the *A. fumigatus*, along with other endoproteases (including alkaline protease, metalloprotease and aspartic protease), which have also been identified [76]. Alkaline serine protease enables epithelium disruption and further fungal colonisation within the airways [81]. APL1, an alkaline serine protease primarily produced by *A. fumigatus* [82], has been shown to be secreted from the apex of the hyphae during infection [83] and degrades collagen, casein, fibrinogen and elastin [30]. The aspartic protease, PEP1, is secreted by the fungal germinal tube [84] and may assist in tissue invasion but it is also thought to act as an allergen of ABPA [85]. However mutant strains deficient in PEP1 have similar virulence to the wild type questioning whether this protease plays a role in tissue invasion. A number of metalloproteases of *A. fumigatus* have been identified including a metalloprotease which was isolated from an alkaline protease deficient mutant and demonstrated considerable proteolytic activity on epithelial cells [86]. A metalloprotease named mep20 which shares 68% sequence similarity to a metalloprotease of *A. flavus* has also been identified [87]. Furthermore an intracellular metalloprotease thought to be associated with small cytoplasmic peptide degradation [88] and a 43 kDa extracellular elastolytic metalloprotease [89] from *A. fumigatus* have also been identified. The extracellular elastolytic metalloprotease possesses the conserved motif (IV-I-H-E-Y-T-H-G-L-S) of the zinc metalloprotease superfamily [90, 91]. Considering the high amount of zinc in biological systems and the high protein composition of host cells, this 43 kDa meltalloprotease has been implicated in host tissue invasion [76, 92].

Proteases target the lung tissue, allowing *A. fumigatus* to invade the tissue causing cellular damage that aids fungal invasion [93]. Proteases also help the spread of allergens through epithelial cells and the production of inflammatory chemokines and cytokines providing conditions for potential initiation of ABPA [94-96]. However there remains no evidence so far that loss of specific protease genes impacts on *A. fumigatus* virulence. Single *ALP, PEP* or *MEP* mutants and a double *ALP, PEP* mutant were not shown to have attenuated virulence in mice [84-86, 97] however it is possible that loss of one protease activity may be compensated by others [4].

1.2.3 Adhesins

There are a number of hypotheses as to how *A. fumigatus* may evade host defences. It has been shown that conidia have structural features which provide sites for development of resistance to antifungal mechanisms of the host and/or the immune system [98]. A key step in colonisation and invasion is the ability of the organism to adhere to the host cells or tissue. *A. fumigatus* may produce several adhesins which may aid in adherence to host tissues. *A. fumigatus* has been shown to bind to respiratory epithelial cells *in vitro*, where they may colonise the airways [39]. These fungal adhesin molecules interact with adhesion components of the host tissue including human complement, fibrinogen and fibronectin and these provide a route of interaction to allow adherence to host tissues [62]. The fungal adhesin AfCalAp, which is present on *A. fumigatus* conidia, was observed to have significant binding to laminin and pulmonary cells [99]. AfCalAp has been to shown to act an allergen, with elevated immunoglobulin E (IgE) reactivity in ABPA sera [99]. Additionally, conidia have been shown to bind and degrade laminin, an extracellular matrix glycoprotein present within basement membranes [62].

1.2.4 Allergens

The conidia surface is made of a complex structure mainly composed of polysaccharides (Figure 1.3), with the majority of allergens being released from the cell wall during growth [30]. A number of *A. fumigatus* allergens have been detected to date, namely Asp f1 to Asp f23 [31] [32] [33] [34]. These allergens all appear to promote a Th-2 mediated hypersensitivity which occurs in ABPA.

1.2.5 Biofilms

Once adhered to the host cells or tissue *A. fumigatus* may be capable of producing a biofilm. A biofilm is where microorganisms bind together on a surface and grow together. Biofilms provide an environment in which bacteria, yeast or fungi can persist in infections and this can contribute to antimicrobial resistance. *A. fumigatus* has been shown to produce an antimicrobial protective biofilm on the surface of human bronchial epithelial cells (HBE) and CF bronchial epithelial cells (CFBE) *in vitro* [100]. Biofilms are often observed as parallel-packed hyphae that run as crosslinking threads which provide a strong biofilm structure. Adherence is crucial for biofilm development and structure; a protein called medA which is produced by the hyphae of *A. fumigatus* has been shown to be important for biofilm formation, since *A. fumigatus* deficient medA strains demonstrated impaired biofilm production [101]. Deletion of medA also resulted in reduced fungal adherence to pulmonary epithelial cells, decreased stimulation of pro-inflammatory cytokines and a diminished fungal burden *in vitro* [101].

1.3 Interactions between *A. fumigatus* and the host immune responses

Several hundred conidia are inhaled on a daily basis (Figure 1.6). The human airway provides a physical barrier to infection and plays a major role in pathogen recognition and innate and adaptive immune responses [102]. Innate immune responses include three areas of defence, (1) anatomical barriers, (2) humoral factors and (3) phagocytic cells and other related microbial products such as cytokines and the complement system [4]. *A. fumigatus* conidia enter the airways through inhalation and are small enough (2-3μm) to reach the alveoli (Figure 1.6).

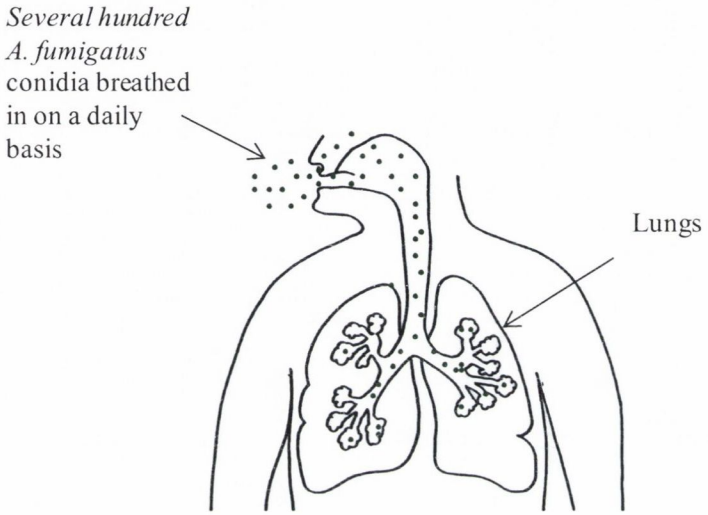


Figure 1.6: Inhalation of airborne *A. fumigatus* conidia

Several hundred conidia are inhaled into the lungs where they are small enough to reach the alveoli.

Here the conidia will meet with a number of anatomical barriers. Airway epithelial cells are ciliated and covered in mucus which both function in clearing the lungs of inhaled microbes [103]. *A. fumigatus* can combat these efforts by releasing toxic molecules for example gliotoxin that inhibit ciliary function [104], and proteases for example elastase that damage the epithelium [104]. Defects in muco-ciliary function are associated with a number of chronic lung diseases [105]. In order to keep any inhaled microorganisms contained within the lung and to prevent invasion to other areas of the body, epithelial cells of the airways are tightly bound together. These tight junctions regulate ions, water and immune cell transport between epithelial cells of the airways and aim to prevent microbial translocation (Figure 1.8) [106]. Mucociliary clearance and tight junctions are an effective method of clearance of and maintenance of conidia within the lungs. The most important mechanisms for the elimination of *A. fumigatus* is most likely phagocytic clearance by alveolar macrophages and neutrophils [73].

Pathogen-Associated Molecular Patterns (PAMPs) on the surface of *A. fumigatus* conidia and hyphae are recognised initially by the immune system through a number of Pathogen Recognition Receptors (PRRs) located on alveolar macrophages, endothelial cells, lymphocytes, immature dendritic cells and mucosal airway epithelial cells [107] and include; Toll-like receptors TLR2 [108], TLR4 [108], Mannose-binding lectin (MBL) and Dectin-1 [108] [109]. Additionally, leukocytes recognise *A. fumigatus* PAMPs such as Beta-D-glucan. Present on the surface of fungi, PAMPs motifs are recognised by host PRRs. As already mentioned phagocytic cells play an important role in erradicating *A. fumigatus* from the airways. Alveolar macrophages are the predominant resident phagocytes in the lung [73]. Alveolar macrophages have been shown to effectively ingest and subsequently kill conidia following the acidification of the phagolysosome and release of reactive oxygen intermediates (ROIs) [110, 111]. However, the ingestion and killing of conidia by macrophages may be slow and appears to be dependent on the swelling of conidia [112]. Philippe *et al* demonstrated the importance of ROIs produced by NADPH oxidase and nitric oxide synthase activity of alveolar macrophages [110]. Notably it has been reported that corticosteroid therapy inhibited alveolar macrophage induces ROI production which prevents conidial killing and hence aids in fungal survival [111]. Furthermore, the rodlet layer on the surface of conidia has been reported in delaying the immune recognition of *A. fumigatus* conidia by dendritic cells and CD4+ T lymphocytes. While immune recognition of conidia might be somewhat weak, germination of conidia and growth of hyphae induces a much stronger immunological response resulting in the recruitment of neutrophils to the site of infection.

Neutrophils circulating in the blood stream are recruited to the airways upon infection and target *A. fumigatus* conidia and hyphae [113]. Neutrophils may kill conidia by phagocytosis and degranulation. Neutrophils adhere to the surface of hyphae, which are too large to be internalized and rapidly kill them via a respiratory burst, neutrophil degranulation, and the release of ROIs [113, 114]. Therefore neutrophils provide another level of defence employed by the immune system to prevent IA and patients with neutropenia show a strong clinical correlation with an increased incidence of IA [115]. These innate immunity mechanisms appear to be sufficient to prevent disease caused by *A. fumigatus*, as demonstrated by mice lacking adaptive immunity who did not show higher susceptibility to invasive aspergillosis [73]. However, adaptive responses work to provide further protection from infection. This was demonstrated by Cenci *et al* and Kurup *et al* who showed that the induction of a Th1 CD4+ lymphocyte response is protective in animal studies of IA [35, 116]. *A. fumigatus* may evade or reduce neutrophil response with the release of gliotoxin which has been shown to impede neutrophil migration to the site of infection [115]. Additionally the *A. fumigatus* toxin fumagillin is able to reduce neutrophil degranulation and inhibit the formation of the NADPH oxidase complex [53]. Surfactant proteins A and D found in the lung surfactant have been found to enhance phagocytosis and killing of conidia by alveolar

macrophages and neutrophils [117]. Pentraxin 3 (PTX3) represents another line of defence against *A. fumigatus*. PTX3 is produced and released by a number of cell types including dendritic cells, fibroblast, phagocytes and endothelial cells in response to inflammatory signals for example TLR activation [118]. Once released it may activate complement and also bind to *A. fumigatus* facilitating pathogen recognition by macrophages and dendritic cells [118]. MBL represents an important immune factor that may be found in the upper airways and buccal cavity and may migrate to the alveolar spaces during inflammation [119]. MBL acts as a PRR where it may bind to *A. fumigatus* and activate complement via the lectin pathway [119], thus providing a defence against *A. fumigatus* in both the upper and lower airways. In an attempt to evade these immune responses *A. fumigatus* releases a number of molecules such as gliotoxin which can inhibit phagocytosis [75], DHN-melanin which can cause a reduction of complement and neutrophil activation [20] and polyketide synthase which confers protection against phagocytosis [120].

The immune system's different responses to *A. fumigatus* conidia or hyphae play an important role in the regulation of inflammatory responses in the host. While a strong immunological response to inhaled conidia would lead to undesirable chronic inflammation, the presence of hyphae indicates a failure to control conidial germination which requires the initiation of a robust proinflammatory response to prevent uncontrolled mycelial growth and development of IA. Immune dysregulation or dysfunction which may cause either an over-stimulated activation or insufficient control of fungal growth may result in significant damage to the host, leading to its common classification as both an allergen and an opportunistic pathogen [121].

In addition to muco-ciliary and immune response clearance of *A. fumigatus*, the conidia may become internalised by epithelial cells or hyphae may penetrate the cell [122]. Internalisation of conidia into epithelial cells may not necessarily kill the conidia, with some remaining viable for nearly 6 hours *in vitro* [122]. Although internalisation of conidia by epithelial cells may be effective at reducing conidia numbers not all are killed and those that remain viable are able to break through the membrane and escape the cell [122]. There have been a number of studies investigating the interaction of *A. fumigatus* with various immune cells [21]. However, there is little research focusing on the direct interaction of *A. fumigatus* with lung epithelial cells [21]. Whether dealing with asymptomatic colonisation, ABPA or IA, the initial point of contact between *A. fumigatus* and the host is almost always the monolayer of epithelial cells in the lung [21]. Therefore, clarifying how these cells react and interact with fungal contact is crucial to our understanding of *Aspergillus* related diseases.

Some studies have been performed on the interaction of live *A. fumigatus* and bronchial epithelial cells; they found that approximately 20-50% of adherent conidia are internalised into the

bronchial epithelial cells where they can remain viable for up to 20 hours thus serving as a possible reservoir of infection (Figure 1.7) [25]. Internalisation of conidia into endosomes of the epithelial cells, are fused via actin to form an acidic phagosome [39, 123]. Those conidia that remain externally adhered to the cell via dectin-1 may germinate and or penetrate or cause damage to the cell, while stimulating release of MyD88 and activation of the NFκB pathway or P13 kinase or MAP kinase pathway which all in turn cause the release of chemokines, cytokines, defensins and causing degranulation (Figure 1.7) [5, 25, 39]. Hope *et al.* recently developed a credible *in vitro* model, consisting of a cellular bilayer system constructed with human alveolar epithelial cells and human pulmonary endothelial cells grown on either side of a semi-permeable polyester membrane with the option to include immune cells [24].

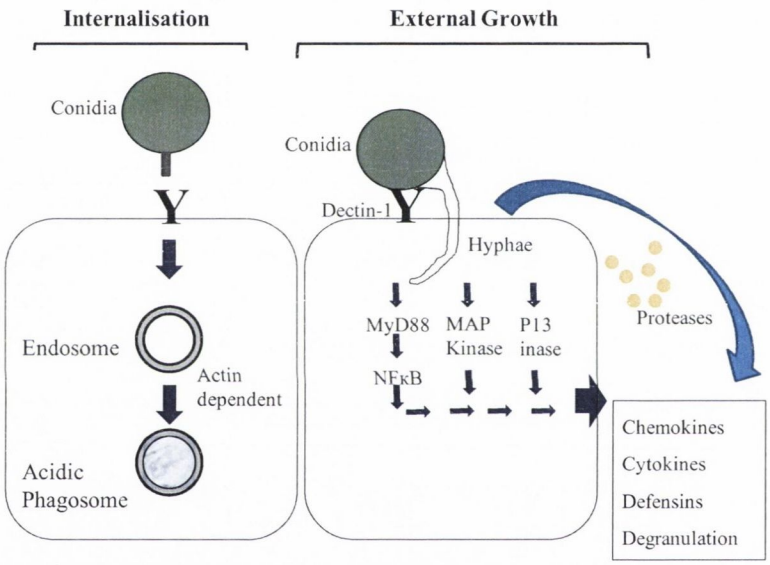


Figure 1.7: Interaction of *A. fumigatus* conidia and hyphae with epithelial cells
Conidia may be internalised into an endosome which fuses with the use of actin to form an acidic phagosome. Alternatively conidia may adhere to bronchial epithelial cells and germinate where hyphae may then penetrate the cell causing a release of cytokines, chemokines and defensins. Adapted from Osherov review [39].

While a limited amount of research has been published on the interaction of *A. fumigatus* and the lung epithelium, remarkably there are only a handful of publications investigating the effect of *A. fumigatus* on the tight junctions of the lung epithelium. The upper airway epithelium acts as the first physical barrier that protects against inhaled pathogens. Epithelial cells form tight junctions providing a highly regulated and impermeable barrier [27]. These tight junctions regulate ions, water and immune cell transport between epithelial cells of the airways and aim to prevent microbial translocation (Figure 1.8) [106]. Recent evidence suggests that tight junctions also play a part in signal transduction pathways that regulate epithelial cell proliferation, gene expression,

differentiation, and morphogenesis [28]. Considering *A. fumigatus* comes into contact with the tight junctions of the lung epithelium on a regular basis, this area requires further investigation as to what signal mechanisms may be triggered and how this may contribute to inflammation. Wan *et al.* demonstrated that the allergens Der p1 and Der p9, both serine proteases, from the house dustmite *Dermatophagoides pteronyssinus* allowed transepithelial migration of allergens by disrupting the epithelial tight junctions. This disruption and transepithelial delivery of allergens led to an enhanced release of pro-inflammatory cytokines from immortalised and primary bronchial epithelial cells [29]. In light of the fact that several hundred conidia are inhaled on a daily basis and *A. fumigatus* is found in up to 80% of CF patient respiratory samples at one time or another [59, 124-126] , an understanding of the effect of *A. fumigatus* on the tight junction of the lung epithelium may provide more insight into *Aspergillus* infections or initiation of inflammation. Failing clearance of *A. fumigatus*, the conidia remain in the lung and alveoli of the lungs. Once in the alveoli and having evaded the immune defence mechanisms, conidia may germinate producing hyphae which may penetrate the epithelial cells and disrupt the tight junctions gaining access to the vascular system where the hyphae and conidia can travel to blood vessels that transport oxygen and carbohydrates. From this point *A. fumigatus* may disseminate to other areas of the body.

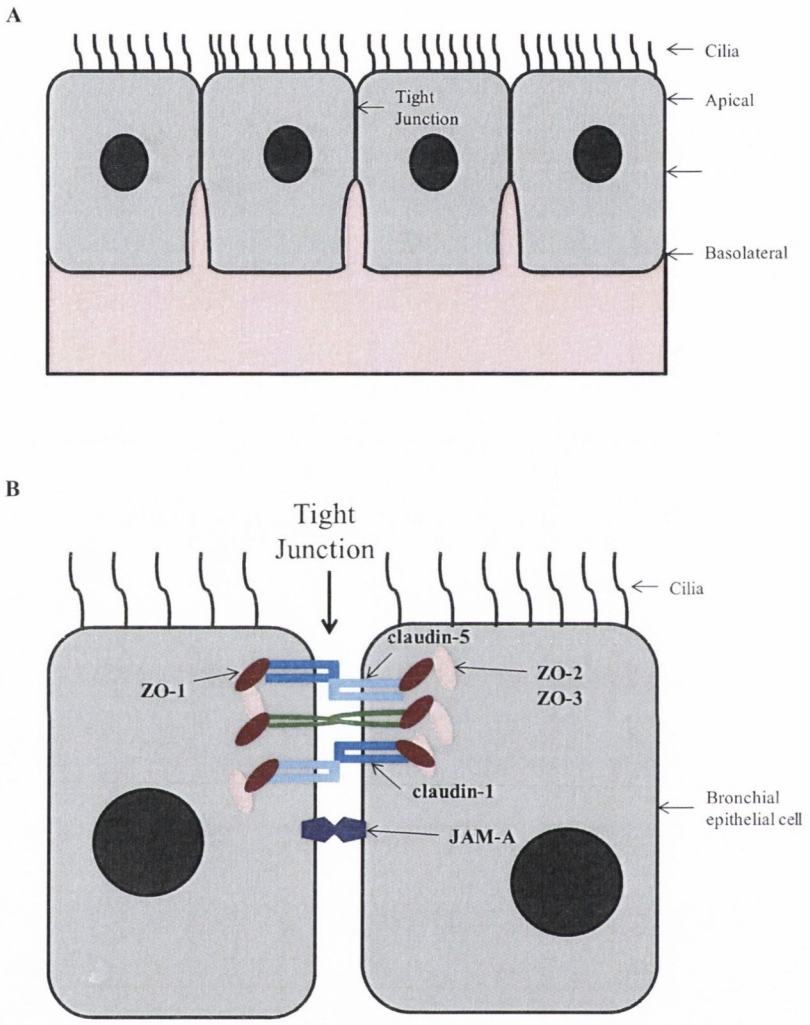


Figure 1.8: The respiratory epithelial barrier and the tight junctions

Ciliated bronchial epithelial cells are tightly bound together by tight junctions. These tight junctions are held together by tight junction proteins (claudins, occludins, zonula occludens (ZO) and junctional adhesion molecules (JAM)) proteins). These proteins regulate tight junctions and transport of water and immune cells and aim to prevent microbial translocation from the lungs.

1.4 Diagnosis of *A. fumigatus*

Diagnosis of aspergillosis often uses a combination of investigative and diagnostic tests. Diagnosis most often utilises computed tomography (CT) scanning or X-ray. Microscopy and culture of clinical samples and /or antigen detection on blood or respiratory fluid are often utilised. Sensitivity to *Aspergillus* may be tested using the skin prick test [127]. Skin prick testing involves directly applying to the skin a commercially prepared application of a solution that detects the presence of *Aspergillus* specific IgE [127].

1.4.1 Detection of *A. fumigatus* by Microscopy and culture methods

Aspergillus may be detected and examined by microscopic analysis and culture of clinical samples. A small proportion of a clinical sample is plated usually in triplicate onto/into a suitable growth medium for *Aspergillus* (malt extract agar/broth, Sabouraud dextrose agar/broth, potato dextrose agar/broth, czapeck Yeast extract agar/broth) and incubated at 25 to 37°C for 1 to 7 days [59, 128-130]. Incubation at 48°C may be used for culture of *A. fumigatus* [6, 7]. Following successful culture of *Aspergillus*, species determination may be made based on macroscopic and microscopic characteristics as outlined in section 1.02. *Aspergillus* bioburden may be assessed by enumeration of colony forming units (CFUs) allowing subsequent calculations to be made to account for the number of CFUs per ml or gram of clinical specimen [59, 130]. Considering *Aspergillus* conidia may be suspended in the air, culturing and handling of clinical samples should ideally be performed in a laminar air flow cabinet to prevent false positive results due to environmental contamination.

1.4.2 Polymerase Chain Reaction (PCR) detection of *A. fumigatus*

Detection and quantification of *A. fumigatus* from patient samples by polymerase chain reaction (PCR), particularly quantitative real time PCR (qPCR), has been shown to be a reliable method of detection. *A. fumigatus* may be detected by qPCR particularly in situations where samples fail to culture *A. fumigatus*. Lack of a positive culture is not always indicative of a sample being negative for *A. fumigatus* and this may be due to the small amount of sample plated for culture [130]. Conversely, DNA extraction from the whole sample or large proportion of the sample followed by qPCR provides the opportunity to test the sample more thoroughly. However it should be kept in mind that qPCR will detect both live and dead *A. fumigatus* DNA. As with culture protocols, handling and processing of clinical samples for DNA extraction and PCR should be carried out in laminar air flow cabinets to prevent false positives by PCR as a result of environmental contamination. Furthermore DNA extractions and PCR should always include a negative control to ensure no reagent contamination. While a number of centres now avail of qPCR for detection of *A. fumigatus* in a variety of types of patient samples (sputum [59, 130], bronchoalveolar liquid (BAL) [131, 132], whole blood [133-135] and serum [136]), there remains

no one gold standard method for either the DNA extraction or qPCR protocols [59, 130, 135, 137-139].

1.4.2.1 European *Aspergillus* PCR Initiative (EAPCRI)

The international society for human and animal mycology (ISHAM) working group called EAPCRI was founded in 2006 by a group of mainly European experts from over 60 centres. This working group was established to research and propose a standardised protocol for *Aspergillus* PCR [131, 133, 140]. When using PCR as for a method of diagnosis, a number of methodological conditions need to be considered. Firstly the type of clinical specimen (sputum, BAL, whole blood, serum) used can affect the ability to reliably extract DNA, particularly with regard to whole blood or serum [135, 136, 140, 141]. For example DNA extraction from whole blood requires additional steps, including red blood cell lysis and white blood cell lysis [135]. Haemoglobin found in red blood cells is known to cause PCR inhibition therefore this must be removed through addition of a red cell lysis step [135]. Furthermore a suitable quantity of sample to be used as starting material for DNA extraction is undefined. The next step for consideration is DNA extraction method which in itself has a number of options to choose from; type of extraction kit and method [137], method of lysis (mechanical (sonication, bead beating, freeze-crushing) or enzymatic)[137]. The DNA extraction also requires other considerations such as sample volume for extraction and volume of elution buffer for final elution of DNA. Once DNA has been extracted the selection of suitable target sequences (*ITS, 28S, 18S*) must be chosen and additionally from this primer and probe sequences must be chosen. Furthermore use of qPCR buffers provides yet another element for consideration. Finally the instrument for qPCR analysis and the model of instrument may introduce bias in results achieved (applied biosystem, Roche, Bio-rad, Thermo scientific). Considering all the methods, reagents and platforms available which have all been published in peer reviewed journals, the choice for one standardised method for PCR from start to finish, requires due consideration. While some of these methods have been published, only a comparison of all methods within one standardised trial would allow accurate comparison of each method. EAPCRI was established for this very purpose to test and compare numerous methods among different centres with the aim of defining one standardised method for DNA extraction and PCR that provides the most accurate and sensitive method for PCR detection and/ or quantification of *Aspergillus*. White *et al* published the first multi-centre trial comparing PCR targeting the *18S* and *28S* region and three different PCR instruments [11]. The authors compared results from ten different centres and concluded that the *28S* region was more sensitive than the *18S* region [11]. EAPCRI has to date performed a number of multi-centre trials testing multiple DNA extraction and PCR methods and still continue research into the topic [11, 135, 136, 140, 142]. Their findings so far have provided a wealth of information. It appears that within the whole PCR method from start to finish the step most likely to bias results is the DNA

extraction process [140]. Furthermore from a number of multi-centre trials, it has been found that mechanical lysis was superior to enzymatic lysis [130, 137, 140]. The group has also found that larger volumes of whole blood (≥3ml) are more effective than smaller volumes (0.2-1ml) [140]. Finally EAPCRI have reported that regardless of the assay used, real-time PCR is superior to standard PCR, which is unsurprising considering real-time PCR is capable of detecting very low quantities of DNA and can detect and report quantities of fungal DNA within a couple of hours.

1.4.3 Lateral Flow Device (LFD)

The LFD was recently released as a new fast detection system for aspergillosis diagnosis. The LFD is a pregnancy test like device (Figure 1.9) with an antibody raised against a protein epitope on an N-linked glycoprotein antigen present in the hyphal cell wall and septa of *A. fumigatus* [143]. It allows testing of both serological samples and BAL and can be used as a point of care test for aspergillosis [143]. Specificity tests showed that the LFD reacted strongly with antigens from species of the genus *Aspergillus* and the closely related species *Eurotium* [143].

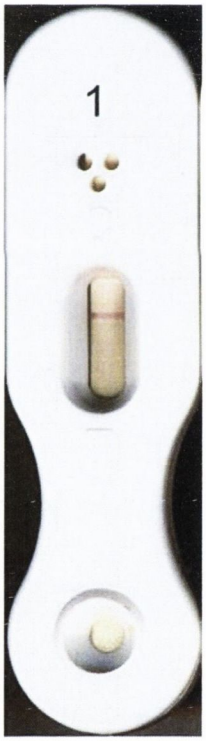


Figure 1.9: Later flow Device for point of care diagnosis of aspergillosis
Image from Thornton publication [143]

1.4.4 β – D- glucan

The cell wall of conidia is composed of β 1, 3- glucan and this has been utilised as a diagnostic marker in both blood and BAL samples [144]. This allows a fast and non-culture based method for the detection of *Aspergillus* and is most often used in the diagnosis of IA.

1.4.5 Galactomanan

Galactomannan (GM) is found in the cell wall of *Aspergillus* and is released during invasive disease. Due to this GM can be detected in blood, cerebrospinal fluid (CSF) [145] and respiratory fluid [132] where it has proved useful as a confirmatory test for infection. GM is most often used in diagnosis by GM ELISA [144] and a number of studies have reported excellent sensitivities and specificity for the test [146-148].

1.5 Cystic Fibrosis

CF is a fatal hereditary autosomal recessive genetic disorder that effects the exocrine (mucus) glands due to a mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene, located on chromosome 7 [149]. CF is the most common life-threatening inherited disease in Ireland [150] with 1 in 19 people carrying a CF causing mutation [150]. Ireland has the highest incidence of CF in the world (1 in 1461) [151] and worldwide the incidence of CF varies from country to country, with an incidence of 1 in 2000-3000 in Europe and 1 in 2000-3500 in the USA [152]. CF affects many organ systems but mainly the respiratory and digestive systems (including the liver and pancreas). The mutation in the *CFTR* gene is characterised by a defect in sodium chloride ion channels. In the airways this *CFTR* mutation causes decreased chloride secretions along with increased sodium absorption at the apical membrane of the airway epithelial cells leading to thick viscous secretions [107, 153, 154]. The altered sodium chloride transport also gives rise to decreased airway surface liquid which causes an altered mucociliary clearance. This impaired mucociliary clearance and viscous mucus in the lungs creates a perfect environment for microorganisms to colonise and persist, making CF patients prone to chronic airway infections by bacteria and fungi [107, 153]. Lung damage and lung failure account for the highest rate of morbidity and mortality in CF patients [155]. Between 2002-2013 82% of Irish CF patient deaths were due to respiratory/cardiac failure [151]. There are over 1000 *CFTR* mutations described and mutation are categorised into six classes (based on the assembly of the *CFTR* due to the mutation phenotype). The $delF_{508}$ *CFTR* mutation is by far the most common *CFTR* mutation worldwide including Ireland and is categorised as class II. The class II mutations account for the largest proportion of *CFTR* mutations (70-80%) [156]. The $delF_{508}$ mutation is caused by a deletion of a single phenylalanine at position 508 and CF patients who are homozygous for $delF_{508}$ ($delF_{508}$ /$delF_{508}$) have a more severe form of the disease including more frequent respiratory infections, an early onset of pancreatic insufficiency and decline in lung function [157]. The classes of *CFTR* mutations are listed below in table 1.1 [158-161].

Table 1.1: The six classes of *CFTR* mutations

***CFTR* mutation class**	
I	Lack of *CFTR* protein production
II	Defective *CFTR* protein processing
III	Defective regulation of *CFTR* protein
IV	Reduced chloride transport through *CFTR* protein
V	Reduced production of *CFTR* protein
VI	Defective stability of *CFTR* protein at cell surfaces, leading to increased turnover of *CFTR*

1.5.1 Prognosis

The prognosis for people with CF has greatly improved over the years. While in the 1950's children with CF were generally not expected to survive long enough to begin school, today the median predicted age of survival for people with CF is in their early 40's [162]. However the predicted mean age of survival in people with CF varies from country to country particularly between developed and underdeveloped countries. For example the predicted mean age of survival in people with CF is mid 30's to 40 years old in the USA, Europe and Ireland while in El Salvador, India and Bulgaria life expectancy for people with CF can drop to as low as 15 years old [151, 163]. The recent release of a drug called Kalydeco/Ivacaftor in 2012 which helps the defective *CFTR* protein work at the surface of the cell will certainly lead to an improved life expectancy in people with CF. This drug is only applicable to CF patients who have *CFTR* protein expression at the cell surface, namely CF patients with the following *CFTR* mutations; G551D (the second most common CFTR mutation), G178R, S549N, S549R, G551S, G1244E, S1251N, S1255P and G1349D [162]. However studies are now investigating whether this drug may have a beneficial effect on CF patients with other *CFTR* mutations [164, 165].

1.5.2 *Aspergillus* in CF

In CF, *Aspergillus* is the most commonly isolated fungal pathogen and is isolated from 6-80% of CF patients at one time or another [59, 124-126]. *Aspergillus* and especially *A. fumigatus* most commonly causes Allergic Bronchopulmonary Aspergillosis (ABPA) [27]. However, many CF patients are found to be colonised with *Aspergillus* without displaying symptoms of ABPA [59, 166]. This group of patients has been poorly studied and the clinical significance of *A. fumigatus* colonisation in the absence of ABPA symptoms remains unclear.

1.5.2.1 ABPA in CF

ABPA effects approximately 7-9% of CF patients [27]. Hemmamm *et al* reported that ABPA patients and those patients sensitive to *Aspergillus* show elevated IgE antibodies to recombinant *Aspergillus* allergens Asp f1, Asp f3, Asp f4 and Asp f6. It has also been reported that thymus and activation-regulated chemokine (TARC), a chemokine that has a ligand on CD4 Th2 cells was elevated in CF patients with ABPA and became further elevated during acute exacerbations of ABPA [167]. In addition to allergens *Aspergillus* produces secondary metabolites, proteins and proteolytic enzymes that contribute to epithelial damage and a subsequent inflammatory response and this has been suggested to cause additional harm to the epithelial cell layer [168]. In CF, ABPA can be difficult to diagnose due to a number of overlapping symptoms between the two diseases. While some patients may become sensitised to *Aspergillus* and develop ABPA, others may be asymptomatically colonised by the fungus. It is unknown why some patients remain chronically colonised without developing ABPA or what factors contribute to the development from asymptomatic colonisation to ABPA.

1.5.2.2 Diagnosis of ABPA in CF

The most up-to-date CF Foundation Consensus Conference outlined the following diagnostic criteria of ABPA; 1) acute or sub acute pulmonary deterioration not attributable to another etiology, 2) total serum IgE >1000 IU/mL, 3) immediate cutaneous reactivity to *Aspergillus* or *in vitro* specific IgE antibodies to *Aspergillus*, and 4) one of the following: *Aspergillus* serum precipitins, elevated specific IgG anti-*Aspergillus* antibodies, new or recent chest radiographic, or chest CT abnormalities that have not cleared with antibiotics and chest physiotherapy [21]. Culture of *Aspergillus* is only a secondary diagnostic criterion for ABPA as many CF patients may be colonised by the fungus in the absence of ABPA [21]. Failure to diagnose or treat ABPA may lead to airway deterioration, bronchiectasis and/or pulmonary fibrosis resulting in significant morbidity and mortality [169].

1.5.2.3 *A. fumigatus* colonisation in CF

Aspergillus colonisation rates vary between different CF centres although a number of studies have reported *Aspergillus* in approximately 6-80% of CF lung cultures [23, 59, 126]. The range in incidence (6-80%) depends on methods of detection across centres. However, several publications have demonstrated that the frequency of *A. fumigatus* isolation from CF sputum does not correlate with rates of ABPA [23, 24, 166]. A subset of asymptomatically colonised CF patients will go on to develop ABPA. In the proportion of patients that continue to be colonised in the absence of ABPA, it is unclear what impact this may have on the patient and lung function. Asymptomatic colonisation has be shown to increase hospitalisations due to pulmonary exacerbations not associated with ABPA [24, 166] and worsen radiological appearances despite

minimal effect on lung function [166]. Despite these findings, there have been few publications focusing on colonisation in the absence of ABPA. Physicians differ in their treatment of this CF patient group with some prescribing antifungal drug therapy for *Aspergillus* colonisation and others only prescribing when ABPA symptoms present. It remains unclear whether treating CF patients that are asymptomatically colonised with *A. fumigatus* would have health benefits for the patient.

1.5.2.4 Diagnosis and Detection of *A. fumigatus* in CF

Diagnosis may be made by a skin prick test or more often *A. fumigatus* is diagnosed by examination of sputum by microscopy, culture methods and/ or molecular methods as outlined in section 1.4.

1.6 Treatment of *Aspergillus*

There are three main classes of antifungal drugs; polyenes, azoles and echinocandins (Table 1.2). At risk patients may be treated with the aim of avoiding *Aspergillus* infection and this is referred to as prophylaxis treatment; however antifungal drugs are often associated with a number of toxic side effects and are high in cost.

Table 1.2: List of antifungals used against *Aspergillus*

Class of antifungal	Name	Formulation
Echinocandin	Micafungin	intravenous
	Anidulafungin	intravenous
	Caspofungin	intravenous
Azoles	Itraconazole	oral, intravenous
	Voriconazole	oral, intravenous
	Posaconazole	oral, intravenous
Polyene	Amphotericin B	oral, intravenous
	Amphotericin B lipid complex	intravenous
	Amphotericin B liposomal	intravenous

1.6.1 Echinocandins

The echinocandins act against *Aspergillus* by non-competitive inhibition of the synthesis of β 1, 3-glucan (a polysaccharide in the cell wall of many pathogenic fungi including *Aspergillus*) (Figure 1.3) and thereby compromising the integrity of the cell wall [170]. They are fungicidal against some fungi and yeasts including *Candida* and fungistatic to others such as *Aspergillus* [171, 172]. Resistance to the echinocandins appears to be rare [170, 173] and casofungin has

been licensed as a treatment drug for IA [170]. Echinocandins have the potential to be either additive or synergistic with polyenes and azoles.

1.6.2 Azoles

Azole drugs can be categorised into two main classes, imidazoles and triazoles. Both classes share the same mechanism of action but are slightly different in structure. However the poor antifungal response and toxicity associated with imidazoles led the development of the new azoles, the triazoles. The antifungal triazoles inhibit the fungal cytochrome P450-mediated synthesis of ergosterol (main component of cell wall membranes) (Figure 1.3) leading to altered cell membrane function, cell death or inhibition of cell growth and replication [170]. The triazoles are often prescribed as they have considerably reduced side effects compared to amphotericin B for example.

1.6.2.1 Itraconazole

Itraconazole has been used successfully to treat ABPA [21, 174]. Itraconazole may be given either intravenously or orally. Itraconazole capsules are generally taken on an empty stomach at least an hour prior to or after a meal and treatment generally continues for approximately 1 to 6 months. In CF the oral form of treatment is preferred due to a higher absorption. The medication has proven to be very effective and has shown minimal side effects. A study of 43 people treated with itraconazole revealed that 93% of them did not have any signs of recurrence for a year [175]. In CF itraconazole has been reported to be effective in reducing *A. fumigatus* bioburden and improving patient symptoms in patients colonised with *A. fumigatus* who did not meet the criteria for ABPA diagnosis [59, 176]. Shoseyov *et al* reported an improvement in six CF patients treated with itraconazole, who had positive sputum cultures but no ABPA [176]. Coughlan *et al.* demonstrated an improvement in patient symptoms and mosaic pattern analysed by 64 slice CT following itraconazole treatment which also correlated with a significant reduction in *A. fumigatus* bioburden [59]. Conversely, one Canadian group reported no significant improvement in symptoms (exacerbation rates, lung function or quality of life) following itraconazole treatment, however no quantification of *A. fumigatus* bioburden was performed pre- or post- treatment so it is hard to determine whether the treatment itself was effective in this study [177].

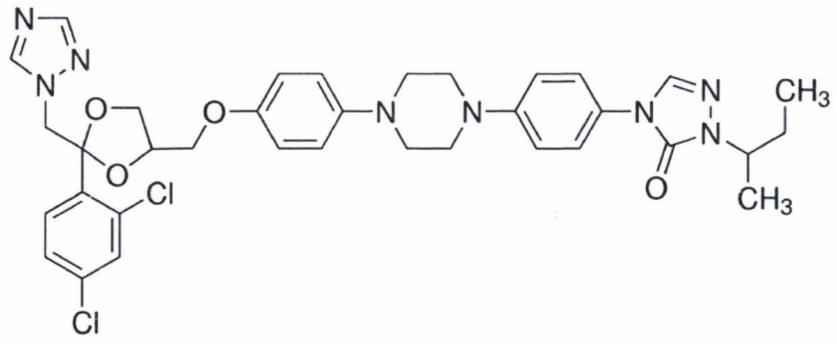


Figure 1.10: Chemical structure of Itraconazole

1.6.2.2 Voriconazole

In 2002 the FDA approved voriconazole for the treatment of IA, *Scedosporium apiospermum* and *Fusarium spp*. It is effective against a variety of microorganisms including *Aspergillus* species [178]. Voriconazole can be taken both orally and intravenously. Voriconazole has some reported side effects however with the most common side effects include vision defects, among them photophobia, blurred vision, and changes in colour vision persisting for the initial few days of treatment and then normalizing [179]. Other side effects include skin rashes and elevated liver enzymes. Generally it is estimated that only 30% of patients experience the vision disorder side effect for longer than 30 minutes after initiation of the therapy. It is mainly due to the blockage of receptor de-excitation during voriconazole therapy.

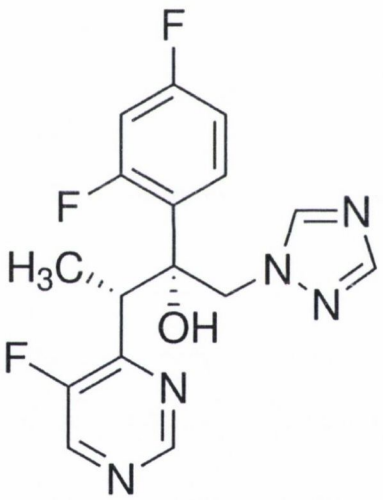


Figure 1.11: Chemical Structure of Voriconazole

1.6.2.3 Posaconazole

Posaconazole is a triazole antifungal drug [180] particularly recommended for treating oral and throat yeast infections as it slows down the fungi growth and treats the infection. Those with weaker capability to fight against the fungal infection are prescribed posaconazole. It is particularly used to treat IA [170]. The duration of the treatment depends upon the general health of the patient and the type and intensity of the infection. Posaconazole also has side effects including; nausea, headaches, blurred vision, diarrhea, dizziness and irregular heart beat [170]. Posaconazole has also been shown to be an excellent triazole for use in prophylaxis. Campoli *et al.* reported an interesting study where fungistatic levels of posaconazole persisted within epithelial cells for up to 48 h in vitro and were confined to the cell membrane which caused an increased concentration of posaconazole at the membrane [181]. The authors concluded that these findings indicated that concentration of posaconazole in mammalian host cell membranes mediated its efficacy in prophylactic regimens and likely explains the observed discrepancy between serum antifungal levels and efficacy [181]. However the authors did state that these findings should be treated with caution when applying the theory to patient treatment *in vivo* [181].

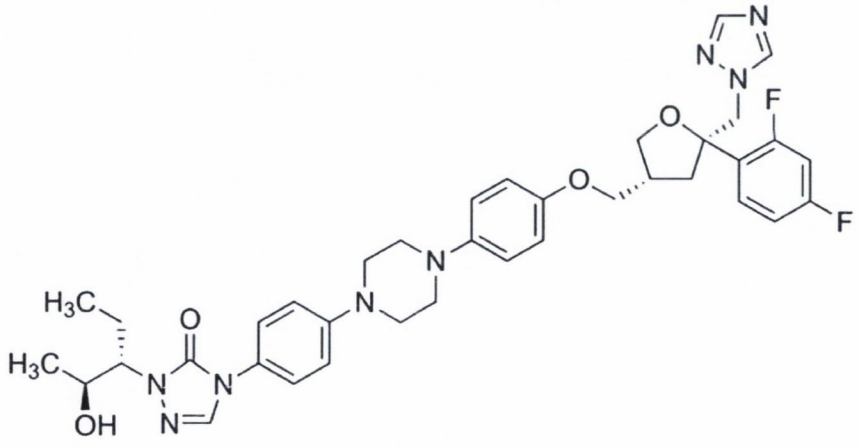


Figure 1.12: Chemical structure of Posaconazole

1.6.3 Amphotericin B

Amphotericin B targets the fungal cell membrane by binding to ergosterol and thus causing the formation of ion channels which leads to monovalent ion leakage and ultimately to cell death [170]. However, these therapies are limited by the occurrence of considerable toxicity, mainly resulting in renal failure and amphotericin B is rarely used on CF patients [170] [182]. Some common side effects of amphotericin B include; chills, dryness of mouth, nausea, vomiting, drowsiness, muscle ache, fever, seizure, jaundice, diarrhoea, weight loss, headache, skin infection, infusion-related toxicity, and renal failure [179]. The lipid formulations of amphotericin B have been shown to be less toxic however they are more expensive. Despite the high cost and serious side effects amphotericin B remains an effective treatment for IA [183].

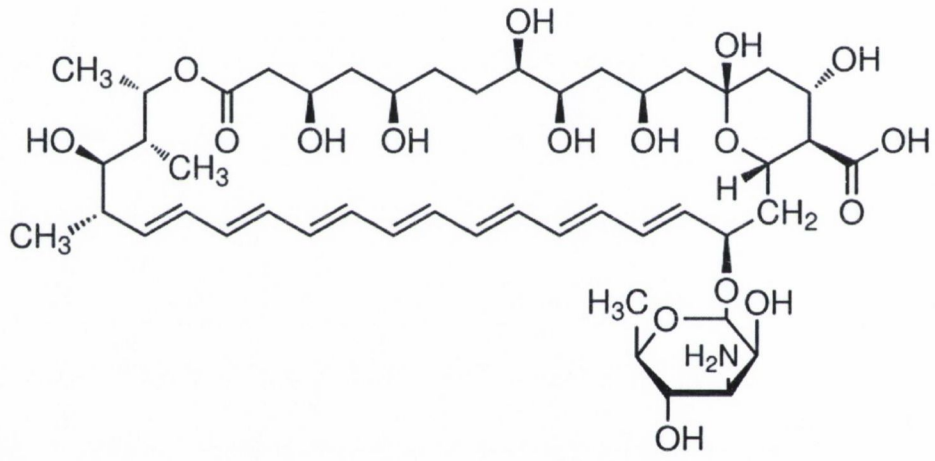


Figure: 1.13: Chemical Structure of Amphotericin B

1.6.4 Resistance

Aspergillus infection can be treated by a number of antifungals. The echinocandins, the azoles and amphotericin B are among the most widely used. Antifungal resistance has been associated with treatment failure in patients with life threatening fungal infections such as aspergillosis. Antifungal resistance can be described as a relative decrease in susceptibility of the fungus to an antifungal agent when compared to other isolates. Standardised test protocols from the Clinical and Laboratory Standards Institute (CLSI, USA) or the European committee on antimicrobial susceptibility testing (EUCAST, Europe) are often used for *in vitro* susceptibility testing to detect resistance [186, 187]. However there is very little data for *Aspergillus* and this remains a hot topic of debate. Furthermore the existence of two different committees for antimicrobial testing results in no one standardised method of testing or use of breakpoints and the cost of purchasing data from CLSI compared to the data which is free of charge from EUCAST may also produce a bias of method in the literature. Considering there are few recognised breakpoints for *Aspergillus* the use of epidemiological cut off values (ECV) provide valuable data which is helpful to identify the acquired resistance in fungi [188]. Resistance to the echinocandins appear to be rare [170]. However, resistance to the triazoles has been reported in a number of continents around the world, including Asia, the USA, Australia and Europe [189-197]. Triazole resistance has been observed in isolates from triazole treated and also triazole naïve patients [198]. As mentioned previously the triazoles inhibit the fungal cytochrome P450-mediated synthesis of ergosterol (main component of cell wall membranes) leading to altered cell membrane function, cell death or inhibition of cell growth and replication [170, 186, 189, 190, 199]. Triazole resistance has been primarily associated with point mutations in the cytochrome P450 sterol 14α-demethylase encoded by *cyp51A* gene and/ or an increased *cyp51A* expression due to the alteration of a tandem repeat in the promoter region [200-203]. There are a number of amino acid substitutions in the *cyp51A* gene such as those at codons G54, G138, P216, M220 and G448 that have been reported in clinical triazole resistant *A. fumigatus* isolates. However there exists a dominant mechanism of resistance to the triazoles, the TR34/L98H mutation. This mechanism involves a 34bp tandem repeat (TR34) in the promoter region in combination with a substitution of leucine for histidine at codon 98 (L98H) of the *cyp51A*-gene and has been found in both environmental and triazole naïve patient samples [192, 194, 195, 200-204]. Countries such as the Netherlands and the United Kingdom (UK) have reported a high incidence of triazole resistance which seems to be increasing [193, 199, 205]. In the Netherlands, the prevalence of triazole resistance was reported to vary between 0.9 to 9.4% [206] and in Manchester triazole resistance has risen from 7% in 1999 to 20% in 2009 [2, 207]. However, the mode of resistance acquisition appears to be different between the two countries [193]. In Nijmegen in the Netherlands, triazole resistance has been attributed to the pressure of

triazole fungicides used in agriculture with predominantly the TR34/L98H mutation [191, 193, 194, 208]. Triazole drugs are one of the recommended antifungal agents for both clinical settings and in agriculture which provides the situation of environmentally acquired resistance as seen in the Netherlands. In Manchester in the UK the high incidence of triazole resistance was reported to be caused by a mutation in the *cyp51A* gene where 18 amino acid alterations were found and acquired during a prolonged triazole treatment of patients with pulmonary aspergillosis [191, 193]. A collection of these isolates underwent genetic analysis of microsatellites and showed the existence of resistant mutants that evolved from originally susceptible strains [191, 193].

1.7 Epidemiology and Genotyping of *A. fumigatus*

The use of phenotype-based methods to distinguish *A. fumigatus* strains has become somewhat rare due to their technical complexity, low discriminatory power and poor reproducibility. Furthermore, a phenotype does not always accurately represent the genotype of a microbe and therefore is unable to provide reliable and stable epidemiological information [209]. Therefore, molecular methods for genotyping have become the method of choice for epidemiological studies. Typing of *Aspergillus* comprises of genetic analysis of isolates below the species level in order to produce strain / genotype specific fingerprints or datasets [209]. Genotyping of fungi, namely *A. fumigatus* can help provide insight into the mode of transmission, spread of the organism, and virulence and for this typing of an organism is mandatory. However, *A. fumigatus* is highly diverse in nature and therefore requires sensitive techniques. There are several genotyping methods available for *A. fumigatus* including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), sequence specific DNA primers (SSPD), multilocus sequence typing (MLST), multilocus enzyme electrophoresis (MLEE) and microsatellites/short tandem repeats (STRs) [210]. Several criteria such as reproducibility, ease of use, ease of interpretation, ease of exchange of data between laboratories, discriminatory power, cost and time need to be considered when choosing a genotyping method [209]. Vanhee *et al* carried out a study comparing RAPD, SSPD, MLST, MLEE and STR, they found the STR method produced the highest resolution, with the greatest reproducibility and discriminatory power [210] [211]. De Valk *et al.* carried out a study comparing all techniques available for molecular typing of *A. fumigatus* and the performance of each method was evaluated with regard to feasibility, ease of interpretation and discriminatory power [212]. Considering *A. fumigatus* isolates have a large extent of genetic variability, the authors concluded that a method with high discriminatory power and high reproducibility were required for typing *A. fumigatus* [212]. The authors concluded that RFLP utilising Afut-1 and microsatellites/STRs showed the greatest discriminatory power of all the methods available for genotyping *A. fumigatus*, however the microsatellites/STRs method

showed the greatest reproducibility and therefore the authors concluded that microsatellites/STRs was the method of choice for typing *A. fumigatus* isolates [212]. A number of genotyping studies of *A. fumigatus* in CF patients show similar trends from country to country [7, 213, 214]. These studies showed examples of colonisation with indistinguishable strains over time, examples of different strains over time, patients sharing indistinguishable strains and patients with dominant genotypes [7, 213, 214]. In the limited number of studies looking at *A. fumigatus* in CF patients, multiple genotypes were found in some patient samples while others contained a unique dominant genotype [7, 213, 214]. Analysis of sequential isolates from CF patients showed those patients who had a history of chronic colonisation demonstrated, a dominant genotype, a limited number of genotypes or a reduction in the diversity of genotypes over time [7, 214]. While there have been a number of genotyping studies of *A. fumigatus* in CF patients in Europe, none have been carried out on an Irish CF cohort [7, 213, 214] so whether Irish CF patients show similar patterns of colonisation remains unclear.

1.8 Project Aims

The asymptomatic colonisation of *A. fumigatus* in CF patients is an understudied area. We sought to;

1. Detect *A. fumigatus* from CF sputum by culture and molecular methods and furthermore monitor the bioburden of *A. fumigatus* following itraconazole treatment
2. CF patients incur a high rate of azole exposure over their lifetime. Additionally microbiology laboratories in Irish hospitals do not routinely test *Aspergillus* susceptibility to antifungals. Therefore whether azole resistance is a problem in Irish CF patients is unclear. Considering this, we sought to monitor the antifungal drug susceptibility of CF and non-CF *A. fumigatus* isolates to a panel of antifungals drugs and investigate the epidemiology of these isolates.
3. After finding a number of different genotypes and different patterns of colonisation we sought to investigate the virulence of different *A. fumigatus* genotypes.
4. *A. fumigatus* is often found colonising the CF lung. It is unclear what effect this asymptomatic colonisation may be having on the CF lung and its integrity. To address this we sought to investigate the interaction of *A. fumigatus* and its culture supernatants with the respiratory epithelium tight junctions.

Chapter 2

Detection and Bioburden of *A. fumigatus* in an Irish Cystic Fibrosis cohort

Chapter 2

Detection and Bioburden of *A. fumigatus* in an Irish Cystic Fibrosis cohort

2.0 Introduction

Aspergillus fumigatus is a filamentous saprophytic fungus found ubiquitously in nature. *A. fumigatus* also plays a role in clinical settings in humans in the form of opportunistic infection, allergic states or colonisation. In CF *Aspergillus* is most commonly associated with Allergic Bronchopulmonary Aspergillosis (ABPA) [27]. However, many CF patients are found to be colonised with *Aspergillus* without displaying symptoms of ABPA [59, 166]. This group of patients has been poorly studied and the clinical significance of *A. fumigatus* colonisation in the absence of ABPA symptoms remains unclear.

A. fumigatus conidia have a complex cell wall that is resistant to lysis [130]. This, alongside the viscous nature of CF sputum, impairs the extraction of DNA by conventional methods. Although, the use of bead beating has been previously published as an effective method of lysing *A. fumigatus* conidia and homogenising CF sputum [137], there is no "gold standard" method for the isolation of *Aspergillus* DNA from CF sputum [130]. We sought to optimise a method for reliably isolating *A. fumigatus* DNA from CF sputum in order to subsequently monitor the bioburden of *A. fumigatus* in an Irish cohort of CF patients colonised with the fungus. This was carried out as part of a collaborative study linking asymptomatic *A. fumigatus* bioburden to CF patient health status [59].

In order to fully understand the clinical impact of symptomatic and asymptomatic *A. fumigatus* colonisation in CF, it is vital to correctly identify the fumigatus species as this is known to be the most virulent in CF. For a long time, identification relied on macroscopic culture and microscopic characteristics. However, more recent identification and quantification of *A. fumigatus* has utilised a combination of morphological characteristics and/or molecular methods although there remains no gold standard. *A. fumigatus* macroscopic characteristics include the growth rate, colony colour and thermo tolerance [6, 215] of the organism. *A. fumigatus* grows at a moderately rapid rate and the rate of *in vitro* growth has been associated with the ability to cause disease [6]. Blue/green colonies, the presence of short smooth colourless/ greenish conidiophores with a round columnar vesicle and uniseriate phalides (Figure 1.2) and the ability to grow at 48°C were

used in this project to make a preliminary identification for all *A. fumigatus* isolates collected throughout the project.

While macroscopic and microscopic characteristics can aid in species identification of *Aspergillus spp*, *A. fumigatus* can be difficult to distinguish morphologically from some other species of *Aspergillus* [9]. In particular, from the recently described *A. lentulus* which has a very similar appearance to *A. fumigatus* [9, 10]. There are a number of target genes used for molecular identification of *Aspergillus* to the species level including the *internal transcriber spacer* (*ITS*) [11-13], *ß-tubulin* [14, 15] and *calmodulin* [15] [16] regions. For the purpose of this study we sought to confirm all isolates as *A. fumigatus*. Patient samples that cultured *Aspergillus* successfully were analysed firstly by macroscopic and microscopic characteristics and then by molecular methods to investigate which species of *Aspergillus* was present. Although *A. fumigatus* has distinct morphological appearances some species may be difficult to distinguish and furthermore, some *Aspergillus* positive samples may fail to culture *Aspergillus*. For these difficult cases molecular identification can be employed and gene copy number can give quantitative information.

In this study, we sought to optimise the DNA extraction protocol for isolation of *Aspergillus* DNA from CF sputum and to subsequently apply this method to clinical sputum samples, with the ultimate goal of using molecular tools to monitor the *A. fumigatus* bioburden in CF patients.

2.1 Materials and Methods

2.1.1 *A. fumigatus* culture and harvesting conditions

Plates of the first genome sequenced *A. fumigatus* strain, AF293 (ATCC293), were grown on Malt Extract Agar (MEA) (Fannin). Sub-cultures were prepared by sweeping a sterile loop over the top of a fully grown AF293 plate to collect conidia. The loop was then pierced into the centre of a fresh MEA plate under aseptic conditions. Plates were incubated at 37°C for 2-3 days. Once growth was present, plates were removed from the incubator and stored at 4°C. No more than 3 passages from the original plate were performed before returning to the original plate/ glycerol stock for subsequent cultures.

To harvest conidia two plates of AF293 were grown on MEA. Each plate was washed with Phosphate Buffered Saline containing 0.1% Tween 80 (Sigma) (v/v) (PBST) and washings collected into a sterile 20ml tube. The universal was centrifuged at 3,500 *x* g for 10 min to pellet the conidia. Supernatant was removed and sample pellets were re-suspended in 1ml Phosphate Buffered Saline (PBS). The wash step was repeated and the final pellet was re-suspended in 1ml PBS.

2.1.2 Enumerating *A. fumigatus* conidia using a haemocytometer

A serial dilution of the harvested conidia was performed from 10^0 to 10^{-3}. The 10^{-3} dilution was loaded onto a Neubauer's haemocytometer and the four corner squares counted. An average of these four squares was multiplied by 1 x 10^4 to give the final count per ml. Considering the results of the count, dilutions were performed to achieve the required number of conidia for future experiments. Triplicate ten-fold dilutions were also made for plating to confirm conidia concentration.

2.1.3 Plasmid preparation of standards for qPCR reactions

2.1.3.1 Isolation of *A. fumigatus* DNA using the Qiagen DNeasy Plant Kit

A serial dilution from 10^6 to 10^0 of A. *fumigatus* conidia per ml was made and centrifuged at 3,000 *x* g for 5 min. DNA was isolated from pelleted conidia using the Qiagen DNeasy Plant template preparation DNA extraction kit (Qiagen, Ireland). The conidia pellet was re-suspended in 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) and vortexed vigorously. The mixture was transferred to a sterile 2ml screw-capped tube containing 1g of 1mm diameter acid washed glass beads (Thistle Scientific). The sample was bead beaten for 180 sec at 2,000 rpm to release the *A. fumigatus* DNA and centrifuged briefly at 1,500 *x* g for 1 min. The sample was transferred to a fresh sterile eppendorf. Beads were washed with 400µl buffer AP1 to remove any remaining DNA. Washes were pooled and added to the bead beaten sample. Samples were incubated while

shaking at 65°C for 10 min. Following incubation, 130µl buffer AP2 was added to the mixture and incubated for 5 min on ice. The samples were then centrifuged at 20,000 *x* g for 5 min. Following centrifugation the supernatant was removed and applied to the QIAshredder mini spin column, placed in a 2ml collection tube and centrifuged at 20,000 x g for 2 min. Flow-through was transferred to a new tube without disturbing the pellet. Buffer AP3/E was then added at a ratio of 1.5:1 and mixed gently. The mixture was then transferred to a DNeasy mini spin column placed in a 2ml collection tube and centrifuged at 6,000 *x* g for 1 min. Following centrifugation the flow-through and collection tube were discarded and replaced with a new collection tube. A volume of 500µl buffer AW was added to the spin column and centrifuged at 6,000 *x* g for 1 min. Flow-through was discarded and 500µl buffer AW was added and centrifuged at 20,000 *x* g for 2 min. Following centrifugation the flow-through and collection tube were discarded and the mini spin column was placed in a sterile 1.5ml eppendorf. To the spin column 100µl of buffer AE was added, incubated at room temperature for 5 min and subsequently centrifuged at 6,000 *x* g for 1 min. The Isolated DNA was then spiked with an internal control of *Meningococcal* capsular transfer gene (*ctrA*) from the bacteria *Neisseria meningitidis*; 1 x 10^4 *ctrA* gene copies from per 100µl (gift from Dr Oliver Morton), aliquoted and stored at -20°C for later use.

2.1.3.2 Amplification of the *28S* rRNA region

AF293 DNA was extracted as previously described (Section 2.1.3.1) and was used as the template for *28S* rRNA PCR. The *28S* rRNA region was amplified using primers ASF1 and ADR1 (Table 2.2) [11]. PCR reactions were performed on the G-Storm GS1 system. The following parameters were used; heated lid 111°C, hot start at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. A final extension step of 72°C for 7 min was added.

2.1.3.3 Purification of PCR product

The resulting *28S* rRNA PCR product was then separated on a 2% agarose gel (w/v) with GelStar (Lonza) as the DNA indicator. Gels were run at 65V for 20 to 30 min. PCR products of 185bp were excised from the agarose gel and transferred to sterile eppendorfs. From this point the Qiaquick Gel extraction Kit was used to purify the PCR product; three volumes of buffer QG was added to one volume of gel and incubated at 50°C for 10 min with intermittent vortexing until the gel slice had completely dissolved. To the mixture one gel volume of isopropanol (Sigma) was added and mixed. The mixture was transferred to a QIAquick spin column placed in a 2ml collection tube and centrifuged at 17,900 *x* g for 1 min. Flow-through was discarded and 500µl buffer QG was added and centrifuged at 17,900 *x* g for 1 min. Flow-through was discarded and 750µl buffer PE was added and centrifuged at 17,900 *x* g for 1 min. Following centrifugation flow-through was discarded and the QIAquick column was centrifuged at 17,900 *x* g for 1 min to

remove any residual buffer. The QIAquick column was then placed into a sterile 1.5ml eppendorf. The purified PCR product was eluted by adding 50µl elution buffer (EB) to the spin column and by centrifuging at 17,900 *x* g for 1 min.

2.1.3.4 Transformation of the *28S* rRNA PCR product into JM109 cells

The PCR product was ligated to the TA-cloning vector (Promega: pGEM-T easy vector system) overnight at 4°C as per the manufacturer's instructions; For each ligation reaction 5 µl of 2X rapid ligation buffer, 1µl pGEM-T easy vector, 3 µl PCR product from section 2.1.3.3 and 1µl T4 DNA ligase were added to a sterile eppendorf. For the positive control 1µl control insert DNA and 2µl deionised water were added in place of the PCR product. For the negative control 3µl deionised water was added in place of PCR product. Samples were mixed and incubated at 4°C overnight. Transformation was performed using the pGEM-T easy vector protocol; a volume of 3µl of ligation mixture was added to a 1.5ml eppendorf on ice. To these tubes 50µl of JM109 cells was added, gently mixed and incubated on ice for 20 min. Following incubation cells were heat shocked for 45-50 sec in a water bath at exactly 42°C and then returned to ice for 2 min. To the transformed cells 950µl super optimal broth with catabolite repression (SOC) medium at room temperature was added and incubated at 37°C for 1.5 hr. Each transformation culture was then plated onto duplicate Luria Burtoni (LB) agar supplemented with 100µg/ml ampicillin, 0.1mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and 60µg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and incubated for 24 hr at 37°C.

2.1.3.5 Purification of transformed *28S* rRNA PCR product

Following incubation, eight white colonies (which represented cells transformed with the PCR product insert) were selected and inoculated into 1-5ml LB medium containing ampicillin. Following incubation for 16 hr at 37°C shaking, 1ml of each culture was transferred to a fresh sterile eppendorf and centrifuged at 15,000 *x* g for 1 min. Supernatant was discarded and another 1 ml of each culture was added to the pellet and centrifuged at 15,000 *x* g for 1 min. Again the supernatant was discarded and from here the Qiagen spin mini prep kit was followed to purify the plasmid; Pelleted bacterial cells were re-suspended in 250µl buffer P1. A volume of 250µl buffer P2 was added and mixed gently. To the mixture 350µl buffer N3 was added, mixed thoroughly and centrifuged at 17,900 *x* g for 10 min. Following centrifugation the supernatant was transferred to a QIAprep spin column and centrifuged at 17900 *x* g for 1 min. Flow-through was discarded and 500µl buffer PB was added and centrifuged at 17,900 *x* g for 1 min. Flow-through was discarded and 750µl buffer PE was added and centrifuged at 17,900 *x* g for 1 min. Following centrifugation, flow-through was discarded and the spin column was centrifuged at 17,900 *x* g for 1 min to remove any residual buffer. The QIAprep column was placed in a sterile 1.5ml eppendorf

and 50µl buffer EB was added to the spin column, let stand for 1 min and then centrifuged at 17,900 *x* g for 1 min to elute the purified plasmid.

2.1.3.6 Restriction digest and qPCR for confirmation of the presence of the *28S* rRNA gene insert

NotI (Promega) digestion was performed as per manufacturer's guidelines; For each reaction 2µl RE 10X buffer (Promega), 0.2µl Acetylated Bovine serum albumin (BSA) (10µg/µl) (Promega), 12.3µl deionised water, 5µl purified plasmid and 0.5µl NotI restriction enzyme (Promega) were added to a sterile eppendorf, mixed gently and incubated at 37°C for 2 hr. Following incubation a 6X loading dye was added to each tube and then separated on a 2% agarose gel (w/v) with GelStar as the DNA indicator. Gels were run at 65V for 20 to 30 min and viewed under UV light exposure.

The purified plasmids were used as the target DNA for the qPCR reactions. The ADR1 and ASF1 primers and the ASP 28p probe (Table 2.2) [11, 216] were employed. The qPCR thermal cycle parameters were as follows; 95°C for 10 min, 45 cycles, 95°C for 15 sec and 60°C for 30 sec. All qPCR reactions were performed using the ABI7000 Prism system SDS version 1.2.

2.1.3.7 Sequencing confirmation of *28S* rRNA insert

The purified plasmids from section 2.1.3.5 were separated on a 2% agarose gel (w/v) with GelStar as the DNA indicator as per section 2.1.3.3 The plasmid bands on the resulting gel were excised and purified as per section 2.1.3.3 and sent to SourceBioScience DNA Sequencing (Dublin, Ireland) for confirmatory nucleotide sequencing.

2.1.3.8 Determination of number of plasmid copies

In order to use the genotyped plasmid as a standard for all subsequent qPCR reactions the number of plasmid copies was determined. All samples were quantified using the Nano2000 system version 1.1.03 and results were input into www.finnzymes.com/java_applets/copy_number_calculation.html to give the copy number per µl.

2.1.4 Quantitative PCR reactions

Serial dilutions of each plasmid were performed to obtain the standard range of 10^6 to 10^1 copies per reaction in 10-fold increments. For every qPCR reaction performed a standard curve of the *28S* rRNA plasmid and a negative control replacing target DNA with molecular grade water (Sigma) was included. The following components were added to each qPCR reaction; 10µl of Taq Man gene expression mastermix (Applied biosystems), 1µl of 20X custom TaqMan gene

expression assay for *28S* containing primers ASF1 and ADR1 and the 28S probe (Table 2.2) (Applied Biosystems), 4µl of molecular grade water (Sigma) and 5µl of plasmid/sample DNA. Samples were then incubated at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec. Equivalent conidia calculations were made based on the fact that *A. fumigatus* has been shown to contain 37 to 90 *28S* gene copies per conidia [130, 217] and we hypothesised an average of 50 *28S* gene copies per *A. fumigatus* conidium. Therefore each gene copy number result from qPCR reactions was divided by 50 to generate an equivalent conidia calculation as represented as equivalent conidia (qPCR).

For all samples a qPCR reaction for the spiked *ctrA* internal control was also performed under the same thermal cycle parameters as above with Taq Man gene expression assay for *ctrA* (Applied biosystems) being used (Table 2.2).

2.1.5 Optimisation of DNA extraction protocol

2.1.5.1 Comparison of the Qiagen DNeasy Plant Kit and the Roche HighPURE PCR kit for extraction of *A. fumigatus* DNA

2.1.5.1.1 Qiagen DNeasy Plant Kit

Serial dilutions of conidia were made in triplicate and from here DNA was extracted as per section 2.1.3.1. Once DNA was extracted, each sample was aliquoted and stored at -20°C for future qPCR as per section 2.1.4.

2.1.5.1.2 Roche HighPURE PCR kit

Serial dilutions of conidia were made in triplicate and centrifuged at 13,000 *x* g for 10 min. Pelleted conidia were re-suspended in 200 µl PBS and the mixture was transferred to a sterile 2ml screw-capped tube containing 1g of 1mm acid washed glass beads. Each serial dilution of conidia was then bead beaten for 180 sec at 2,000 rpm to release the *A. fumigatus* DNA. Samples were centrifuged briefly at 1,500 *x* g for 1 min and transferred to a fresh sterile eppendorf. Beads were washed twice with molecular grade water to remove any remaining DNA. Washes were pooled and the Roche Diagnostic High Pure PCR template preparation DNA extraction kit was used from here. Manufacturer's guidelines were adhered to; a volume of 300µl of binding buffer and 40µl proteinase K were added to the 300µl of bead beaten sample and incubated at 70°C for 10 min. Following incubation the sample was transferred to a sterile eppendorf and 200µl isopropanol was added and mixed. The mixture was transferred to a High filter column placed in a collection tube and centrifuged at 8,000 *x* g for 1 min. The flow-through and collection tube were discarded and 500µl inhibitor removal buffer was added and then centrifuged at 8,000 *x* g for 1 min. The flow-through and collection tube were discarded, the column was placed in a new collection tube

and centrifuged at 8,000 *x* g for 1 min. The flow-through and collection tube were discarded, the column placed in a new collection tube. The sample was then washed by adding 500µl wash buffer and centrifuged at 8,000 *x* g for 1 min (this step was repeated once again). After the final wash step, flow-through was discarded and the column was centrifuged at 13,000 *x* g for 10 sec to remove any residual wash buffer. The column was placed in a sterile eppendorf and 100µl elution buffer (pre-warmed to 70°C) was added and then centrifuged at 8,000 *x* g for 1 min to elute DNA. Once DNA was extracted, each sample was aliquoted and stored at -20°C for future qPCR as per section 2.1.4.

2.1.5.2 Optimisation of the bead beating duration for extraction of *A. fumigatus* DNA using the Roche HighPURE PCR kit

Serial dilutions (10^6 to 10^0) of conidia were made in triplicate and DNA was extracted using the Roche HighPURE PCR kit as per section 2.1.5.1.2 with the following exceptions; bead beating times of either 90 sec, 180 sec or 300 sec were performed. Once DNA was extracted, each sample was aliquoted and stored at -20°C for future qPCR as per section 2.1.4.

2.1.5.3 Determination of the benefits of using PBST to harvest conidia from CF samples

Dilutions of conidia were made from 10^5 to 10^0 in duplicate. To one serial dilution PBST was added to a final concentration of 0.1% (v/v) and centrifuged at 13,000 *x* g for 10 min. The other serial dilution had PBS added and from here DNA extraction was performed as per section 2.1.5.1.2. Once DNA was extracted, each sample was aliquoted and stored at -20°C for future qPCR as per section 2.1.4.

2.1.6 Determination of *Aspergillus* bioburden in an Irish CF patient group undergoing itraconazole therapy for asymptomatic *Aspergillus* colonisation

2.1.6.1 Collection of CF sputum samples

For this study, CF patients with *A. fumigatus* positive sputum cultures and lacking symptoms of ABPA were recruited by our collaborators in the Royal College of Surgeons Ireland (RCSI) at Beaumont hospital (Patients 1-13). Inclusion criteria required a confirmed diagnosis of CF by sweat chloride test [59, 158, 218] and subsequent confirmatory genotyping [59, 218]. Patients included had to be colonised with *A. fumigatus* on two separate and consecutive occasions at least 12 weeks apart in the year before the study commenced. Prior to recruitment, patients were required to be exacerbation free for the proceeding 6 weeks and have no prior diagnosis of ABPA (according to classical consensus conference criteria [21]) [59]. Exclusion criteria included; suspected or diagnosis of ABPA, allergy to any azoles, prior lung transplantation or prior administration of corticosteroid therapy [59]. Itraconazole therapy was administered orally at 400mg once daily for 6 weeks. Sputum samples from pre- and post- itraconazole treatment were collected by Dr. Sanjay Chotirmall.

2.1.6.2 Processing of sputum for downstream *A. fumigatus* quantification

All sputum samples were processed within 24 hr of collection. Any samples waiting 24 hr to be processed were stored at 4°C. Samples were weighed and coded. Sputasol (Oxoid) was added to each sputum sample at a ratio of 1:1. Samples were incubated for 15 min at 37°C shaking. Once samples were homogenised, a small aliquot was taken to perform serial dilutions for Colony Forming Unit (CFU) counts. The remaining sample was treated with RNALater (Qiagen) at a ratio of 1:2 overnight at 4°C and then stored at -80°C until further processing. RNALater is a high salt based solution which inactivates enzymes harmful to DNA structure.

2.1.6.3 Colony Forming Unit (CFU) counts of *A. fumigatus*

An aliquot of Sputasol treated sputum from each patient sample was used to perform CFU counts. A serial dilution from 10^0 to 10^{-3} was performed on each sample and plated on to MEA. Plates were incubated at 37°C for 18 to 35 hr. Colonies were counted and recorded per gram of original sputum. This was performed in triplicate for each patient sample.

2.1.6.4 Isolation of DNA from CF sputum samples

Each patient sample stored at -80°C was thawed on ice and a 1ml volume was taken for DNA extractions. PBST was added to each 1ml sample to a final concentration of 0.1% (v/v). This was to aid in pelleting the hydrophobic *Aspergillus* conidia. From here DNA was extracted from samples using the Roche HighPURE PCR kit as per section 2.1.5.1.2 using a bead beating time

of 180 sec. Once DNA was extracted, each sample was spiked with an internal *ctrA* control plasmid at a concentration of 10^4 gene copies per 100µl, aliquoted and stored at -20°C for future qPCR.

DNA extractions were carried out in duplicate for each patient sample. For each extraction a positive extraction from 100 *A. fumigatus* conidia (293 strain (AF293)) and a negative extraction from molecular grade water were carried out.

2.1.6.5 Quantification and purity of isolated DNA

A small aliquot of DNA was thawed on ice and quantified using the nano2000 system version 1.1.03. Quantification was performed alongside A260/A280 and A260/A230 measurements to ensure the purity of DNA samples before proceeding to qPCR. All readings were carried out in triplicate.

All samples used for qPCR had high yields of DNA. Only DNA samples with A260/A280 and A260/A230 readings above 1.8 were deemed eligible for further qPCR reactions.

2.1.6.6 Quantitative PCR reactions

DNA from each patient sample was quantified in triplicate by qPCR as previously described (section 2.1.4) with the following exceptions; 10µl of Taq Man gene expression mastermix (Applied biosystems), 1ul of 20X custom TaqMan gene expression assay (Applied Biosystems), 2µl of molecular grade water and 7µl of patient sample DNA, a positive reaction control of DNA extracted from 100 *A. fumigatus* AF293 conidia and a negative control of all reaction components replacing target DNA with molecular grade water were added to each reaction. Equivalent conidia calculations were made as per section 2.1.4 and represented as equivalent conidia per gram of sputum. DNA extraction for each patient sample was performed in duplicate.

2.1.6.7 The Limit of Detection (LOD) and Limit of Quantification (LOQ) of *Aspergillus* using the optimised DNA extraction protocol

In order to define the minimum number of equivalent conidia that are detectable with reasonable accuracy and quantified with reproducible results from CF sputum, a LOD and LOQ experiment was performed.

A. fumigatus conidia were isolated as previously described (section 2.1.1). Conidia at increasing concentrations were spiked into *Aspergillus* negative CF sputum (these are theoretical conidia numbers from dilution series). DNA was isolated as per section 2.1.5.1.2 and 28S rRNA qPCR was performed as per section 2.1.4. This experiment was carried out in triplicate.

2.1.6.8 Quantitative PCR quality control measures

Absolute quantification of DNA copies was performed using the standard curve method (section 2.1.6.6). The standard curve was calculated based on Ct values plotted for every gene copy concentration entered. From this the slope and R^2 values were calculated. Only qPCR results generated using standard curves with R^2 values of >0.98 and slopes between -3.3 and -3.6 were used in this study. The PCR efficiency for the 28S rRNA plasmid standards was calculated using the following equation [219];

$$\text{PCR efficiency} = 10^{-1/\text{slope of the standard curve}} - 1$$

2.1.6.9 Ethics Committee Approval

Ethics approval from the Royal College of Surgeons Ireland institutional review board was obtained for the clinical study (section 2.1.6) and informed consent obtained from all study participants (n=13).

2.1.6.10 Statistical analysis of data

All statistical analysis throughout this chapter was performed on GraphPad Prism version 5. Statistical significance was tested between two DNA extraction kits (section 2.1.5.1) using a student t-test (unpaired). Statistical significance between bead beating times (section 2.1.5.2) was determined using the analysis of variance (ANOVA) test. Addition of PBST versus no PBST in DNA isolation (section 2.1.5.3) was tested for statistical significance using the student t-test (unpaired). Statistical significance between Aspergillus bioburden pre- and post-treatment, measured by CFU counts (CFU/g) and qPCR (section 2.1.6) was determined using Friedman's test. P values of <0.05 were considered significant.

2.1.7 Confirmation of all *Aspergillus* isolates as *A. fumigatus*

2.1.7.1 Harvesting of *Aspergillus* conidia from patient isolates

All *Aspergillus* isolates grown from section 2.1.6 (a subset of CF centre Hospital 1) (Table 2.1) were subcultured on MEA. Conidia were harvested as per section 2.1.1. Conidia counts were performed as per section 2.1.2. Considering this count, a volume of 10^6 conidia per ml was prepared.

2.1.7.2 Isolation of DNA from *Aspergillus* isolates

DNA was isolated from all *Aspergillus* isolates as per section 2.1.5.1.2 with the following exceptions; a 10^6 concentration of conidia from each patient isolate was used for DNA isolation

and 100µl of PBST was added to each sample prior to the first centrifugation to aid the pelleting of the hydrophobic conidia.

2.1.7.3 Amplification of the *Internal Transcribed Spacer* (*ITS*) region

The DNA isolated from each isolate was used as a template for PCR amplification of the ITS region. The following components were added to each 30µl PCR reaction: 15µl of AmpliTaq Gold mastermix (Applied biosystems), 2µM ITS4 reverse primer [47] (Table 2.2), 2µM *ITS5* forward primer [11] (Table 2.2), 6µl of molecular grade water (Sigma) and 5µl of *Aspergillus* isolate DNA. For every PCR reaction performed the following were included; a positive reaction control of DNA extracted from 100 *A. fumigatus* AF293 conidia and a negative control replacing target DNA with molecular grade water (Sigma). The following thermal cycle parameters were used: heated lid 111°C, 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 53°C for 15 sec and elongation at 72°C for 1 min and a final elongation step at 72°C for 7 min.

2.1.7.4 Gel electrophoresis of PCR products

PCR products from section 2.1.7.3 were separated by gel electrophoresis alongside a DNA ladder (Sigma) to establish the presence of a PCR product of approximately 500bp. A 6X loading buffer (Promega) was added to each PCR product and separated on a 2% agarose gel (w/v) containing gel star as the DNA indicator as per section 2.1.3.3 alongside a 25 to 1,000bp DNA ladder. The gel was exposed under UV light to view the PCR products.

2.1.7.5 PCR product Purification

All successful PCR products showing one distinct 500bp band were purified using the Promega Wizard SV gel and PCR clean up kit; an equal volume of membrane binding solution was added to the PCR product. The mixture was added to a SV mini column placed in a collection tube, incubated for 1min at room temperature and centrifuged at 16,000 *x* g for 1 min. Flow-through was discarded. Washing was performed by adding 700µl wash solution to the mini column and centrifuged at 16,000 *x* g for 1 min. A repeat wash step was performed with 500µl wash solution and centrifuged at 16,000 *x* g for 1 min. The flow-through was discarded and the mini column centrifuged at 16,000 *x* g for 1 min to remove any residual buffer. The mini column was transferred to a sterile 1.5ml eppendorf and 50µl nuclease free water was added and incubated at room temperature for 1 min. Following incubation the tube was centrifuged at 16,000 *x* g for 1 min to elute the purified PCR product. Purified PCR products were sent to SourceBioScience DNA Sequencing (Dublin, Ireland) for nucleotide sequencing. Sequence results were analysed using the Basic Local Alignment Search Tool (BLAST) version 2.3.25 on the NCBI website (www.ncbi.nlm.nih.gov/BLAST/) for identification of the isolate sequence.

2.1.7.6 Gel extraction of PCR product from samples that amplified two products

In some cases, sample PCR products showed two bands. The 500bp band was the band of interest. To isolate the band of interest the entire PCR product of each of these samples was separated on a 2% agarose gel (w/v) containing gel star as the DNA indicator as per section 2.1.3.3 and the 500bp band of interested was cut from the gel using a sterile scalpel. The Qiagen QIAquick Gel Extraction kit was used to excise the product from the gel as per section 2.1.3.3. The purified 500bp PCR products were quantified on the Nanodrop (Nano 2000 version 1.1.03) and sent to SourceBioScience DNA Sequencing (Dublin, Ireland). Sequence results were analysed using the Basic Local Alignment Search Tool (BLAST) version 2.2.25 on the NCBI website (www.ncbi.nlm.nih.gov/BLAST/) for sequence identification of the isolates.

2.1.7.7 Confirmation of unculturable isolates as *A. fumigatus* by qPCR

Some patient samples (patients 11, 12, 13) did not culture *Aspergillus* but *Aspergillus* 28S rRNA gene copies were detected and quantified by qPCR as part of the collaborative study with RCSI. In order to confirm these samples contained *A. fumigatus* a qPCR was carried out using *A. fumigatus* specific primers and probes, targeting the *ITS* region [13, 220] (Table 2.2). DNA isolated in section 2.1.6.4 was employed as target DNA. qPCR was carried out as per section 2.1.4 with the following exceptions; the following components were added to each 20µl PCR reaction for every sample; 10µl of Taq Man gene expression mastermix (Applied biosystems), 0.5µM *A. fum* Reverse primer, 5µM *A. fum* forward primer, 7.5 µM A. fum probe (Table 2.2), 5.8µl of molecular grade water and 3µl of sample DNA. A qPCR Ct value of <40 qualified as a positive result for the presence of *A. fumigatus.*

2.1.7.8 Restriction digest to differentiate between *A. fumigatus* and *A. lentulus*

One isolate displayed an atypical phenotype when grown on MEA. *A. fumigatus* and *A. lentulus* have been known to be difficult to distinguish morphologically [9, 10] and even by sequencing. A restriction digest of the *RodA* region (487bp) can distinguish between these two *Aspergillus* species. Restriction digest with the *Sty1* enzyme cuts the *A. fumigatus RodA* region at base pair 209 where a Cytosine is present. Therefore, the *A. fumigatus RodA* gene will separate into two bands following agarose gel electrophoresis. *A. lentulus* will show only one band due to a change of Cytosine to Thymine at position 209 of the *RodA* gene [9].

2.1.7.8.1 PCR amplification of the *RodA* region for restriction digestion

A PCR was performed with the RodA1 forward and RodA2 reverse primers (Table 2.2). The following components were added to each 30µl PCR reaction; 15µl of AmpliTaq Gold mastermix (Applied biosystems), 2µM RodA1 forward primer, 2µM RodA2 reverse primer, 6µl of molecular grade water and 5µl of patient 3's *Aspergillus* DNA. The following thermal cycle parameters were

used; heated lid 111°C, hot start automatic 95°C for 5 min, 35 cycles of denaturation at 95°C for 15 sec, annealing at 53°C for 15 sec, elongation 72°C for 1 min and a final elongation step at 72°C for 7 min. A 2% agarose gel (w/v) of the PCR products was performed to confirm successful amplification of the *RodA* region.**Restriction Digest of the 487bp *RodA* region**

The PCR products (section 2.1.7.8.1) were used as the template DNA. To each reaction 12.3µl of sterile deionised water, 2µl RE 10x Buffer (Promega), 0.2µl Acetylated BSA (10µg/µl) (Promega), 5µl PCR product DNA (1ug/ µl of DNA) (section 2.1.7.8.1) and 0.5µl Sty1 restriction enzyme (Promega) were added to a sterile eppendorf, mixed gently and incubated at 37°C for 2 hr. Following incubation a 6X loading dye was added to each tube and then separated on a 2% agarose gel (w/v) with GelStar as the DNA indicator and viewed under UV light exposure.

2.1.8 CF *Aspergillus* isolate Information for chapter

Table 2.1: Sample information for CF patient samples from chapter

Sample Patient no (p), sample no (s), colony no (c)	Patient Type	Hospital no.	Cultured positive for *Aspergillus* (✓/×)
p1 s1	CF	1	✓
p1 s2	CF	1	✓
p2 s1	CF	1	✓
p2 s2	CF	1	✓
p3 s1	CF	1	✓
p4 s1	CF	1	✓
p5 s1	CF	1	✓
p6 s1	CF	1	✓
p6 s2	CF	1	✓
p6 s3	CF	1	✓
p7 s1	CF	1	✓
p7 s2	CF	1	✓
p7 s3	CF	1	✓
p8 s1	CF	1	✓
p9 s1	CF	1	✓
p10 s1	CF	1	✓
p10 s2	CF	1	✓
p10 s3	CF	1	✓
p10 s4	CF	1	✓
p11 s1	CF	1	×
p11 s2	CF	1	×
p11 s3	CF	1	×
p11 s4	CF	1	×
p11 s5	CF	1	×
p11 s6	CF	1	×
p12 s1	CF	1	×
p12 s2	CF	1	×
p12 s3	CF	1	×
p12 s4	CF	1	×
p12 s5	CF	1	×
p13 s1	CF	1	×
p13 s2	CF	1	×
p13 s3	CF	1	×
p13 s4	CF	1	×

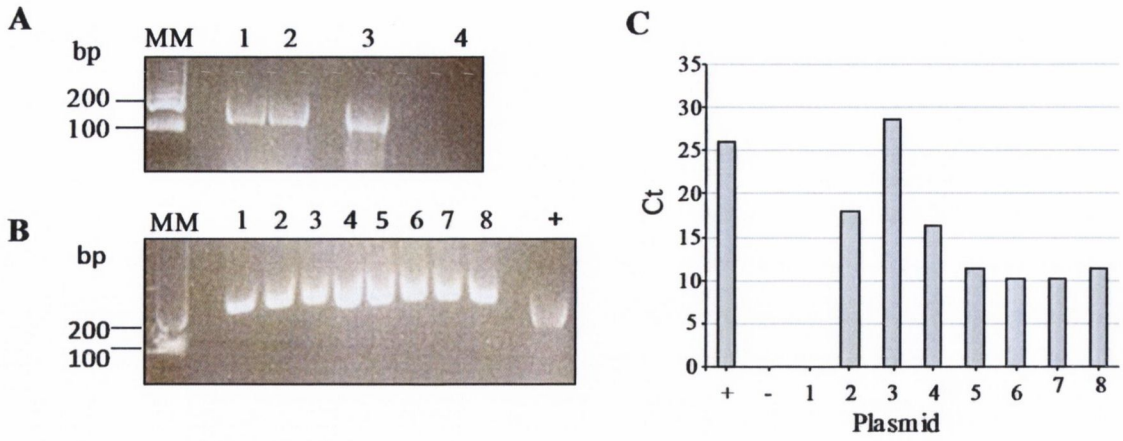
Table 2.2: Primer and probe Sequence information used for chapter

Primer/ Probe	Reverse / Forward	Sequence	Supplier
ASF1	Forward	5'-GCACGTGAAATTGTTGAAAGG-3'	Applied Biosystems
ADR1	Reverse	5'-CAGGCTGGCCGCATTG-3'	Applied Biosystems
ASP 28p probe		5'-FAM-CATTCGTGCCGGTGTACTTCCCCG-TAMRA-3'	Applied Biosystems
CTRA	Forward	5'-GCTGCGGTAGGTGGTTCA-3'	Applied Biosystems or Eurofins MWG Operon
CTRA	Reverse	5'- TTGTCGCGGATTTGCAAC -3'	Applied Biosystems or Eurofins MWG Operon
CTRA probe		5'-FAM-CATTGCCACGTGTCAGCTGCACAT-TAMRA-3'	Applied Biosystems or Eurofins MWG Operon
ITS5	Forward	5'- GGAAGTAAAAGTCGTAACAAG G-3'	Eurofins MWG Operon
ITS4	Reverse	5'- TCCTCCGCTTATTGATATGC-3'	Eurofins MWG Operon
A. fum For	Forward	5'- GCCCGCCGTTTCGAC-3'	Eurofins MWG Operon
A. fum Rev	Reverse	5'-CCGTTGTTGAAAGTTTTAACTGATTAC-3'	Eurofins MWG Operon
A. fum Probe	Probe	5'- CCCGCCGAAGACCCCAACATG-3'	Eurofins MWG Operon
RodA1	Forward	5'-GCTGGCAATGGTGTTGGCAA-3'	Eurofins MWG Operon
RodA2	Reverse	5'- AGGGCAATGCAGGAAGACC-3'	Eurofins MWG operon

2.2 Results

2.2.1 Construction of the *28S* rRNA plasmid for use as a standard in qPCR reactions

The preparation of *28S* rRNA plasmid for qPCR standards was performed (Section 2.1.3). PCR amplification of the *28S* rRNA amplified successfully with a band of 185bp observed on the agarose gel (Figure 2.1A). This 185bp amplicon was then used to prepare the *28S* rRNA plasmid. Confirmation of the successful preparation of the plasmid was performed by NotI restriction digest, qPCR and nucleotide sequencing. The restriction digest performed did not clearly show whether the plasmid preparation had been successful (Figure 2.1 B). Bands in each lane were of slightly different molecular weight (MW) and not all bands were identical to the positive control (Figure 2.1B). This would indicate that not all samples were the correct *28S* rRNA plasmid. Therefore, qPCR for the *28S* rRNA region (Figure 2.1C) where a Ct value corresponds to a positive result and nucleotide sequencing of the plasmids (Figure 2.1D) was performed to confirm the presence of the *28S* rRNA amplicon. Both qPCR and sequencing confirmed successful amplification (on par with a previously prepared *28S* rRNA plasmid). This plasmid was used to prepare a standard curve for qPCR (Figure 2.2) and the plasmid was subsequently used for all qPCR reactions in order to allow absolute quantification of *28S* rRNA copies in our patient samples. Standard curves used as part of this study had slopes between -3.3 and -3.6 and R^2 values >0.98, which are within the acceptable quality assurance levels required for publication [55].



D

Sequence: M13 forward primer

NNNNNNNNNNNNNNNNNNGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCG
CGGGAATTCGATTGCACGTGAAATTGTTGAAAGGGAAGCGTTTGCGACCAGACTCGCCCGCGG
GGTTCAGCCGGCATTCGTGCCGGTGTACTTCCCCGTGGGCGGGCCAGCGTCGGTTTGGGCGGCC
GGTCAAAGGCCCTCGGAATGTATCACCTCTCGGGGTGTCTTATAGCCGAGGGTGCAATGCGGC
CAGCCTGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC
GCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCA
TAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCA
TAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACT
GCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGG
GAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCA
GGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAA
AGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACG
CTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGNNAA
GCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGNGCTTTCTCATAGCTCACGCTGTNNTATCTCAGTTCGGNGTAGGTCGTTCGC
TCCAGCTGGGNTGTGTGCACGAACCCCCCGTNCAGCCCGACGCTGCGCCTTATCCNGTAACTA
NCGNCTTGAGTCNNCCNGTANANACGANTNNTCNCCNCTGNNGCANNNCTGGNANNNGNATT
ANCNNNANNNNNNNNGNAGNNGNGCTNNNNANNNTNGNANNNNNNNNCNNCNGNNTNNNCT
NNNNNANNNNNNTNNNNNTNGNNNNTGNTNNNNNNNNNNNNNCNNNGTAAAANNNNNNNGN
NNNANNNNNNNNN

Figure 2.1: Preparation of the *28S* rRNA plasmid for qPCR standards

A) Agarose gel of *28S* rRNA amplicons (185 bp). Lanes 1, 2 and 3 contain the *28S* rRNA amplicon and lane 4 is a negative control. **B)** Electrophoresis separation of the NotI restriction digest of the purified *28S* plasmid. Lanes 1 to 8 contain restriction digests of plasmids 1 to 8 respectively, the positive control (+). **C)** Quantitative PCR Ct values for the *28S* rRNA sequence transformed in to JM109 cells. The positive control (+) is previously constructed *28S* rRNA plasmids and the negative control (-) is molecular grade water. Numbers 1 to 8 on the x-axis denote the eight transformed colonies. Plasmids 2, 3 and 4 were sent for nucleotide sequencing to confirm that the *28S* rRNA insert had been taken up. **D)** Representative nucleotide sequence result confirming the *28S* rRNA insert. The red text highlights the forward primer sequence. The sequence between the green highlighted plasmid overhangs is the *28S* rRNA insert of interest.

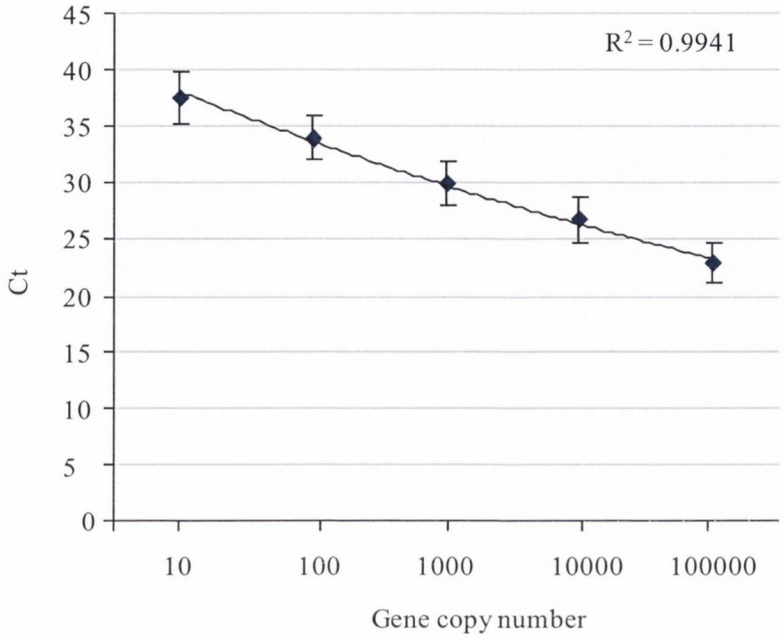


Figure 2.2: Standard curve generated using the *28S* rRNA plasmid.

Using the finnzymes website

(http://www.finnzymes.fi/java_applets/copy_number_calculation.html).

The plasmid stock was diluted to obtain concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 gene copies per qPCR reaction. This experiment was performed in triplicate and results are averages of this triplicate (error bars represent standard deviations).

2.2.2 Optimisation of the extraction of *A. fumigatus* DNA

To optimise the DNA extraction protocol two different DNA extraction kits, three different bead beating times and the addition of PBST to aid in pelleting the conidia were explored. Comparison of the Qiagen DNeasy Plant Kit and the Roche HighPURE PCR kit for extraction of *A. fumigatus* DNA demonstrated that lower concentrations of DNA gave unexpectedly high readings with the Qiagen kit (Figure 2.3A). For instance the 10^1 and 10^2 conidia concentrations extracted using the Qiagen kit resulted in 245 and 434 equivalent conidia, respectively. It should be noted that a negative control for DNA extraction resulted in a negative yield with Ct values of over 40. While a Ct value of 40 is considered the cut off point for reproducible detection [221], it may discount some low level contamination. At 10^4 and 10^5 prepared conidia concentrations the Qiagen kit yielded 7072.89 and 7052.04 equivalent conidia, respectively. This was below the expected conidia concentration and both the 10^4 and 10^5 prepared conidia concentrations gave a very similar result, despite the 10-fold increase in conidia numbers prepared.

In contrast the 10^1 and 10^2 conidia concentrations extracted with the Roche kit gave 23.70 and 85.34 equivalent conidia, respectively. At 10^3, 10^4 and 10^5 prepared conidia concentrations the Roche kit yielded 1393.68, 10367.4 and 86217.4 equivalent conidia, respectively. Although no statistical significance was found between kits overall (student t-test), the Roche kit isolated equivalent conidia numbers that were on par with original concentrations of conidia from which the DNA was extracted. Therefore the Roche kit was employed for all further DNA extractions. All bead beating times extracted *A. fumigatus* DNA when analysed by qPCR and there was no statistical significance found between the 90 sec, 180 sec and 300 sec bead beating times (ANOVA) (Figure 2.3B). In view of this, the median of these times, a bead beating duration of 180 sec was used in all further DNA extractions.

The addition of PBST prior to first centrifugation to aid in pelleting the hydrophobic conidia revealed no significant advantage over PBS alone (student t-test) (Figure 2.3C). However, a pellet was observed following centrifugation only in the PBST treated samples. For this reason PBST was included in all future DNA extractions. A final protocol for the extraction of *Aspergillus* DNA from CF sputum used the Roche HighPURE PCR kit, a bead beating time of 180 sec and addition of PBST prior to first centrifugation.

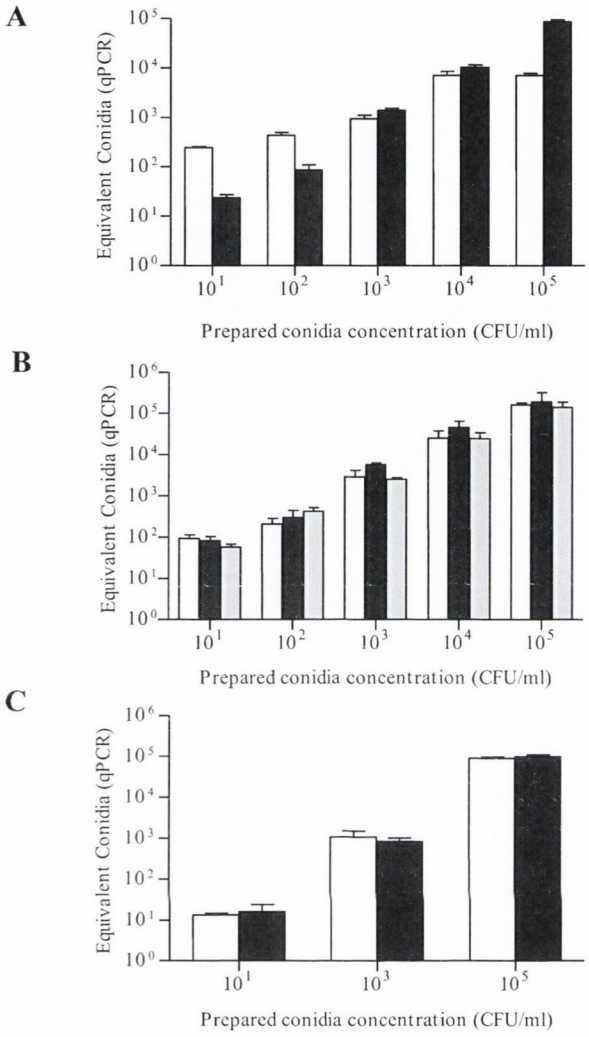


Figure 2.3: Optimisation of *A. fumigatus* DNA isolation technique

A) Histogram illustrating the efficiency of two DNA extraction kits at accurately isolating *A. fumigatus* DNA from all conidia in a series of prepared concentrations. The Roche High pure PCR template kit (■) and the Qiagen DNeasy plant kit (□) were used. The y-axis represents equivalent conidia numbers as calculated from *28S* rRNA gene copy numbers and the x-axis represents the prepared conidia concentrations. **B)** Three different bead beating times were compared for their efficiency at lysing *A. fumigatus* conidia and yielding higher DNA concentrations. A 90 seconds (□), 180 seconds (■) and 300 seconds (■) bead beating times were explored. Axes are represented as in A above. **C)** Improving DNA yield by pelleting of the hydrophobic conidia was explored by adding PBST (■) to a final concentration of 0.1% (v/v) or PBS (□) to a serial dilution of *A.fumigatus* conidia. Axis are represented as in A and B above. All experiments were performed in triplicate and error bars are representative of standard deviations from the mean.

2.2.3 The Limit of Detection (LOD) and Limit of Quantification (LOQ) of *Aspergillus* using the optimised DNA extraction protocol

The minimum concentration at which the conidia could be identified in a reproducible fashion or the limit of detection in CF sputum was 5 conidia/ml (Figure 2.4A). The minimum concentration of conidia that could be quantified with an acceptable level of precision and accuracy in CF sputum was 50 conidia/ml (Figure 2.4A). For the LOD and LOQ the Cv was 0.27% and 0.12%, respectively.

2.2.3.1 qPCR quality control measures

Absolute quantification of DNA copies was performed using the standard curve method. The standard curve was calculated based on Ct values plotted for every gene copy concentration entered (Figure 2.2). From this, the slope and R^2 values were calculated. Only qPCR results generated using standard curves with R^2 values of >0.98 and slopes between -3.3 and -3.6 were used in this study. The PCR efficiency is a measure of how well the amplification is working and thus critical to accurate data interpretation [219]. The PCR efficiency for the 28S rRNA assay used in this study was 95.95%. A PCR efficiency of 90% to 100% is considered a good PCR efficiency [219, 222].

The Coefficient of variance (Cv) is a representation of the ratio of the standard deviation to the mean, and it is useful for comparing the degree of variation from one data set to another. A Cv value of <1% is considered optimal [219]. The optimised DNA extraction method was performed in triplicate on a serial dilution of *A. fumigatus* conidia and the resulting *A. fumigatus* DNA was quantified using qPCR. For 10^1, 10^2, 10^3, 10^4 and 10^5 the coefficient of variance seen at each conidia concentration was 0.02%, 0.01%, 0.015%, 0.1% and 0.05%, respectively (Figure 2.4 B). Six replicates of this experiment were performed and the coefficient of variance between technical replicates did not fall above 0.05% at any conidia concentration.

All negative controls included throughout the study did not amplify any products indicating that all qPCR reaction components did not contain any contamination that may cause increased amplification. All positive controls amplified successfully confirming that the each reaction amplified *A. fumigatus* DNA when present. Each patient sample was spiked with $1x10^4$ copies of the *ctrA* plasmid as an internal control and on average patients samples amplified 8838.471 gene copies per 100µl with an overall Cv of 0.7%. This indicates PCR inhibition was minimal in our qPCR reactions.

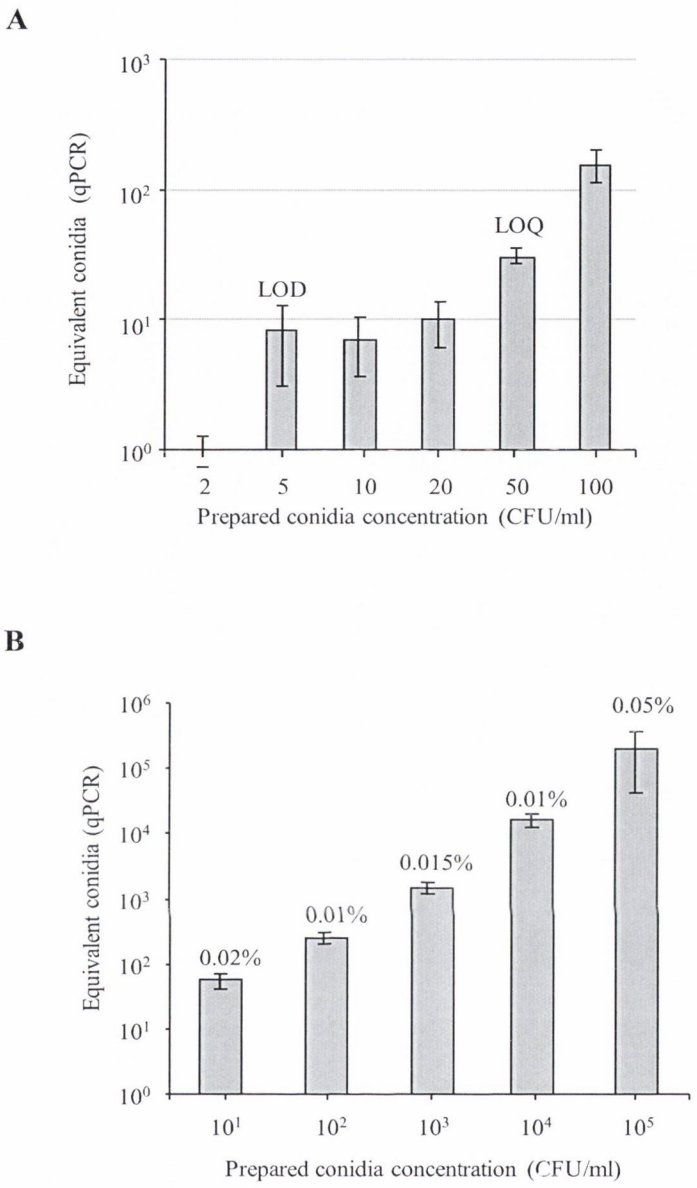


Figure 2.4: Quantitative PCR quality control Measures

A) Histogram of the Limit of Detection (LOD) and Limit of Quantification (LOQ). *Aspergillus* conidia at increasing concentrations were spiked into *Aspergillus* negative CF sputum. DNA was isolated and *28S* rRNA *q*PCR was performed. The y-axis represents equivalent conidia numbers as calculated from *28S* rRNA gene copy numbers and the x-axis represents the prepared conidia concentrations. Experiment was performed in triplicate and error bars are representative of standard deviations from the mean. **B)** Histogram illustrating the efficiency of the final DNA extraction protocol in a series of prepared concentrations used downstream on clinical sputum samples. Axes are represented as in A above. Percentages above each bar represent coefficient of variance seen at each conidia concentration. Experiment was performed six times and error bars are representative of standard deviations from the mean.

2.2.4 Itraconazole effectively reduced the bioburden of *A. fumigatus* in the airways of CF patients

For this study CF patients with *A. fumigatus* positive sputum cultures and lacking symptoms of ABPA were recruited. Other inclusion and exclusion criteria for this study were outlined in section 2.1.6.1. Sputum samples from pre- and post-itraconazole treatment were collected and the *A. fumigatus* bioburden determined by CFU counts and qPCR. Three patients (patients 11, 12 and 13) did not culture *A. fumigatus* but were qPCR positive for *A. fumigatus* (Figure 2.5A), indicating the importance of molecular methods in combination with standard culture for effective detection of *A. fumigatus* in CF sputum. In a number of cases patient samples were culture negative but qPCR positive (Figure 2.5A). Two patients were culture positive both pre- and post-itraconazole treatment (Figure 2.5A), however the bioburden was significantly reduced post-itraconazole treatment for all patients as measured by CFU counts and qPCR (Figure 2.5B and 2.5C).

Overall CFU counts gave higher *Aspergillus* counts compared to qPCR results (Figure 2.5 B and 2.5C). Pre- treatment, *A. fumigatus* counts reported 3.5 x 10^4 CFU /g sputum compared to qPCR results of 6.45 x 10^3 conidia /g sputum. Post-treatment *A. fumigatus* counts reported 2 x 10^3 CFU /g sputum compared to 1.5 x 10^3 conidia /g sputum found by qPCR (Figure 2.5B and 2.5C). In order to investigate the effects of itraconazole on *A. fumigatus* bioburden in CF sputum we took a pre-treatment time point at month 0, a post-treatment time point at month 4 and a 6 month and 12 month follow-up from all patients for comparison. CFU counts revealed a significant reduction in *A. fumigatus* bioburden post itraconazole treatment ($p<0.001$; Friedman's test, $Q=20.59$) (Figure 2.6B) and qPCR analysis also showed a significant reduction in *A. fumigatus* post-itraconazole treatment ($p<0.01$; Friedman's test, $Q=15.12$) (Figure 2.6C). Although CFU counts remained low at the 12 month follow-up, qPCR results showed a minor increase when compared to the 6 month follow-up.

This work was carried out as part of a larger clinical study, lead by Prof. Noel G. McElvaney at the RCSI, investigating the health status of this patient group pre- and post-itraconazole treatment [59]. We found that decreases in *A. fumigatus* bioburden due to itraconazole therapy correlated with improved lung structure monitored by CT scans, decreased infective exacerbations and produced an overall improvement in respiratory symptoms [59]. The clinical findings of this study were submitted in the PhD of Dr Sanjay Chotirmall.

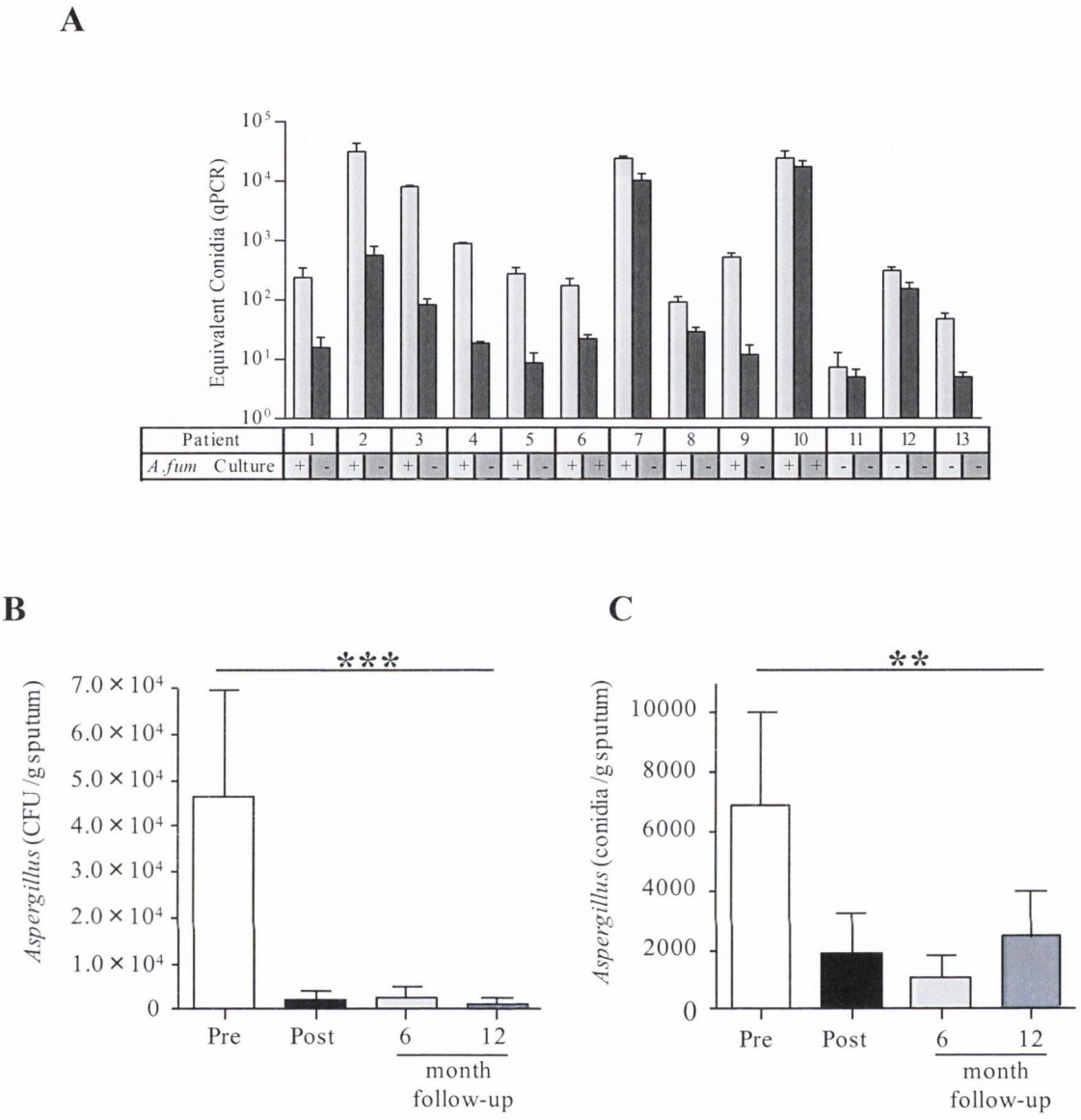


Figure 2.5: Monitoring *Aspergillus* bioburden pre- and post-itraconazole treatment.

A) Histogram illustrating the equivalent conidia for each patient of the clinical study, pre- and post itraconazole treatment. The y-axis represents equivalent conidia numbers as calculated from *28S* rRNA gene copy numbers. The x-axis represents each patient from the study (n=13) at pre (▫) and post (■) with a table indicating whether the sample was *A. fumigatus* culture positive (+) or negative (-). **B)** Colony forming units (CFU) of *A. fumigatus* per gram of spontaneously expectorated sputum in patients with CF (n=13) pre- (□) and post- (■) treatment with itraconazole (400mg orally once daily for six weeks) and at 6(▫) and 12(▪) months. All CFU counts were performed in triplicate for each patient sample. ***p<0.001(Friedman's test; Q=20.59). **C)** The equivalent *A. fumigatus* conidia number was determined in the same patient group (n=13) using qPCR targeting the 28S rRNA region. **P<0.01(Friedman's test; Q=15.12).

2.2.5 Amplification of the *ITS* region from cultured *Aspergillus* isolates

As part of this study CF *Aspergillus* isolates from the clinical study (from section 2.1.6) were grown and the DNA extracted for amplification of the *ITS* region to confirm isolates as fumigatus or non-fumigatus. Agarose gels of these amplicons are shown in Figure 2.6. All but two PCR amplifications produced a single 500bp PCR product (Figure 2.6C lanes 1 and 7) as can be seen from the 500bp band present and only the 500bp band was processed and sent for sequencing. Positive and negative controls for these PCRs indicated successful amplification and the absence of contaminating factors, respectively. Sequence analysis results of PCR products identified all isolates as *A. fumigatus* with 99 to 100% identity (Appendix I).

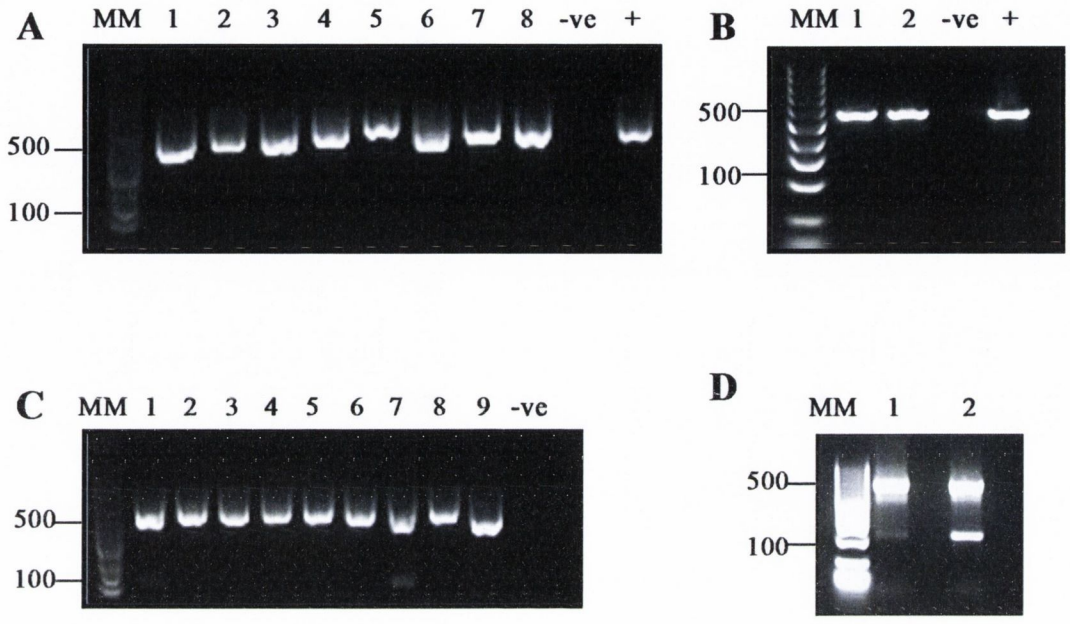


Figure 2.6: Amplification of the *ITS* region from Clinical study CF isolates

Represent *ITS* rRNA regions amplified from *A. fumigatus* clinical isolates (section 2.2.8.3). *ITS* PCR products are represented by the 500bp band. MM is the molecular weight marker and –ve is the negative PCR control, positive control (+). **A)** Lanes 1 = p1 s1, 2 = p1 s2, 3 = p2 s1, 4 = p2 s2, 5 = p3 s1, 6 = p4 s1, 7 = p5 s1, 8 = p6 s1. **B)** Lanes 1 = p6 s2, 2 = p6 s3. **C)** Lanes 1 = p7 s1, 2 = p7 s2, 3 = p7 s3, 4 = p8 s1, 5 = p9 s1, 6 = p10 s1, 7 = p10 s2, 8 = p10 s3, 9 = p10 s4. **D)** Two isolates produced more than one PCR product illustrated by two or more bands per sample on the gel (figure 2.6 (C) lanes 1 and 7). These complete PCR products were separated on an agarose gel for subsequent gel extraction excising the 500bp band of interest.

2.2.6 Confirmation of unculturable isolates as *A. fumigatus* by qPCR

Three patient's samples failed to culture *Aspergillus* (patients 11, 12 and 13; Table 2.1) (Figure 2.5A), throughout the study but gene copies were quantified by qPCR. In order to confirm these samples contained *A. fumigatus* a qPCR was carried out using *A. fumigatus* primers specific to the *ITS* region. All samples amplified with Ct values of <40 (Figure 2.7) indicating the presence of *A. fumigatus* in all samples.

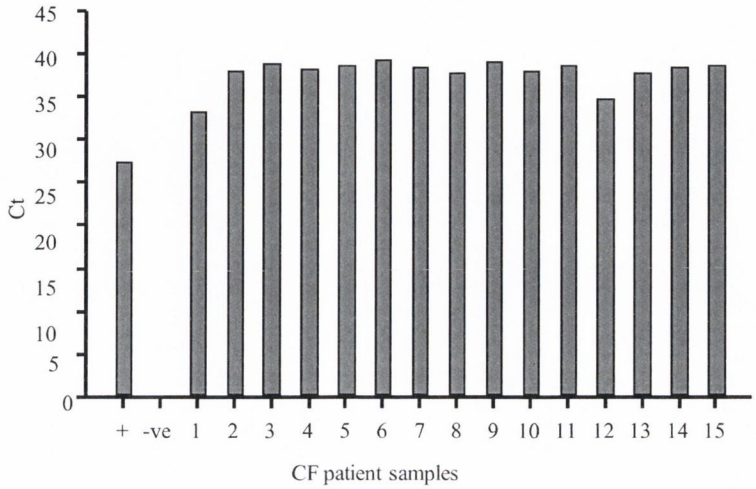


Figure 2.7: Real time PCR confirmation of the presence of *A. fumigatus* in patients 11, 12 and 13.

Histogram of Ct values of qPCR reactions to confirm the presence of *A. fumigatus*. The y-axis represents the Ct value for qPCR reaction of each sample and x-axis represents each sample (1 to 15) analysed. Lanes represent patient samples; 1 = p11 s1, 2 = p11 s2, 3 = p11 s3, 4 = p11 s4, 5 = p11 s5, 6 = p11 s6, 7 = p12 s1, 8 = p12 s2, 9 = p12 s3, 10 = p12 s4, 11 = p12 s5, 12 = p13 s1, 13 = p13 s2, 14 = p13 s3, 15 = p13 s4 and positive control (+) and negative controls (-ve) were included. Ct values of <40 qualified as a positive result for the presence of *A. fumigatus*.

2.2.7 Restriction digest to differentiate between *A. fumigatus* and *A. lentulus*

One *Aspergillus* isolate's growth was distinct from *A. fumigatus* (Figure 2.8A). This isolate required a longer duration for growth and displayed a markedly reduced sporulation. *A. fumigatus* and *A. lentulus* have similar morphology with the exception that *A. lentulus* is associated with poor sporulation [10] and therefore can be hard to distinguish. A restriction digest of the *RodA* region (487bp) that can distinguish between these two *Aspergillus* species, was performed [9]. DNA was extracted from this isolate and a PCR amplifying the *RodA* region was successfully carried out (Figure 2.8B). The *StyI* restriction digest was performed on these PCR products. Following amplification of the *RodA* region and subsequent Styl restriction digest the sample produced two distinct bands as did the AF293 reference strain (Figure 2.8C). This confirmed the isolate as *A. fumigatus* as the *RodA* region of *A. fumigatus* is cleaved at base pair 209 where a cytosine is present following Styl digestion, forming two distinct bands upon electrophoresis. *A. lentulus* has a Thymine instead of a Cytosine at this site and therefore is not cleaved into two distinct bands. [9].

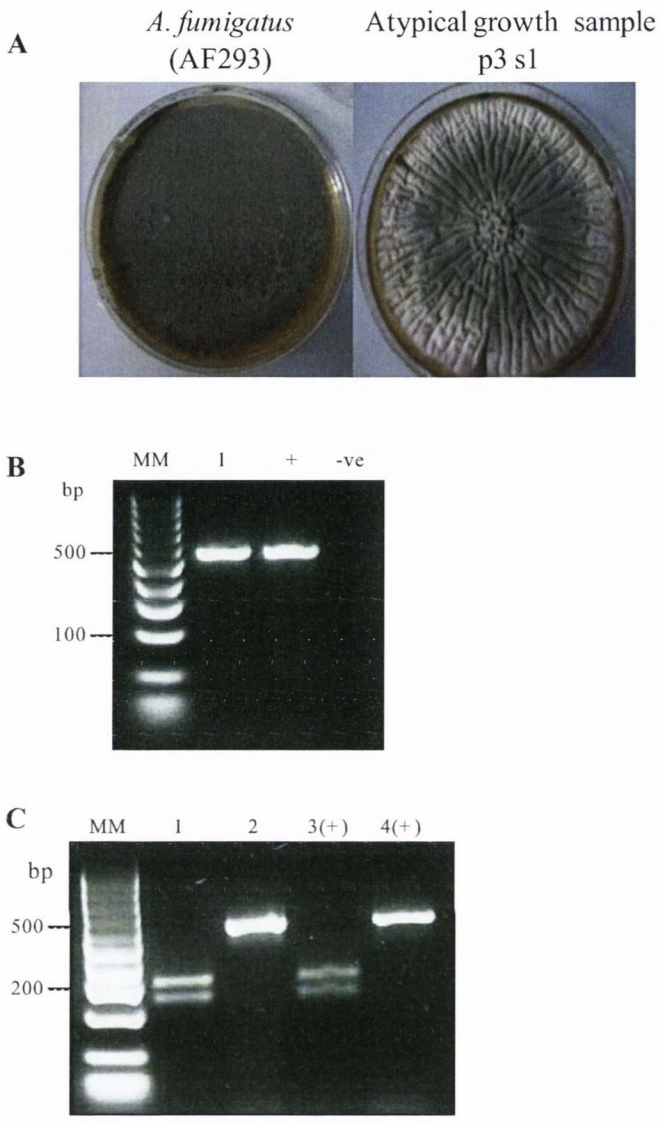


Figure 2.8: Differentiation between *A. fumigatus* and *A. lentulus*.

A) Shows the typical growth pattern of *A. fumigatus* (AF293) and the atypical phenotype observed in one clinical CF isolate. **B)** Agarose gel of amplified *RodA* gene from *A. fumigatus* isolate p3 s1 (Table 2.1), positive control (+) and negative (-ve) controls. **C)** *A StyI* restriction digest of isolate p3 s1 lane 1, sample p3 s1 without restriction digest lanes 2 and positive control with and without restriction digest in lanes 3 and 4 respectively.

2.3 Discussion

This study sought to optimise a method for reliably isolating *A. fumigatus* DNA from CF sputum. To aid in the homogenisation of CF sputum, sputasol was added to chemically homogenise the sputum. Two different DNA extraction kits, namely the Roche HighPURE PCR kit and the Qiagen DNeasy Plant kit, were tested for their ability to reliably isolate *Aspergillus* DNA from CF sputum. These kits were chosen based on suggestions from colleagues involved in The European *Aspergillus* PCR Initiative (EAPCRI) programme. This program consists of a large number of centres that compare PCR protocols with the aim of ultimately providing a gold standard for *Aspergillus* PCR [138, 142]. In the work presented here, standards containing known low conidia concentrations resulted in unexpectedly high qPCR yields when using the Qiagen DNA extraction kit. These same low concentration standards gave expected low qPCR yields using the Roche kit. These results suggest that low level contamination may be a possible factor causing low level contamination which may be masked at higher concentrations. It would be advisable to carry out DNA extractions on each reagent from this kit to identify if the kit is a source of low level contamination. Considering these higher DNA yields were only observed at the lower conidia concentrations in the Qiagen kit, low level reagent contamination was suspected. Other studies have also noted complications posed by fungal contamination within DNA extraction kits [4, 139]. Although no statistical significance was found between kits overall, the Roche kit consistently isolated equivalent conidia numbers that were on par with original concentrations of conidia from which the DNA was extracted. The Roche kit has been utilised in numerous publications [136, 141, 223-225]. Rantakokko-Jalav *et al* compared a number of kits for the extraction of *A. fumigatus* DNA including DNA-Pure yeast genomic kit, the QIAmp DNA mini kit, the Masterpure DNA purification kit, and the Roche High Pure PCR template preparation kit [223]. From a comparison of these kits the authors found the Roche High Pure PCR template kit resulted in the best detection limit for *A. fumigatus* DNA of all the kits that were compared [223]. Therefore the Roche kit was employed for all further DNA extractions.

Three different bead beating times were explored for the isolation of *Aspergillus* DNA (Figure 2.3B). While bead beating is necessary in the extraction of DNA from *Aspergillus*, the duration of bead beating must be defined. Although a number of papers include bead beating in their DNA extraction protocol, no bead beating duration is disclosed [11, 130, 136, 137, 141, 142, 223, 224, 226]. No significant difference in DNA yields from the three different bead beating times tested was observed in this study (ANOVA) therefore, the median of these times, a bead beating duration of 180 sec, was used.

To harvest conidia from patient samples the first step is usually to centrifuge the sample to pellet the conidia. *Aspergillus* conidia are hydrophobic [227] and this may lead to some conidia remaining on the liquid meniscus or sticking to the sides of tubes. The addition of PBST to the sample prior to the first centrifugation may aid in pelleting the hydrophobic conidia. Results revealed no significant difference (Student t-test) in DNA yield between PBST treated and untreated samples (Figure 2.3C). However, a pellet was observed following centrifugation only in the PBST treated samples. For this reason PBST was included in all future DNA extractions from CF sputum.

The incidence of *A. fumigatus* in CF patients has a wide variation between different studies with 6-80% of CF patients reported to be positive for *A. fumigatus* [59, 124-126], however the incidence of APBA in CF is only 7-9%. Many CF patients are colonised with *A. fumigatus* without displaying any symptoms of ABPA. It is not currently known what effect this colonisation in the absence of ABPA may have on the patient's health. Physicians differ in their treatment of CF patients who are culture positive for *Aspergillus* but show no ABPA symptoms with some prescribing antifungals and others not. Furthermore the efficiency of antifungal drugs at reducing *A. fumigatus* bioburden in the CF airways is not fully understood. Antifungal treatments also face additional challenges due to the fact that the dosage administered to the CF patient often does not result in therapeutic levels in the blood or airways [177, 228]. The concentration of a drug at which it is expected to be effective without causing any serious problem or side effects is referred to as the therapeutic level of a drug. Itraconazole has limited oral bioavailability and the capsule form requires an acidic environment for dissolution (often taken with orange juice to aid absorption), however this may be inhibited by antacid therapies. Liquid forms of itraconazole are also available and have been found to be better absorbed but are unpalatable [177, 228]. Poor absorption, as a complication of CF, presents further challenges in reaching therapeutic levels of drugs in the patient. Bentley *et al* carried out a small study investigating therapeutic levels of azoles in children with CF [228]. They reported only 4 out of 8 patients reached therapeutic levels of itraconazole (5-15mg/L) and 2 out of 8 reached therapeutic levels for voriconazole (1.3-5.7mg/L) in the blood. Although the authors followed dosages recommended in several clinical guidelines they concluded that these treatment levels were likely underdosing their CF cohort [228]. Likewise, Aaron *et al* found that only 57% of CF children and adults (8 out of 14) given oral itraconazole for *A. fumigatus* colonisation in the absence of ABPA reached therapeutic levels (0.3mg/L) [177]. Other studies have also found a marked inter-patient variability in the absorption of itraconazole [229, 230]. Despite all of these challenges itraconazole remains the gold standard for treatment of ABPA [21]. The treatment of CF patients who are asymptomatically or silently colonised with *A. fumigatus* remains at the discretion of the attending physician in most Irish CF centres as there is no standard for treatment of this group of CF patients.

Here we took a different approach to monitoring the efficacy of itraconazole treatment. We monitored the bioburden of *A. fumigatus* in the airways of asymptomatically colonised adults with CF pre- and post-itraconazole treatment (400mg orally once daily for 6 weeks). Firstly, CF patients recruited for this study were all confirmed positive for *Aspergillus* colonisation by macro- and microscopic inspection, culture and molecular techniques outlined in the methods section of this chapter. Patients displayed no symptoms of ABPA as defined by the most up-to-date CF Foundation Consensus Conference [21]. *Aspergillus* bioburden, measured by CFU counts (CFU/g) and qPCR (equivalent conidia/g), was significantly reduced following itraconazole treatment ($p<0.05$, Friedman's test) (Figure 2.5B & C).

CFU counts produced higher readings overall compared to qPCR results, which has also been observed in other studies [130]. Baxter *et al* [130] compared qPCR results and CFU counts in CF sputum and found higher CFU counts for some but not all samples. They suggest this discrepancy is likely due to sampling error as only a small proportion of the whole sample is used for the CFU count. There is also a potential for loss of DNA during the numerous processing steps for DNA extraction. Additionally *A. fumigatus* conidia are hydrophobic and clump which may lead to an over-representation in a small sample aliquot for CFU sampling or conversely a negative culture result depending on the aliquot taken, whereas the DNA extraction process homogenises the sample very thoroughly. Although CFU counts remained low at the 12 month follow-up, qPCR results showed a minor increase when compared to the 6 month follow-up, suggesting qPCR may be a more sensitive method of *Aspergillus* detection and quantification in CF sputum. [130]. Interestingly 62% of the cohort were all culture positive pre-itraconazole treatment and culture negative post-treatment (Figure 2.5A). However the qPCR results were positive both pre- and post-treatment (Figure 2.5A). This is likely due to sensitivity of the qPCR method but may take into effect the fact that qPCR will pick up dead organisms. There were three patients who failed to culture *A. fumigatus* but gene copies were detected for these samples by qPCR. This illustrates that standard culture alone may miss *A. fumigatus* present in the sample which has also been reported in other studies. Baxter *et al* found a number of CF sputum samples that were culture negative but PCR positive in every extraction method they tested [130]. However, it should be noted that while molecular methods are very sensitive they do not distinguish between live and dead organisms. Therefore, we suggest that both, standard culture and molecular methods should be used for the detection of *Aspergillus* in CF sputum.

A. fumigatus represents an important coloniser within the CF airway and has been shown to increase hospitalisations and worsen radiological appearances despite minimal effect on lung function [24, 166]. The significant reduction of *A. fumigatus* bioburden post-itraconazole treatment in this study illustrates the effectiveness of itraconazole at reducing *A. fumigatus* in the

airways of CF patients. Treating *A. fumigatus* colonised patients may lead to a reduction in hospitalisations a theory that has also been suggested by other studies [24]. The efficacy of itraconazole treatment has been carried out in other studies with a focus on ABPA and these found that treatment with itraconazole was associated with a reduction in ABPA exacerbations [28]. While other studies have linked itraconazole treatment to improved patient health or reduced lung exacerbations, no studies have directly linked this to a reduction in *A. fumigatus* bioburden in the airways [28, 50]. Therefore, these studies cannot draw conclusions on the antifungal properties of itraconazole being responsible for reduced lung exacerbations. The study presented here has clearly shown that treating asymptomatic *A. fumigatus* colonised CF patients reduced *Aspergillus* bioburden in the airways and this correlated with improved lung structure (a significant reduction in mosaic pattern following itraconazole treatment, measure by by sixty-four-slice-high-resolution computed tomography (HRCT)), decreased infective exacerbations and an overall improvement in respiratory symptoms, as measured by CFQR scoring system [59]. Due to a lack of patient symptoms many physicians do not treat CF patients who are culture positive for *A. fumigatus* and lacking ABPA symptoms. Studies have now shown that colonising *A. fumigatus* conidia are not innocent bystanders in the CF airways [24] [166]. McMahon *et al.*, [166] reported that patients with *A. fumi*gatus colonisation and no ABPA showed greater radiological abnormalities compared to patients not colonised by *A. fumigatus*. Our study further supports this finding as the reduction of *A. fumigatus* bioburden due to itraconazole treatment was associated with an improvement in radiological findings [59]. CF patients asymptomatically colonised with *A. fumigatus* may be acquiring lung damage which could contribute to the overall decline in lung capacity over their lifetime. Physicians should consider treating asymptomatic *Aspergillus* colonised CF patients.

Chapter 3

Antifungal Susceptibility and Epidemiological Analysis of *A. fumigatus*

Chapter 3

Antifungal Susceptibility and Epidemiological Analysis of *A. fumigatus*

3.0 Introduction

Aspergillus infection can be treated by a number of antifungals. The echinocandins, the triazoles and amphotericin B are among the most widely used. Resistance of *A. fumigatus* to the echinocandins or amphotericin B appear to be rare [170, 182]. The triazoles have been found to be active both *in vitro* and *in vivo* against *Aspergillus fumigatus* with the exception of fluconazole. However, resistance to the triazoles has been reported in a number of continents around the world, including Asia, the USA, Australia and Europe [189-197]. Triazole resistance has been observed in isolates from triazole treated and also triazole naïve patients [198]. Countries such as the Netherlands and the United Kingdom (UK) have reported a high incidence of triazole resistance which seems to be increasing [193, 199, 205]. However, the mode of resistance acquisition appears to be different between the two countries [193]. In Nijmegen in the Netherlands, triazole resistance in Dutch isolates has been attributed to the pressure of triazole fungicides used in agriculture with predominantly the TR34/L98H mutation [191, 193, 194, 208]. In Manchester in the UK the high incidence of triazole resistance was reported to be acquired after a prolonged triazole treatment of patients with pulmonary aspergillosis [191, 193]. In Ireland, Cystic Fibrosis (CF) patients are often treated with triazoles for fungal infections and when antibiotic treatment for a pulmonary exacerbation is unsuccessful. Therefore CF patients may incur a high rate of exposure to triazoles over their lifetime. Furthermore, like in the Netherlands, triazoles are also used as fungicides in Irish agriculture thus the emergence of triazole resistance in Ireland remains a possibility [194, 197, 208, 231, 232]. However, there are no publications reporting the presence or absence of triazole resistance of *Aspergillus* in Ireland and these gaps in the literature need to be addressed. Additionally, microbiology laboratories in Irish hospitals do not routinely test for antifungal drug susceptibility and therefore the presence or absence of resistance remains unclear and accurate determination of which antifungal drug to use is usually not defined. *A. fumigatus* isolates from our CF and non-CF cohorts were tested to assess the susceptibility of the strains to a panel of nine antifungal drugs.

Genotyping of fungi, namely *A. fumigatus* can help provide insight into colonisation patterns and possible sources and routes of transmission of isolates. Microsatellite typing, in particular the STR*Af* assay developed by deValk *et* al in 2005, has become the genotyping method of choice for most laboratories due to its high reproducibility, ease of exchange of data between laboratories,

suitability for large scale epidemiology studies and its high discriminatory power [7, 233]. Microsatellite typing is a genotyping method that can be used to distinguish between individual *A. fumigatus* isolates. Microsatellites or Short Tandem Repeats (STR) are short repetitive sequences that are abundantly found in genomes of eukaryotes [233]. In microsatellite typing, STRs are amplified by PCR using fluorescently labelled primers and the size of the fragments can then be accurately determined with capillary electrophoresis using allelic ladders [233]. Differences between isolates can be distinguished from each other based on the differences in numbers of STRs. In this study a panel of nine STRs for high resolution fingerprinting of *A. fumigatus* were used [233]. The nine markers are divided into 3 di-nucleotide repeat markers referred to as STR*Af*2 assay, 3 tri-nucleotide repeat markers known as the STR*Af*3 assay and 3 tetra-nucleotide repeat markers known as the STR*Af*4 assay [233]. Genotypes with similar STR numbers are more closely related and dendrograms are created from the STR genotyping results to reflect the phylogenetic relationship between strains. However, *A. fumigatus* can exist as asexual conidia but has also been shown to have a sexual cycle [234], hence this should be kept in mind as an organism that is reproducing sexually can produce new combinations of alleles that could produce a low percentage of similarity even though the organism is closely related to the parent organisms [235]. In contrast organisms that reproduce asexually do not share alleles among lineages and therefore a low percentage of similarity is indicative of distinguishable genotypes [235]. It should be noted however that while *A. fumigatus* has the ability to reproduce sexually, it requires specific parameters and tends to be rare [234]. O'Gorman *et al* discovered the sexual cycle in *A. fumigatus* in 2009, they found that sexual reproduction required; (1) isolates of complimentary types to be present (MAT-1 and MAT-2), (2) specific growth medium (oatmeal agar), (3) specific incubation temperature (30°C) and (4) in the absence of light [234]. The authors stated that although air sampling revealed that complimentary mating types were in close proximity to each other in the environment, sexual reproduction of *A. fumigatus* required environmental parameters rarely encountered in nature and therefore this method of reproduction is likely rare [234]. If the strains studied are not propagating via the sexual pathway but rather mostly asexually, then the dendrogram would best represent the phylogenetic relationship. Our aim was to assess the epidemiology of the *A. fumigatus* isolates collected in four Irish CF cohorts and other non-CF patients from two hospitals.

3.1 Materials and Methods

3.1.1 Confirmation of *Aspergillus* isolates as *A. fumigatus*

3.1.1.1 Harvesting of *Aspergillus* conidia from patient isolates

All *Aspergillus* isolates were cultured and harvested as per section 2.1.7.1.

3.1.1.2 Isolation of DNA from *Aspergillus* isolates

DNA was isolated from all *Aspergillus* isolates as per section 2.1.7.2.

3.1.1.3 Amplification of the Internal Transcribed Spacer (*ITS*) region

Amplification of the *ITS* region was performed as per section 2.1.7.3.

3.1.1.4 Gel electrophoresis of PCR products

PCR products from section 3.1.1. were separated by gel electrophoresis as per section 2.1.7.4.

3.1.1.5 PCR product Purification

PCR products from section 3.1.1.4 were purified and sent for nucleotide sequencing as per section 2.1.7.5.

3.1.2 Determination of *A. fumigatus* susceptibility by Minimum Inhibitory Concentration (MIC) or Minimum effective concentration (MEC) to a panel of antifungal drugs

Plates of each *A. fumigatus* isolate were grown on MEA and sub-cultured as per section 2.1.1. Conidia from each isolate were collected from fully grown plates with a cotton swab, suspended in PBST and left to settle for 3 to 5 min. Turbidity was adjusted to 80-82% transmittance at 530nm (equivalent to inoculum of 0.6-5 x 10^6 CFU/ml). From this 100µl of the inoculum was added to 11ml of YeastOne inoculum broth (Sensititre) at room temperature and mixed, to give a final inoculum of 0.5-5 x 10^4 CFU/ml. A volume of 100µl of the mixed broth was transferred into each well of a 96-well Yeastone plate (Trek Sensititre). The Sensititre method is comparable to the CLSI method which uses a 96 well, RPMI broth medium, 0.4-5 x 10^4 CFU/ml, 48 hr incubation and a MIC endpoint of complete inhibition of growth determined by visual inspection [236]. A colony count was performed by plating 10µl from the positive control well onto MEA. A CFU count of 50-500 qualified as a correct inoculum. The 96 well plates were covered with an adhesive seal and incubated at 35°C for 48 hr along with the colony count plates as per manufacturer's instructions [237]. After 48 hr plates were read visually using a reading mirror and CFU counts were determined. The echinocandin antifungals tend to produce a trailing growth on the Trek Sensititre plate system which makes visual reading of results problematic. Therefore,

echinocandin minimum effective concentration (MEC) were determined microscopically by the appearance of a stunted growth of hyphae showing short stubby hyphae and also a reduced turbidity compared to the control well [238]. Epidemiological cut-off values (ECV) from recent publications were used [154, 173, 186, 239]; caspofungin 1μg/ml, posaconazole 0.25μg/ml, itraconazole 1μg/ml, voriconazole 1μg/ml and amphotericin B 2μg/ml.

3.1.3 Genotyping of all CF and Non-CF *A. fumigatus* isolates

DNA was isolated from each *A. fumigatus* isolate collected. Additionally two A. *fumigatus* environmental isolates were collected by a Trinity College undergraduate student named Shane Gahan as part of his 4th year research project and these were also genotyped. The two environmental triazole resistant isolates were identified by the student. Collection, identification, antifungal sensitivity testing and mutation screening of the two environmental isolates were performed and submitted in fulfilment of the undergraduate final year research report for Shane Gahan. DNA from all isolates was extracted as per section 2.1.5.1.2 with the following exceptions; a 10^8 concentration of conidia from each isolate was used for DNA isolation and 100μl of PBST was added to each sample prior to the first centrifugation.

3.1.3.1 Multiplex PCR – the STR*Af* assay

Three multiplex PCRs were performed; the STR*Af*2 assay, STR*Af*3 assay and the STR*Af*4 assay [233]. For each multiplex PCR forward primers (Table 3.1) were labelled with FAM, HEX and TET respectively [233]. The following components were added to each 25μl PCR reaction; 13.8μl of molecular grade water (Sigma), 2.5μl of 10x buffer (Roche Diagnostics), 2μl of dNTP's (Roche Diagnostics), 0.5μM STR*Af*2 or STR*Af*3 or STR*Af*4 primer mix (Table 3.1, 0.2μl of Fast start Taq at 5U/μl (Roche Diagnostics), 3μl of MgCl-2 at 25 mM (Roche Diagnostics) and 1μl of template DNA. All primers used were constructed by Roche Diagnostics.

The following thermal cycle parameters were used; heated lid 111°C, hot start denaturation at 95°C for 10 min, 35 cycles, denaturation 95°C for 30 sec, annealing 60°C for 30 sec, elongation 72°C for 1 min, end cycle, elongation 72°C for 10 min and then cooled to room temperature.

3.1.3.2 DNA sequence analysis

DNA sequence analysis of the STR*Af* assay PCR products (section 3.1.2.1) was performed on the ABI 3500 XL Genetic Analyser. For each sequence analysis carried out, 1μl of diluted PCR product (1 in 200 dilution) and 9μl of diluted size standard CC500 ROX (Promega) (1 in 10 dilution) was added to a 48 well plate. Samples were heated to 95°C for 1min to produce single stranded DNA and then cooled to 4°C for 1min. Each plate of samples was then analysed on an ABI 3500 XL Genetic Analyser utilising gene mapper software version 4.0 (Applied Biosystems) according to manufacturer guidelines.

3.1.3.3 Construction of Dendrograms

Results were analysed and dendrograms and spanning trees produced. The repeat numbers of the nine markers of all isolates were analyzed by using BioNumerics, version 3.5, and the unweighted pair group method with arithmetic averages with the multistate categorical similarity coefficient. All markers were given an equal weight. In the resulting dendrogram, the indicated percentages reflect the number of corresponding markers. For example, two strains with six of the nine corresponding markers are 66.7% identical. Isolates with 100% identity were considered indistinguishable.

3.1.4 CF and Non-CF *Aspergillus* isolate Information

Table 3.1: Sample information for CF and non-CF patient samples from chapter

Sample Patient no (p), sample no (s), colony no (c)	Patient Type	Hospital no.	Cultured positive for *Aspergillus* (✓/×)
P14 s1 c1	CF	1	✓
p14 s1 c2	CF	1	✓
p14 s1 c3	CF	1	✓
p14 s1 c4	CF	1	✓
p14 s1 c5	CF	1	✓
p14 s2 c1	CF	1	✓
p14 s2 c2	CF	1	✓
p14 s2 c3	CF	1	✓
p14 s2 c4	CF	1	✓
p14 s2 c5	CF	1	✓
p15 s1 c1	CF	1	✓
p15 s1 c2	CF	1	✓
p15 s1 c3	CF	1	✓
p15 s1 c4	CF	1	✓
p15 s1 c5	CF	1	✓
p15 s2 c1	CF	1	✓
p15 s2 c2	CF	1	✓
p15 s2 c3	CF	1	✓
p15 s2 c4	CF	1	✓
p15 s2 c5	CF	1	✓
p16 s1 c1	CF	1	✓
p16 s1 c2	CF	1	✓
p16 s1 c3	CF	1	✓
p16 s1 c4	CF	1	✓
p16 s1 c5	CF	1	✓
p17 s1 c1	CF	1	✓
p17 s1 c2	CF	1	✓
p17 s1 c3	CF	1	✓
p17 s1 c4	CF	1	✓

Sample Patient no (p), sample no (s), colony no (c)	**Patient Type**	**Hospital no.**	**Cultured positive for *Aspergillus* (✓/×)**
p17 s1 c5	CF	1	✓
p18 s1 c1	CF	1	✓
p18 s1 c2	CF	1	✓
p18 s1 c3	CF	1	✓
p18 s1 c4	CF	1	✓
p18 s1 c5	CF	1	✓
p19 s1 c1	CF	1	✓
p19 s1 c2	CF	1	✓
p19 s1 c3	CF	1	✓
p19 s1 c4	CF	1	✓
p19 s1 c5	CF	1	✓
p20 s1	CF	2	✓
p21 s1	CF	2	✓
p22 s1	CF	2	✓
p23 s1	CF	2	✓
p23 s2	CF	2	✓
p24 s1	CF	2	✓
p25 s1	CF	2	✓
p26 s1	CF	2	✓
p27 s1	CF	2	✓
p28 s1	CF	2	✓
p29 s1	CF	2	✓
p30 s1	CF	2	✓
p30 s2	CF	2	✓
p31 s1	CF	2	✓
p32 s1	CF	2	✓
p33 s1	CF	2	✓
p34 s1	CF	2	✓
p35 s1	CF	2	✓
p36 s1	CF	2	✓
p37 s1	CF	2	✓
p38 s1	CF	2	✓

Sample Patient no (p), sample no (s), colony no (c)	**Patient Type**	**Hospital no.**	**Cultured positive for *Aspergillus* (✓/×)**
p39 s1	CF	2	✓
p40 s1	CF	2	✓
p41 s1	CF	2	✓
p42 s1	CF	2	✓
p43 s1	CF	2	✓
p44 s1	CF	2	✓
p45 s1	CF	2	✓
p46 s1	CF	2	✓
p47 s1	CF	2	✓
p48 s1	CF	2	✓
p49 s1	CF	2	✓
p50 s1	CF	2	✓
p51 s1	CF	3	✓
p52 s1	CF	3	✓
p53 s1	CF	3	✓
p54 s1	CF	3	✓
p55 s1	CF	3	✓
p56 s1	CF	4	✓
p56 s2	CF	4	✓
p56 s3	CF	4	✓
p56 s4	CF	4	✓
p57 s1	CF	4	✓
p57 s2	CF	4	✓
p57 s3	CF	4	✓
p58 s1	CF	4	✓
p58 s2	CF	4	✓
p59 s1	CF	4	✓
p59 s2	CF	4	✓
p60 s1	CF	4	✓
p61 s1	CF	4	✓
p61 s2	CF	4	✓
p61 s3	CF	4	✓

Sample Patient no (p), sample no (s), colony no (c)	**Patient Type**	**Hospital no.**	**Cultured positive for *Aspergillus* (✓/×)**
p62 s1	CF	4	✓
p63 s1 c1	CF	4	✓
p63 s1 c2	CF	4	✓
p63 s1 c3	CF	4	✓
p63 s1 c4	CF	4	✓
p63 s1 c5	CF	4	✓
p63 s2	CF	4	✓
p63 s3	CF	4	✓
p64 s1	CF	4	✓
p65 s1	CF	4	✓
p65 s2	CF	4	✓
p66 s1	Non-CF	1	✓
p67 s1 c1	Non-CF	1	✓
p67 s1 c3	Non-CF	1	✓
p67 s1 c4	Non-CF	1	✓
p67 s1 c5	Non-CF	1	✓
p68 s1	Non-CF	1	✓
p69 s1 c1	Non-CF	1	✓
p69 s1 c2	Non-CF	1	✓
p69 s1 c3	Non-CF	1	✓
p69 s1 c4	Non-CF	1	✓
p69 s1 c5	Non-CF	1	✓
p70 s1 c1	Non-CF	1	✓
p70 s1 c2	Non-CF	1	✓
p70 s1 c3	Non-CF	1	✓
p70 s1 c4	Non-CF	1	✓
p70 s1 c5	Non-CF	1	✓
p71 s1	Non-CF	5	✓
p72 s1	Non-CF	5	✓
p72 s2	Non-CF	5	✓
p73 s1	Non-CF	5	✓
p74 s1	Non-CF	5	✓

Sample Patient no (p), sample no (s), colony no (c)	**Patient Type**	**Hospital no.**	**Cultured positive for *Aspergillus* (✓/×)**
p74 s2	Non-CF	5	✓
p75 s1	Non-CF	5	✓
p75 s2	Non-CF	5	✓
p76 s1	Non-CF	5	✓
p77 s1	Non-CF	5	✓
p78 s1	Non-CF	5	✓
p79 s1	Non-CF	5	✓
p79 s2	Non-CF	5	✓
p80 s1	Non-CF	5	✓
p81 s1	Non-CF	5	✓
p82 s1	Non-CF	5	✓
p83 s1	Non-CF	5	✓
p84 s1	Non-CF	5	✓
p84 s2	Non-CF	5	✓
p85 s1	Non-CF	5	✓
p86 s1	Non-CF	5	✓
p87 s1	Non-CF	5	✓
p88 s1	Non-CF	5	✓
p89 s1	Non-CF	5	✓
p90 s1	Non-CF	5	✓
p91 s1	Non-CF	5	✓
p92 s1	Non-CF	5	✓
p93 s1	Non-CF	5	✓
p94 s1	Non-CF	5	✓
p95 s1	Non-CF	5	✓
p96 s1	Non-CF	5	✓
p97 s1	Non-CF	5	✓
p98 s1	Non-CF	5	✓
p99 s1	Non-CF	5	✓
p100 s1	Non-CF	5	✓
p101 s1	Non-CF	5	✓
p102 s1	Non-CF	5	✓

Sample Patient no (p), sample no (s), colony no (c)	**Patient Type**	**Hospital no.**	**Cultured positive for *Aspergillus* (✓/×)**
p103 s1	Non-CF	5	✓
p104 s1	Non-CF	5	✓
p105 s1	Non-CF	5	✓
p106 s1	Non-CF	5	✓
p107 s1	Non-CF	5	✓
p108 s1	Non-CF	5	✓
p109 s1	Non-CF	5	✓
p110 s1	Non-CF	5	✓

Table 3.2: Primer Sequence Information for STR*Af* assay

STR*Af* primer	Primer Label	Forward	Reverse	Repeat Unit
STR*Af*2 2A	FAM	AAGGGTTATGGCCATTAGGG	GACCTCCAGGCAAAATGAGA	GA
STR*Af*2 2B	HEX	TATTGGATCTGCTCCCAAGC	GAGATCATGCCCAAGGATGT	AG
STR*Af*2 2C	TET	TCGGAGTAGTTGCAGGAAGG	AACGCGTCCTAGAATGTTGC	CA
STR*Af*3 3A	FAM	GCTTCGTAGAGCGGAATCAC	GTACCGCTGCAAAGGACAGT	TCT
STR*Af*3 3B	HEX	CAACTTGGTGTCAGCGAAGA	GAGGTACCACAACACAGCACA	AAG
STR*Af*3 3C	TET	GGTTACATGGCTTGGAGCAT	GTACACAAAGGGTGGGATGG	TAG
STR*Af*4 4A	FAM	TTGTTGGCCGCTTTTACTTC	GACCCAGCGCCTATAAATCA	TTCT
STR*Af*4 4B	HEX	CGTAGTGACCTGAGCCTTCA	GGAAGGCTGTACCGTCAATCT	CTAT
STR*Af*4 4C	TET	CATATTGGGAAACCCACTCG	ACCAACCCATCCAATTCGTAA	ATGT

3.2 Results

3.2.1 Amplification of the *ITS* region from cultured *Aspergillus* isolates

CF (n=107) and non-CF (n=61) *Aspergillus* isolates were grown and the DNA extracted for amplification of the *ITS* region to confirm isolates as fumigatus or non-fumigatus. Agarose gels of these amplicons are shown in Figure 3.1 and 3.2 All PCR amplifications produced a single 500bp PCR product (Figure 3.1 and 3.2) as can be seen from the 500bp band present. Positive and negative controls for these PCRs indicated successful amplification and the absence of contaminating factors, respectively. Sequence analysis results of PCR products identified all isolates as *A. fumigatus* with 99 to 100% identity (Appendix I).

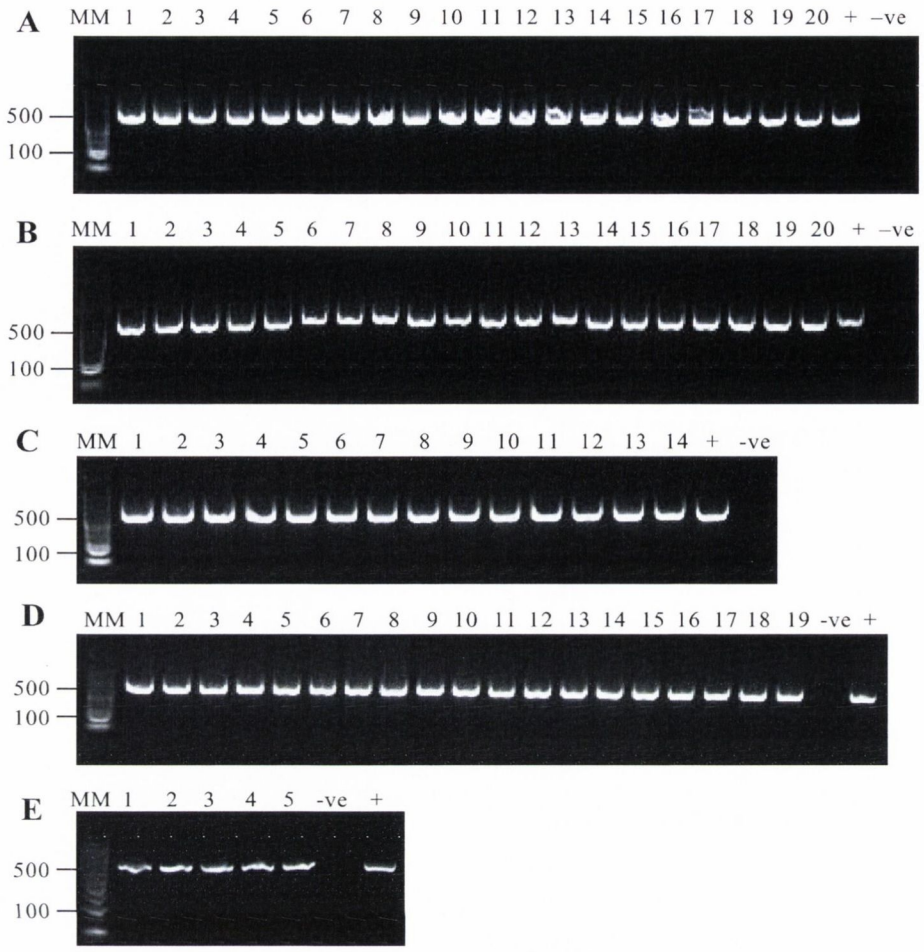


Figure 3.1: Amplification of the *ITS* region from Clinical *Aspergillus* isolates

Represent *ITS* rRNA regions amplified from *A. fumigatus* clinical isolates. **A)** Hospital 1 CF isolates; Lanes 1= p14 s1 c1, 2= p14 s1 c2, 3= p14 s1 c3, 4= p14 s1 c4, 5= p14 s1 c5, 6= p14 s2 c1, 7= p14 s2 c2, 8= p14 s2 c3, 9= p14 s2 c4, 10= p14 s2 c5, 11= p15 s1 c1, 12= p15 s1 c2, 13= p15 s1 c3, 14= p15 s1 c4, 15= p15 s1 c5, 16= p15 s2 c1, 17= p15 s2 c2, 18= p15 s2 c3, 19= p15 s2 c4 and 20= p15 s2 c5 . **B)** Hospital 1 CF isolates; Lanes 1= p16 s1 c1, 2= p16 s1 c2, 3= p16 s1 c3, 4= p16 s1 c4, 5= p16 s1 c5, 6= p17 s1 c1, 7= p17 s1 c2, 8= p17 s1 c3, 9= p17 s1 c4, 10= p17 s1 c5, 11= p18 s1 c1, 12= p18 s1 c2, 13= p18 s1 c3, 14= p18 s1 c4, 15= p18 s1 c5, 16= p19 s1 c1, 17= p19 s1 c2, 18= p19 s1 c3, 19= p19 s1 c4 and 20= p19 s1 c5 . **C)** Hospital 2 CF isolates; Lanes 1= p20 s1, 2= p21 s1, 3= p22 s1, 4= p23 s1, 5= p23 s2, 6= p24 s1, 7= p25 s1, 8= p26 s1, 9= p27 s1, 10= p28 s1, 11= p29 s1, 12= p30 s1, 13= p30 s2 and 14= p31 s1. **D)** Hospital 2 CF isolates; Lanes 1= p32 s1, 2= p33 s1, 3= p34 s1, 4= p35 s1, 5= p36 s1, 6= p37 s1, 7= p38 s1, 8= p39 s1, 9= p40 s1, 10= p41 s1, 11= p42 s1, 12= p43 s1, 13= p44 s1, 14= p45 s1, 15= p46 s1, 16= p47 s1, 17= p48 s1, 18= p49 s1 and 19= p50 s1. **E)** Hospital 3 CF isolates; Lanes 1= p51 s1, 2= p52 s1, 3= p53 s1, 4= p53 s1 and 5= p55 s1.*ITS* PCR products are represented by the 500bp band. MM is the molecular weight marker, lanes 1-n represent patient samples (section 3.1.4) and –ve is the negative PCR control, positive control (+).

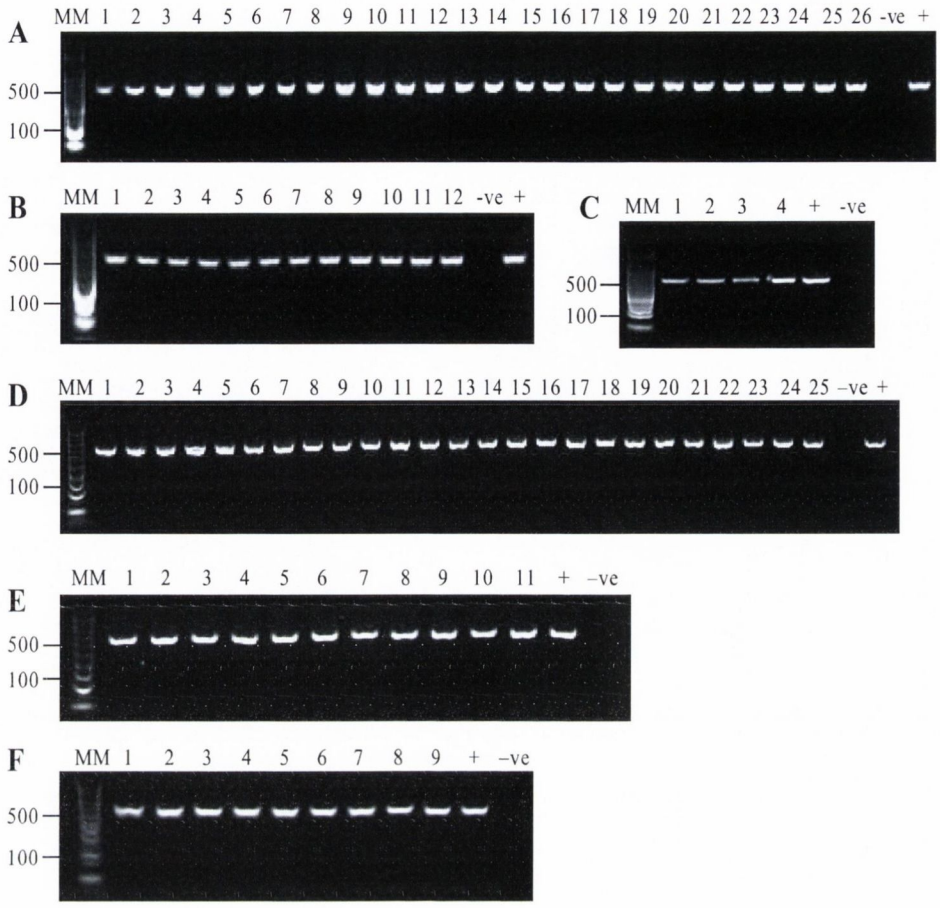


Figure 3.2 Amplification of the *ITS* region from Clinical *Aspergillus* isolates

Represent *ITS* rRNA regions amplified from *A. fumigatus* clinical isolates, **A)** Hospital 4 CF isolates; Lanes 1= p56 s1, 2= p56 s2, 3= p56 3, 4= p56 s4, 5= p57 s1, 6= p57 s2, 7= p57 s3, 8= p58 s2, 9= p58 s2, 10= p59 s1, 11= p59 s2, 12= p60 s1, 13= p61 s1, 14= p61 s2, 15= p61 s3, 16= p62 s1, 17= p63 s1 c1, 18= p63 s1 c2, 19= p63 s1 c3, 20= p63 s1 c4, 21= p63 s1 c5, 22= p63 s2, 23= p63 s3, 24= p64 s1, 25= p65 s1 and 26= p65 s2. **B)** Hospital 1 non-CF isolates; Lanes 1= p66 s1, 2= p67 s1 c1, 3= p67 s1 c3, 4= p67 s1 c4, 5= p67 s1 c5, 6= p68 s1, 7= p69 s1 c1, 8= p69 s1 c2, 9= p69 s1 c3, 10= p69 s1 c4, 11= p69 s1 c5 and 12= p70 s1 c1. **C)** Hospital 1 non-CF isolates; Lanes 1= p70 s1 c2, 2= p70 s1 c3, 3= p70 s1 c4 and 4= p70 s1 c5. **D)** Hospital 5 non-CF isolates; Lanes 1= p71 s1, 2= p72 s1, 3= p72 s2, 4= p73 s1, 5= p74 s1, 6= pp74 s2, 7= p75 s1, 8= p75 s2, 9= p76 s1, 10= p77 s1, 11= p78 s1, 12= p79 s1, 13= p79 s2, 14= p80 s1, 15= p81 s1, 16= p82 s1, 17= p83 s1, 18= p84 s1, 19= p84 s2, 20= p85 s1, 21= p86 s1, 22= p87 s1, 23= p88 s1, 24= p89 s1 and 25= p90 s1 . **E)** Hospital 5 non-CF isolates; Lanes 1= p91 s1, 2= p92 s1, 3= p93 s1, 4= p94 s1, 5= p95 s1, 6= p96 s1, 7= p97 s1, 8= p98 s1, 9= p99 s1, 10= p100 s1 and 11= p101 s1. **F)** Hospital 5 non-CF isolates; Lanes 1= p102 s1, 2= p103 s1, 3= p104 s1, 4= p105 s1, 5= p106 s1, 6= p107 s1, 7= p108 s1, 8= p109 s1 and 9= p110 s1. *ITS* PCR products are represented by the 500bp band. MM is the molecular weight marker, lanes 1-n represent patient samples (section 3.1.4) and –ve is the negative PCR control, positive control (+).

3.2.2 Determination of antifungal drug susceptibility of all *A. fumigatus* isolates

All *A. fumigatus* isolates were grown in the presence of a panel of nine antifungal drugs: the echinocandins (anidulafungin, micafungin and caspofungin), the triazoles (posaconazole, voriconazole, itraconazole and fluconazole), 5-flucytosine and amphotericin B, and the MICs were established. Considering the Trek Sensititre plate system used is comparable to the CLSI method [236] and CLSI breakpoints were not available ECVs from recent publications were used [173, 186, 239]. A MIC or MEC value exceeding the ECV would be suggestive of the presence of resistance. For this study we used ECVs from recent publications [173, 186, 239]; caspofungin 1μg/ml, posaconazole 0.25μg/ml, itraconazole 1μg/ml, voriconazole 1μg/ml and amphotericin B 2μg/ml. Within our CF and non-CF cohorts no antifungal drug resistance was observed for the panel of echinocandins (Figure 3.3 and Appendix II). For the echinocandin antifungals; Minimum effective concentrations (MEC's) of anidulafungin ranged between <0.015 and 1 μg/ml (Figure 3.3A), micafungin from <0.008 to 1 μg/ml (Figure 3.3B) and caspofungin from <0.008 to 1 μg/ml (Figure 3.3C) (Appendix II). The Minimum inhibitory concentrations (MIC's) for 5-flucytosine ranged from 1 to 64μg/ml (Figure 3.5A and Appendix II). No triazole resistance was found within any of our *A. fumigatus* isolates with the exception of fluconazole (Figure 3.4 and Apendix II) which has been reported to be inactive against *Aspergillus* [170]. For the triazoles; MIC's of posaconazole ranged from <0.008 to 0.25 μg/ml (Figure 3.4A), voriconazole from 0.015 to 1 μg/ml (Figure 3.4B), itraconazole from <0.015 to 1 μg/ml (Figure 3.4C) and fluconazole from 128 to >256 μg/ml (Figure 3.4D) (Appendix II). For amphotericin B MIC's of 0.25 to 2 μg/ml were recorded (Figure 3.5B and Appendix II). From our results 30 of the 187 isolates tested were equal to the ECV value of 1μg/m for the echinocandin, caspofungin (Figure 3.3C and Appendix II). For posaconazole 19 out of the 187 isolates tested were equal to the ECV and all remaining isolates had MICs below the ECV (Figure 3.4 A and Appendix II). For itraconazole 5 of the 187 isolates tested were equal to the ECV and all remaining isolates were below the ECV (Figure 3.4C and Appendix II). Three isolates of the 187 tested had voriconazole MIC's equal to the ECV and all remaining isolates below the ECV value (Figure 3.4B and Appendix II). Finally for amphotericin B 22 of the 187 isolates tested were equal to the ECV and the remaining isolates had MICs below that of the ECV value (Figure 3.5B and Appendix II).

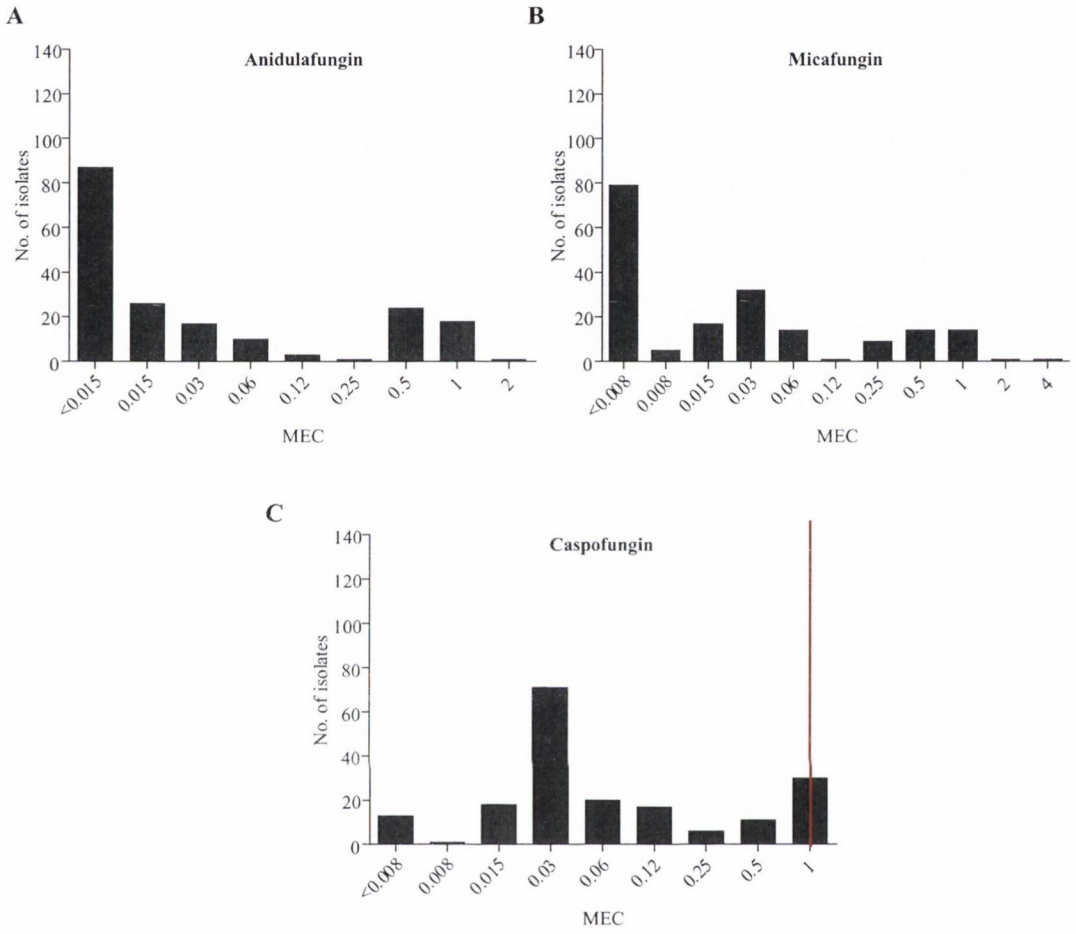


Figure 3.3: Minimum Effective Concentration (MEC) of the echinocandin antifungals

The MEC of 184 clinical and 3 reference strains of *A. fumigatus* against the echinocandins; **A)** anidulafungin, **B)** micafungin and **C)** caspofungin. The red line (-) indicates the epidemiological cut off value (ECV) for caspofungin [239]. The y-axis represents the number of *A. fumigatus* isolates and the x-axis represents the MEC for the antifungal drug.

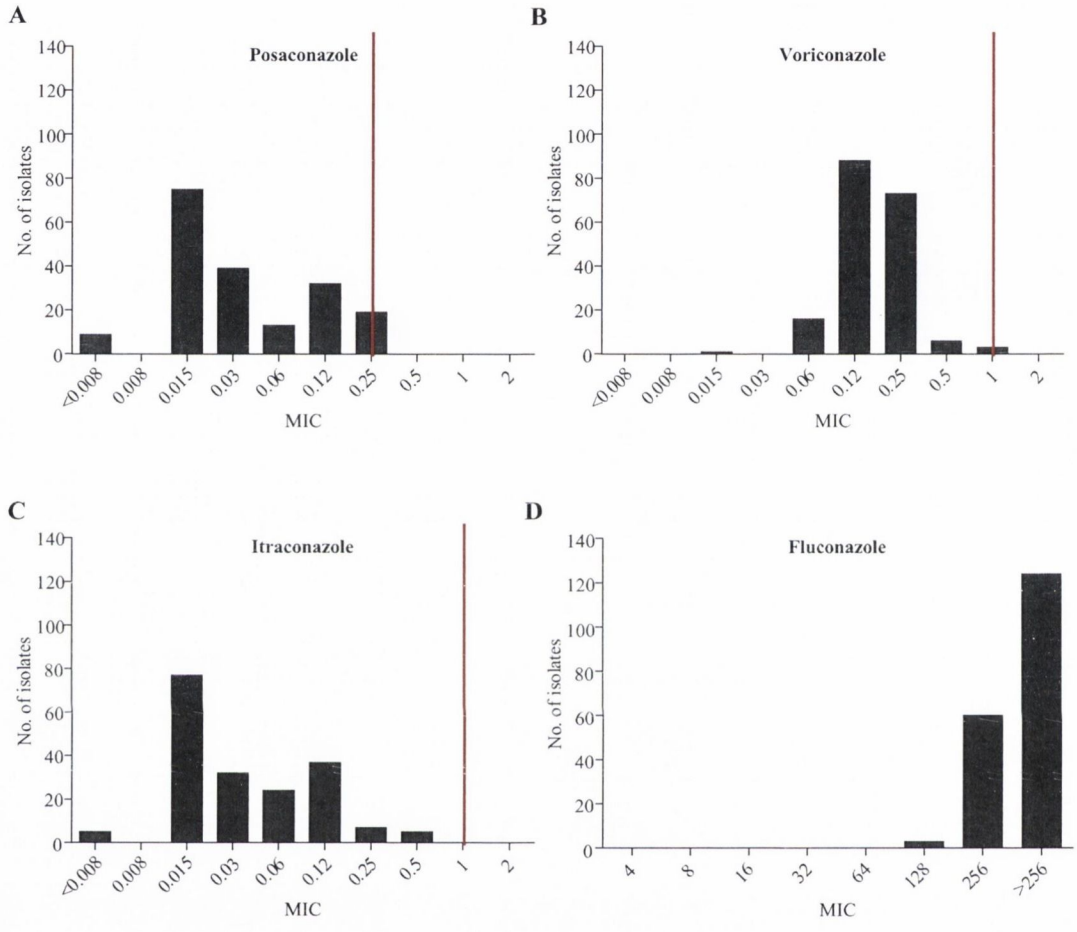


Figure 3.4: Minimum Inhibitory Concentration (MIC) of the triazole antifungals

The MIC of 184 clinical and 3 reference strains of *A. fumigatus* against the triazoles; **A)** posaconazole, **B)** voriconazole, **C)** itraconazole and **D)** fluconazole. The red line (-) indicates the epidemiological cut off value (ECV) for the antifungal drug [173, 186]. The y-axis represents the number of *A. fumigatus* isolates and the x-axis represents the MIC for the antifungal drug.

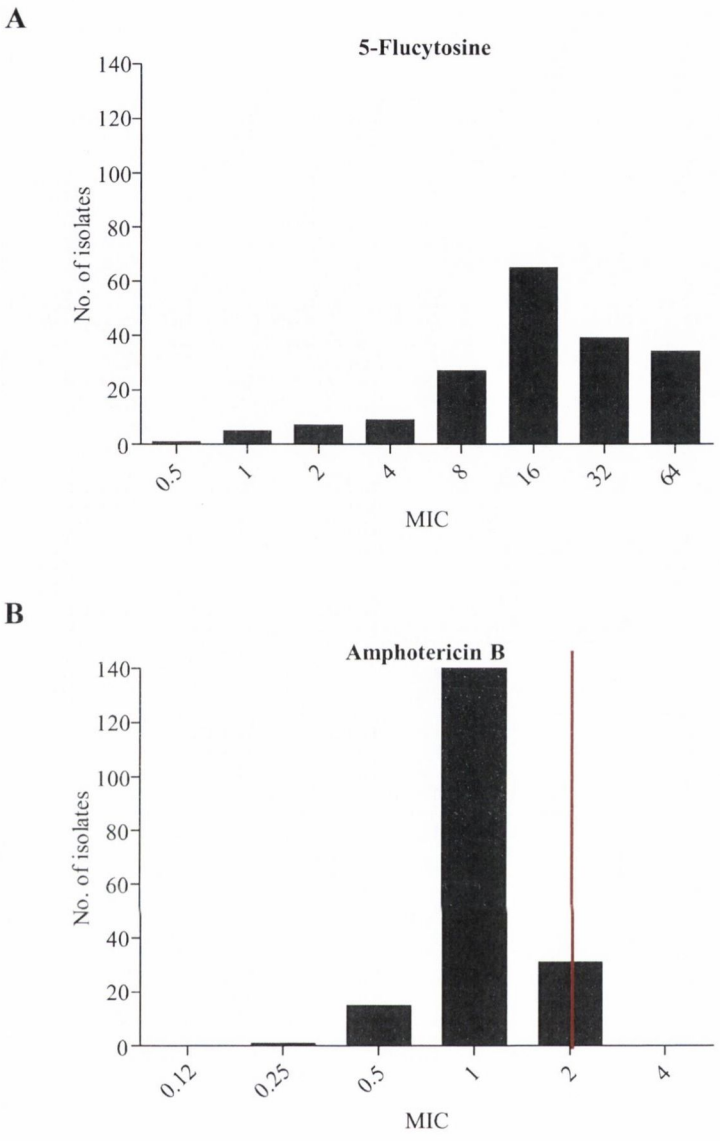


Figure 3.5: Minimum Inhibitory Concentration (MIC) of 5-flucytosine and Amphotericin B
The MIC of 184 clinical and 3 reference strains of *A. fumigatus* against **A)** 5-flucytosine and **B)** amphotericin B. The red line (-) indicates the epidemiological cut off value (ECV) for amphotericin B [154]. The y-axis represents the number of *A. fumigatus* isolates and the x-axis represents the MIC for the antifungal drug.

3.2.3 Multiplex PCR – the STRAf assay

DNA was isolated from each *A. fumigatus* isolate and three multiplex PCRs were performed [233]. All PCRs were carried out successfully. DNA sequence analysis of the STR*Af* assay PCR products was performed and results were analysed by BioNumerics version 3.5 software. The repeat numbers of the nine markers of all isolates were analyzed by using BioNumerics, version 3.5, and the unweighted pair group method with arithmetic averages with the multistate categorical similarity coefficient. All markers were given an equal weight. In the resulting dendrogram, the indicated percentages reflect the number of corresponding markers.

3.2.3.1 Epidemiological analysis of *A. fumigatus* isolates from four CF Centres

In order to assess the association of *A. fumigatus* genotypes with different variables (hospital, colonisation over time and patient type) the same dendrogram is depicted in figure 3.7 and 3.8 with the associated colour-coded table differing based on the variable being analysed. CF isolates from four CF centres displayed a large diversity of genotypes (Figure 3.6). This is illustrated in figure 3.6 where 66 genotypes were found in the 85 isolates investigated. Additionally the spanning tree showed a wide genotypic diversity with numerous branches (Figure 3.6).

From the four CF centres only one example of an indistinguishable isolate (p64 s1 and p14 s1) shared between two hospitals was found (Arrow in Figure 3.7). There did not appear to be any clustering of genotypes within each hospital cohort. Two patterns of colonisation were observed in our CF cohorts; persistent colonisation defined here as colonisation with an indistinguishable isolate (100% similarity) over time (identification of an indistinguishable genotype from two or more consecutive samples) and non-persistent colonisation defined here as colonisation with distinguishable isolates over time (no occurrence of the same isolate in consecutive samples). Furthermorepatients sharing an indistinguishable genotype (indistinguishable genotype present in more than one patient) was also observed (Figure 3.8). In total we had consecutive isolates from 16 CF patients and samples were collected from patients ranging from 1-24 months apart. Two patients (Figure 3.8; p10 and p57) fell into the persistent colonisation pattern. Three patients (p1, p2 and p59) showed examples where consecutive samples were very closely related (90% similarity). One patient (p61) showed evidence of persistent colonisation, where indistinguishable isolates were identified from the first two consecutive samples, followed by clearance, where on the third consecutive sample a distinguishable genotype was found (Figure 3.8). Two patients (p56 and p63) showed recurrence of a genotype, where the genotype identified from their first sample (p56 s1 and p63 s1) and their last sample (p56 s4 and p63 s3) were indistinguishable however their intermittent samples were distinguishable from the first and last sample. The remaining eight patients with consecutive samples were found to have non-persistent colonisation with distinguishable genotypes over time (Figure 3.8).

A number of incidences of patients sharing an indistinguishable isolate were observed (Figure 3.8). Some patients who were persistently colonised also shared isolates with other patients; one patient (p10) was colonised with an indistinguishable genotype over four consecutive samples and this indistinguishable genotype was also found in three other CF patients (p5, 6 and 17) (Figure 3.8). Interestingly one CF isolate from patient 36 (p36 s1) appeared to be very different from all other isolates in the CF cohorts. There did not appear to be any specific clustering of isolates within the CF groups as illustrated by the large diversity of CF genotypes demonstrated by a number of different branches connecting the 66 genotypes found (Figure 3.6).

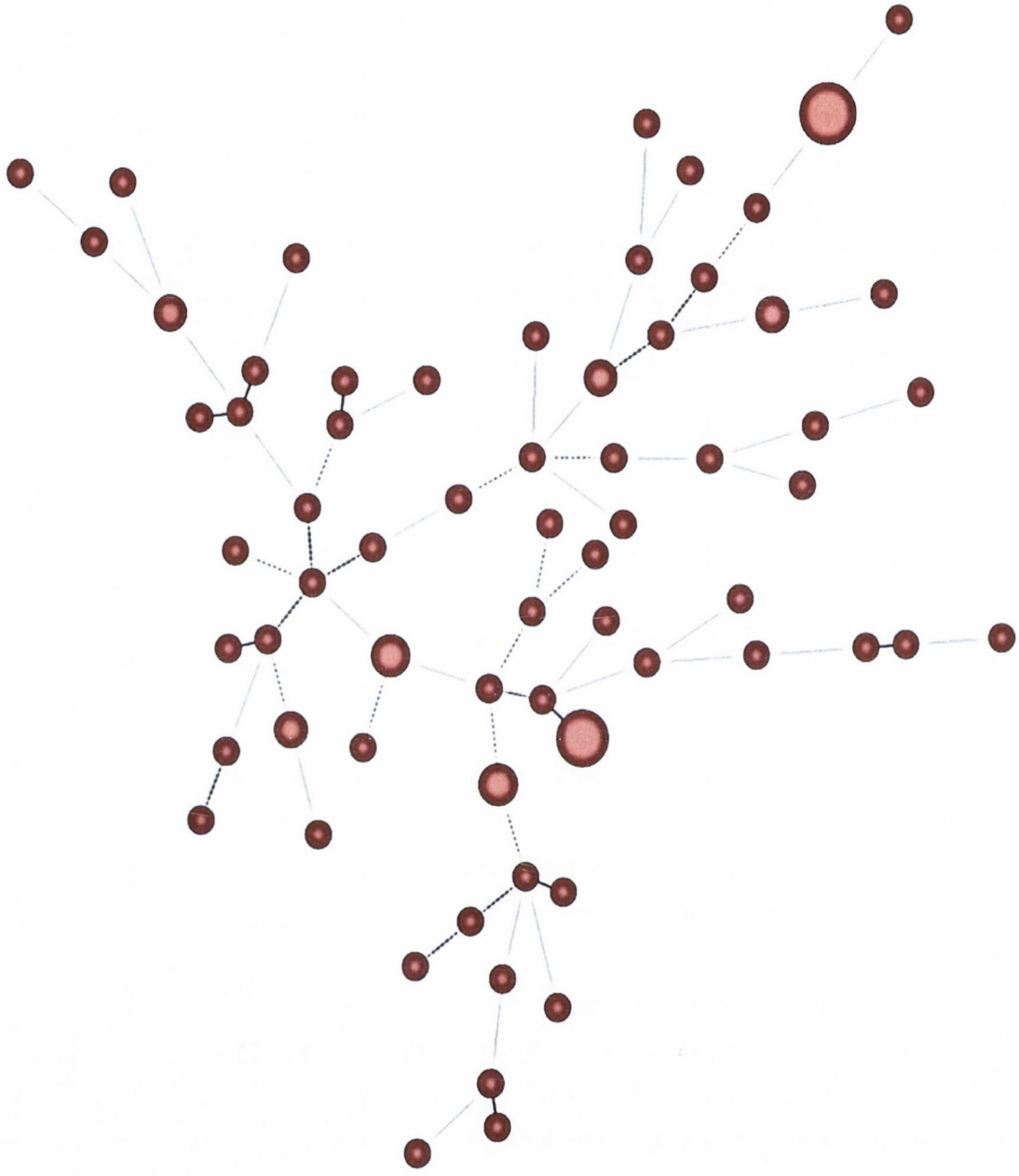
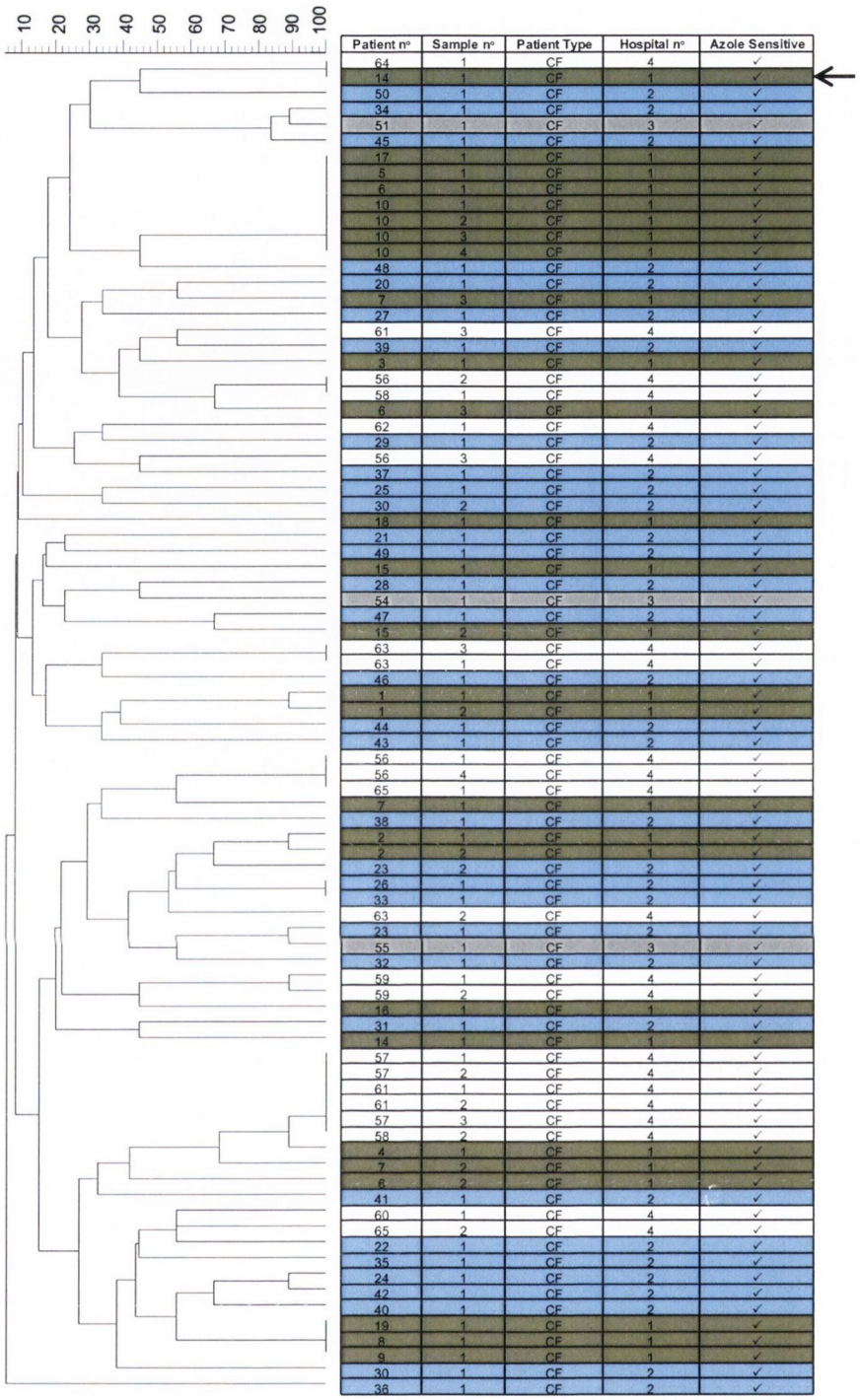


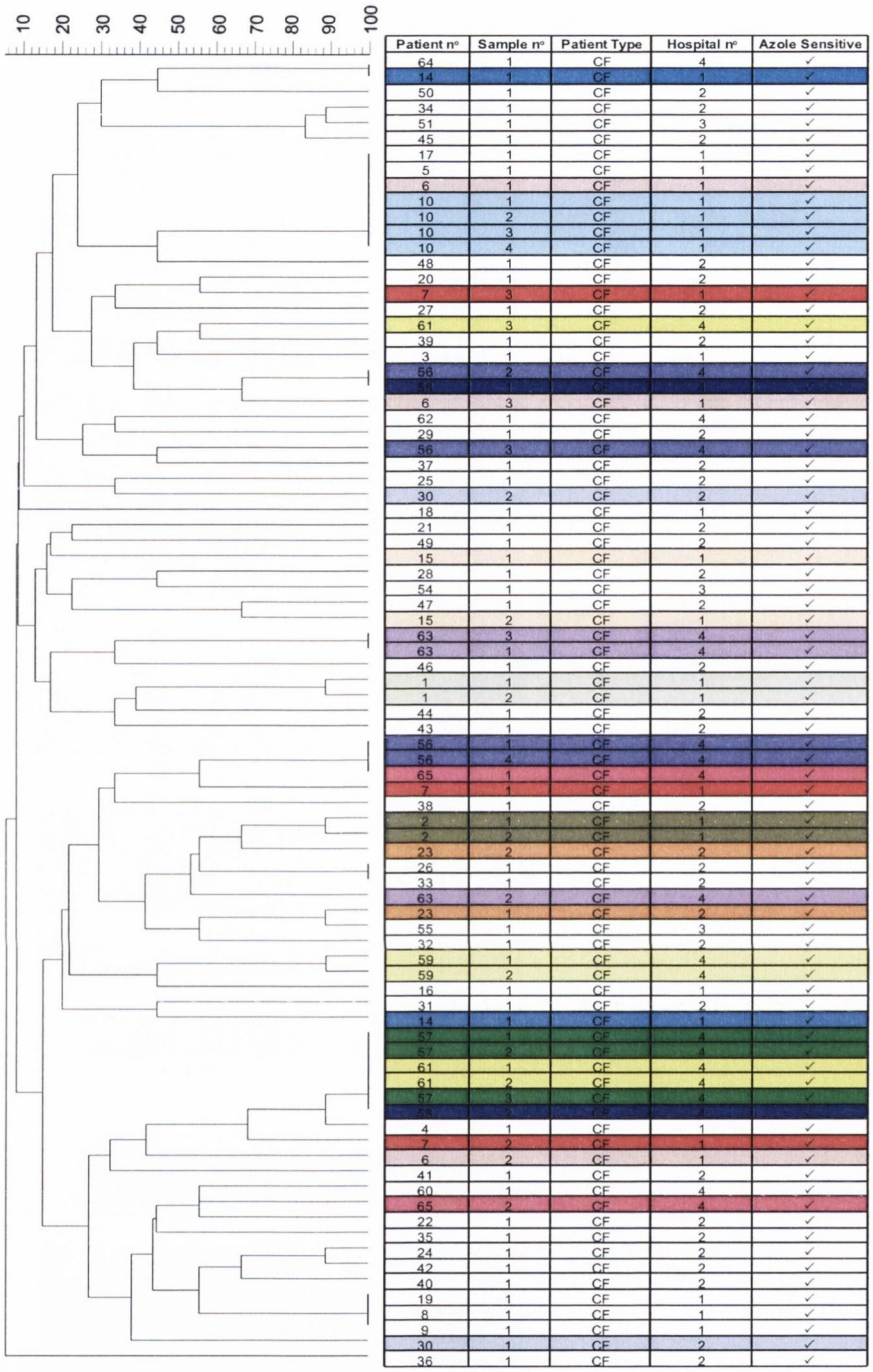
Figure 3.6: Spanning Tree of microsatellite typing of CF isolates

Spanning tree representation of figure 3.7/3.8; the figure shows the 66 genotypes (red circles) of the 85 CF *A. fumigatus* isolates investigated and the number of strains belonging to the same genotype (sizes of the circles). Each small red circle represents 1 single CF *A. fumigatus* isolate, with each circle increasing in size representing 2, 3, 4 etc. A solid thick black line (-) denotes a 1 marker difference out of 9 STR markers. A thin grey solid line (-) denotes 2 markers difference out of 9 markers. A thick black dashed line (---) represents 3 markers difference out of 9 markers. A thin grey dashed line (---) represents >4 markers difference out of 9 markers.



Patient n°	Sample n°	Patient Type	Hospital n°	Azole Sensitive
64	1	CF	4	✓
14	1	CF	1	✓
50	1	CF	2	✓
34	1	CF	2	✓
51	1	CF	3	✓
45	1	CF	2	✓
17	1	CF	1	✓
5	1	CF	1	✓
6	1	CF	1	✓
10	1	CF	1	✓
10	2	CF	1	✓
10	3	CF	1	✓
10	4	CF	1	✓
48	1	CF	2	✓
20	1	CF	2	✓
7	3	CF	1	✓
27	1	CF	2	✓
61	3	CF	4	✓
39	1	CF	2	✓
3	1	CF	1	✓
56	2	CF	4	✓
58	1	CF	4	✓
8	3	CF	1	✓
62	1	CF	4	✓
29	1	CF	2	✓
56	3	CF	4	✓
37	1	CF	2	✓
25	1	CF	2	✓
30	2	CF	2	✓
18	1	CF	1	✓
21	1	CF	2	✓
49	1	CF	2	✓
15	1	CF	1	✓
28	1	CF	2	✓
54	1	CF	3	✓
47	1	CF	2	✓
15	2	CF	1	✓
63	3	CF	4	✓
63	1	CF	4	✓
46	1	CF	2	✓
1	1	CF	1	✓
1	2	CF	1	✓
44	1	CF	2	✓
43	1	CF	2	✓
56	1	CF	4	✓
56	4	CF	4	✓
65	1	CF	4	✓
7	1	CF	1	✓
38	1	CF	2	✓
2	1	CF	1	✓
2	2	CF	1	✓
23	2	CF	2	✓
26	1	CF	2	✓
33	1	CF	2	✓
63	2	CF	4	✓
23	1	CF	2	✓
55	1	CF	3	✓
32	1	CF	2	✓
59	1	CF	4	✓
59	2	CF	4	✓
16	1	CF	1	✓
31	1	CF	2	✓
14	1	CF	1	✓
57	1	CF	4	✓
57	2	CF	4	✓
61	1	CF	4	✓
61	2	CF	4	✓
57	3	CF	4	✓
58	2	CF	4	✓
4	1	CF	1	✓
7	2	CF	1	✓
6	2	CF	1	✓
41	1	CF	2	✓
60	1	CF	4	✓
65	2	CF	4	✓
22	1	CF	2	✓
35	1	CF	2	✓
24	1	CF	2	✓
42	1	CF	2	✓
40	1	CF	2	✓
19	1	CF	1	✓
8	1	CF	1	✓
9	1	CF	1	✓
30	1	CF	2	✓
36	1	CF	2	✓

Figure 3.7: Dendrogram of microsatellite typing of CF isolates from 4 hospitals colour coded based on hospital. Metadata associated with the dendrogram is colour coded based on hospital number; hospital 1(■), hospital 2 (■), hospital 3(■) and hospital 4 (□). Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate. Arrow indicates a genotype shared between two CF centres. A larger scale of this dendrogram is available in appendix III.



Patient n°	Sample n°	Patient Type	Hospital n°	Azole Sensitive
64	1	CF	4	✓
14	1	CF	1	✓
50	1	CF	2	✓
34	1	CF	2	✓
51	1	CF	3	✓
45	1	CF	2	✓
17	1	CF	1	✓
5	1	CF	1	✓
6	1	CF	1	✓
10	1	CF	1	✓
10	2	CF	1	✓
10	3	CF	1	✓
10	4	CF	1	✓
48	1	CF	2	✓
20	1	CF	2	✓
7	3	CF	1	✓
27	1	CF	2	✓
61	3	CF	4	✓
39	1	CF	2	✓
3	1	CF	1	✓
58	2	CF	4	✓
58	3	CF	4	✓
6	3	CF	1	✓
62	1	CF	4	✓
29	1	CF	2	✓
58	3	CF	4	✓
37	1	CF	2	✓
25	1	CF	2	✓
30	2	CF	2	✓
18	1	CF	1	✓
21	1	CF	2	✓
49	1	CF	2	✓
15	1	CF	1	✓
28	1	CF	2	✓
54	1	CF	3	✓
47	1	CF	2	✓
15	2	CF	1	✓
63	3	CF	4	✓
63	1	CF	4	✓
46	1	CF	2	✓
1	1	CF	1	✓
1	2	CF	1	✓
44	1	CF	2	✓
43	1	CF	2	✓
56	1	CF	4	✓
56	4	CF	4	✓
65	1	CF	4	✓
7	1	CF	1	✓
38	1	CF	2	✓
2	1	CF	1	✓
2	2	CF	1	✓
23	2	CF	2	✓
26	1	CF	2	✓
33	1	CF	2	✓
63	2	CF	4	✓
23	1	CF	2	✓
55	1	CF	3	✓
32	1	CF	2	✓
59	1	CF	4	✓
59	2	CF	4	✓
16	1	CF	1	✓
31	1	CF	2	✓
14	1	CF	1	✓
57	1	CF	4	✓
57	2	CF	4	✓
61	1	CF	4	✓
61	2	CF	4	✓
57	3	CF	4	✓
58	1	CF	4	✓
4	1	CF	1	✓
7	2	CF	1	✓
6	2	CF	1	✓
41	1	CF	2	✓
60	1	CF	4	✓
65	2	CF	4	✓
22	1	CF	2	✓
35	1	CF	2	✓
24	1	CF	2	✓
42	1	CF	2	✓
40	1	CF	2	✓
19	1	CF	1	✓
8	1	CF	1	✓
9	1	CF	1	✓
30	1	CF	2	✓
36	1	CF	2	✓

Figure 3.8: Dendrogram of microsatellite typing of CF isolates from 4 hospitals, colour coded based on consecutive CF isolates over time. Metadata associated with the dendrogram is colour coded based on patients with a number of samples over time. Patient; p1-(■), p2-(■), p6-(■), p7-(■), p10-(■), p14-(■), p15-(■), p23-(■), p30-(■), p56-(■), p57-(■), p58- (■), p59-(■), p61-(■), p63-(■) and p65-(■). All unshaded boxes (□) are CF samples where only one sample was taken. Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate. A larger scale of this dendrogram is available in appendix IV.

3.2.3.2 Epidemiology of CF and non-CF *A. fumigatus* isolates

In addition to the CF isolates, a collection of non-CF *A. fumigatus* isolates from non-CF patients (a non-CF bronchiectasis cohort from hospital 1 and any non-CF clinical *A. fumigatus* isolate received into the medical laboratory from hospital 5) were genotyped as a comparative group.

From the 107 *A. fumigatus* isolates (61 CF, 44 non-CF and 2 environmental isolates) tested, 91 genotypes were found (Figure 3.9). Only one example the of a CF and a non-CF patient sharing an indistinguishable isolate was found (Figure 3.11), however a number examples of a CF and a non-CF patient isolate being closely related (90% similarity) was observed (Figure3.11). Like the CF cohorts, the non-CF cohorts showed examples of patients sharing indistinguishable isolates.

Notably the triazole resistant environmental isolates collected by Shane Gahan were distinguishable from all clinical isolates (Figure 3.9, 3.10 and 3.11). These environmental isolates were not closely related (15% similarity) and were distributed over two clades in the dendrogram (Fig 3.10). There did not appear to be any specific clustering based on CF or non-CF isolates as CF and non-CF isolates where found within each clade and branch of isolates in the dendrogram and spanning tree respectively (Figure 3.9 and 3.11).

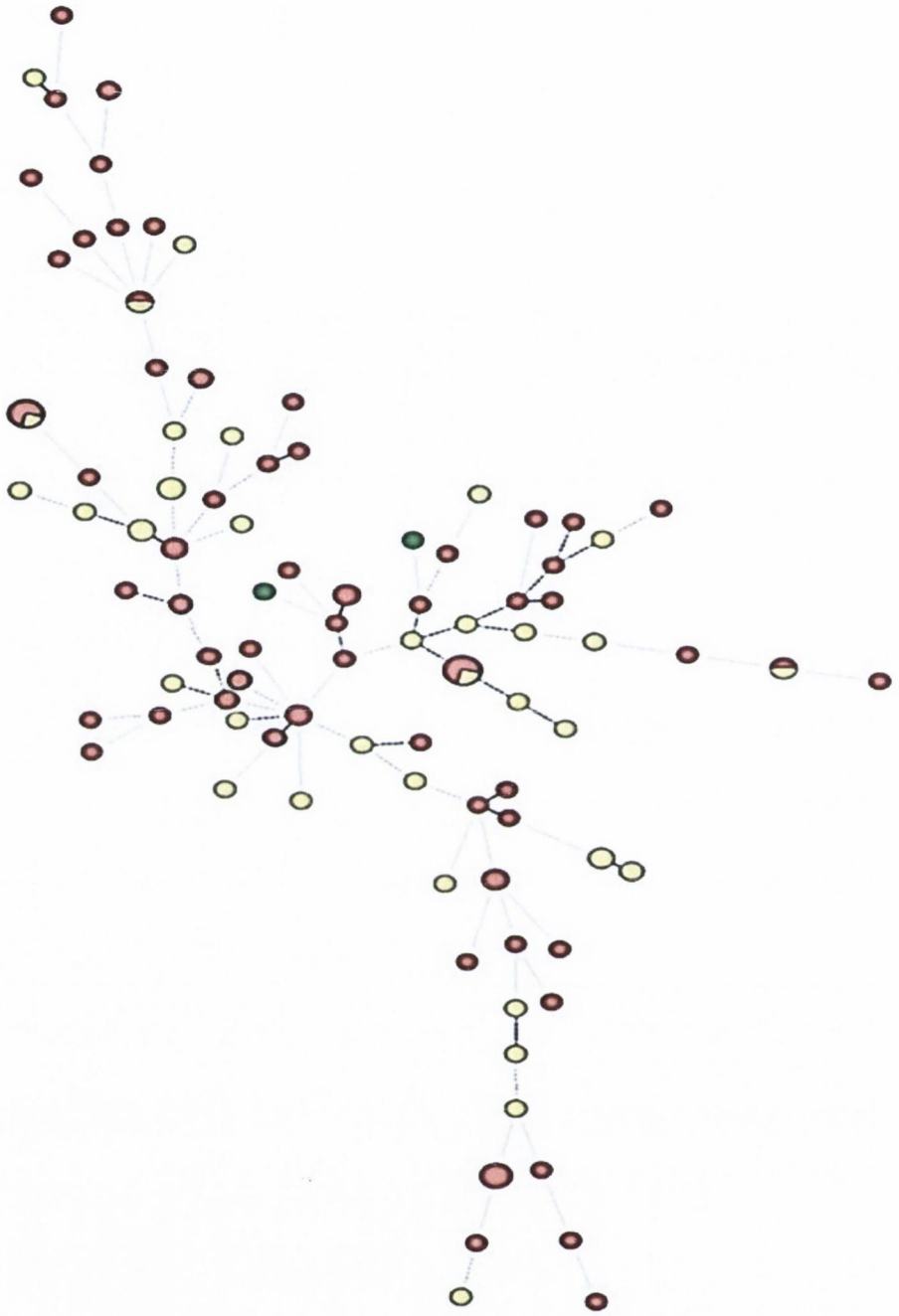


Figure 3.9: Spanning Tree of microsatellite typing of CF isolates

Spanning tree representation of figure 3.10/3.11; the figure shows the 91 genotypes (circles) of the 61 CF (red circles), the 44 non-CF and the 2 environmental (green circles) *A. fumigatus* isolates investigated and the number of strains belonging to the same genotype (sizes of the circles). Each small circle represents 1 single *A. fumigatus* isolate, with each circle increasing in size representing 2, 3, 4 etc. isolates with the same genotype. A solid thick black line (-) denotes a 1 marker difference out of STR 9 markers. A thin grey solid line (-) denotes 2 markers difference out of 9 markers. A thick dashed line (---) represents 3 markers difference out of 9 markers. A thin grey dashed line (---) represents ≥4 markers difference out of 9 markers.

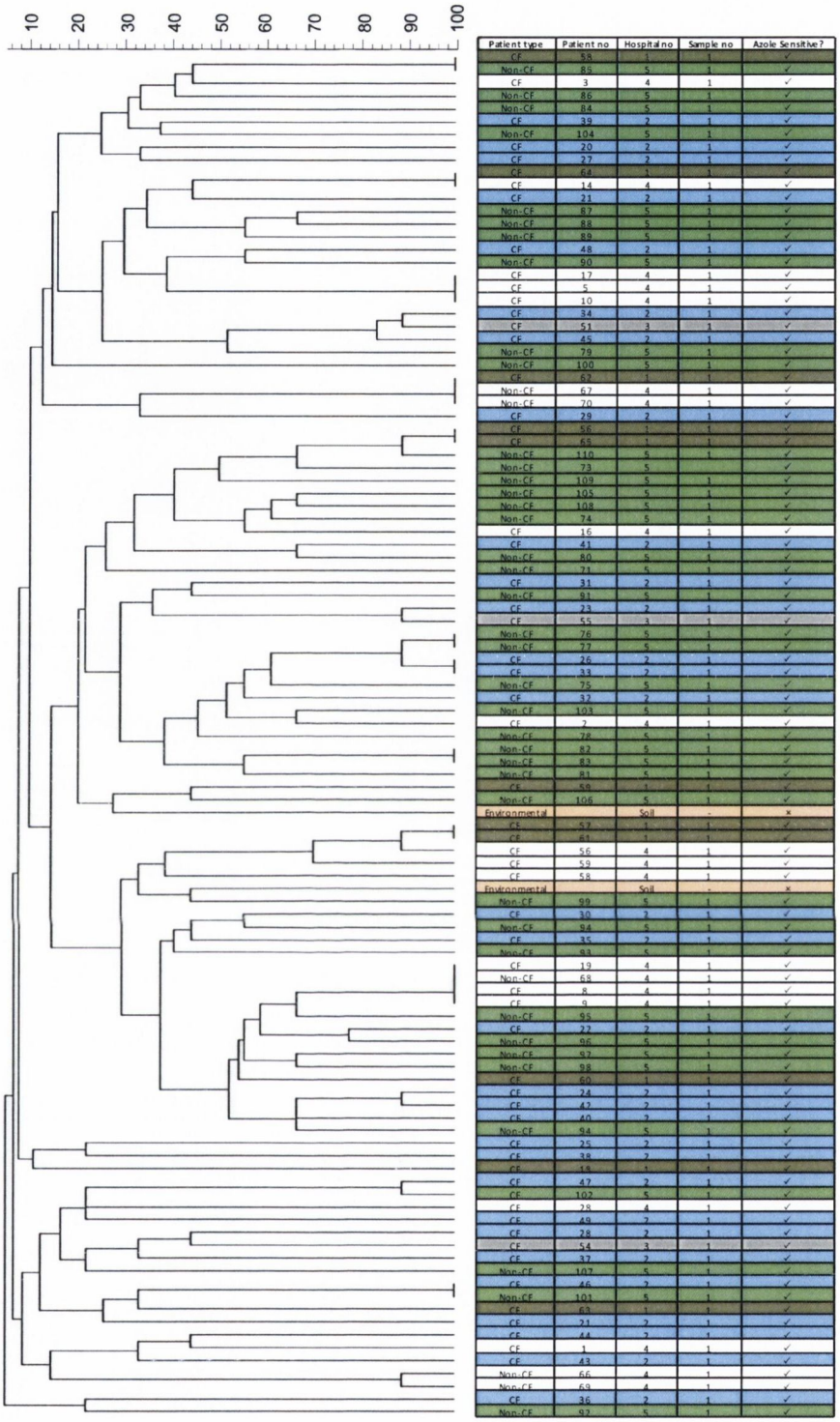
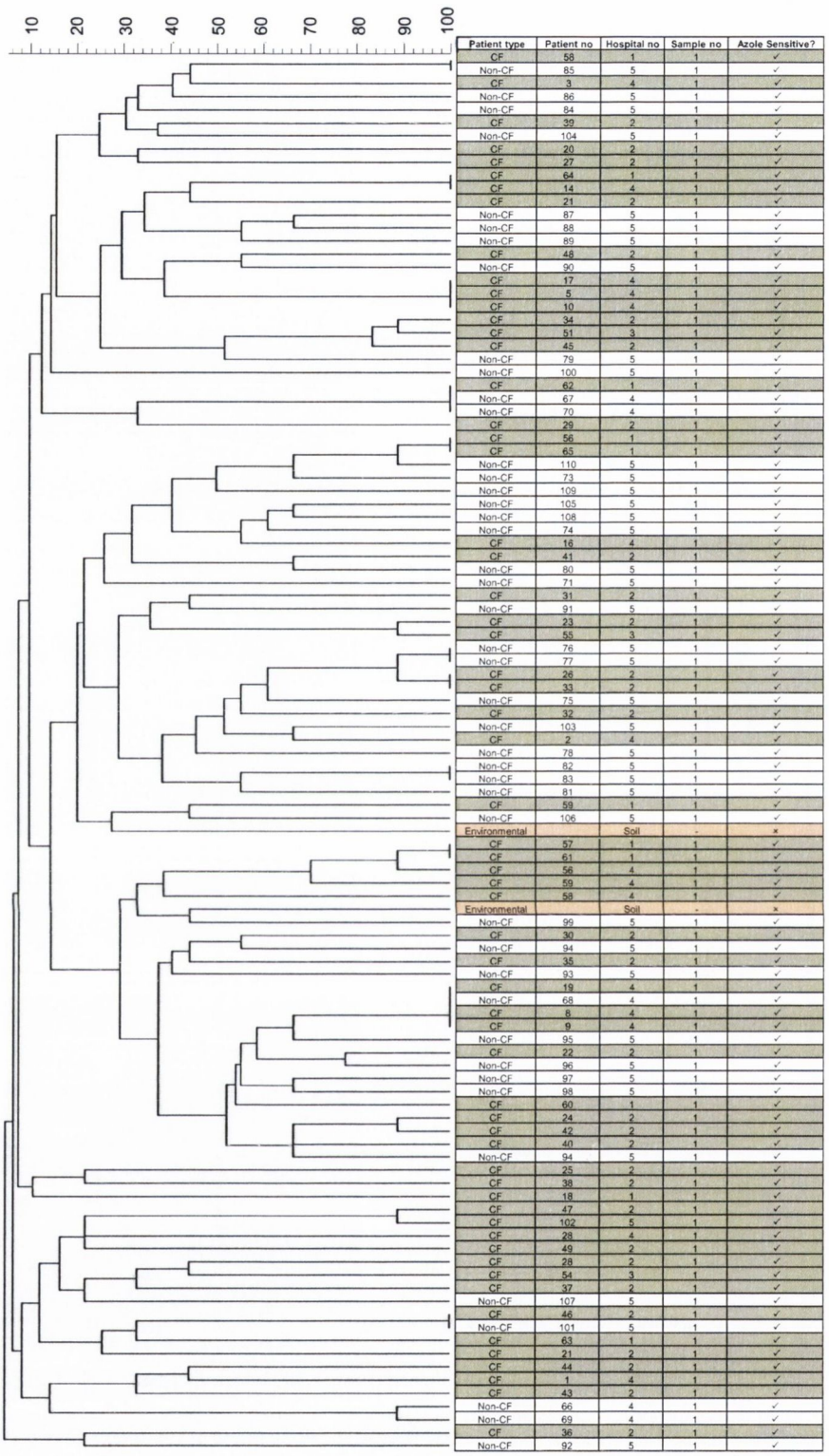


Figure 3.10: Microsatellite typing of CF and Non-CF *A. fumigatus* isolates from 5 Hospitals

Metadata associated with the dendrogram is colour coded based hospital number; hospital 1(■), hospital 2 (■), hospital 3(■) and hospital 4 (□).Two environmental isolates colour coded (■). Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate. A larger scale of this dendrogram is available in appendix V.



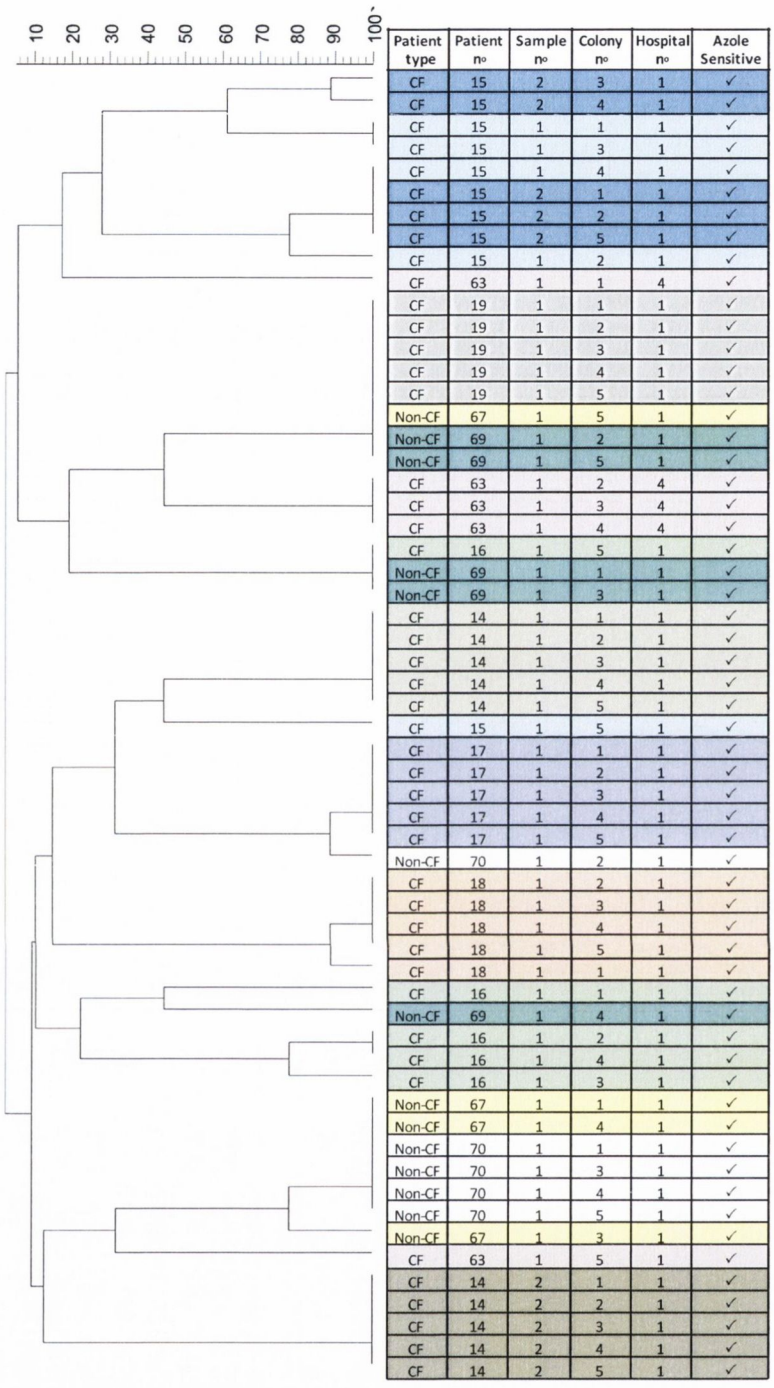
Patient type	Patient no	Hospital no	Sample no	Azole Sensitive?
CF	58	1	1	✓
Non-CF	85	5	1	✓
CF	3	4	1	✓
Non-CF	86	5	1	✓
Non-CF	84	5	1	✓
CF	39	2	1	✓
Non-CF	104	5	1	✓
CF	20	2	1	✓
CF	27	2	1	✓
CF	64	1	1	✓
CF	14	4	1	✓
CF	21	2	1	✓
Non-CF	87	5	1	✓
Non-CF	88	5	1	✓
Non-CF	89	5	1	✓
CF	48	2	1	✓
Non-CF	90	5	1	✓
CF	17	4	1	✓
CF	5	4	1	✓
CF	10	4	1	✓
CF	34	2	1	✓
CF	51	3	1	✓
CF	45	2	1	✓
Non-CF	79	5	1	✓
Non-CF	100	5	1	✓
CF	62	1	1	✓
Non-CF	67	4	1	✓
Non-CF	70	4	1	✓
CF	29	2	1	✓
CF	56	1	1	✓
CF	65	1	1	✓
Non-CF	110	5	1	✓
Non-CF	73	5		✓
Non-CF	109	5	1	✓
Non-CF	105	5	1	✓
Non-CF	108	5	1	✓
Non-CF	74	5	1	✓
CF	16	4	1	✓
CF	41	2	1	✓
Non-CF	80	5	1	✓
Non-CF	71	5	1	✓
CF	31	2	1	✓
Non-CF	91	5	1	✓
CF	23	2	1	✓
CF	55	3	1	✓
Non-CF	76	5	1	✓
Non-CF	77	5	1	✓
CF	26	2	1	✓
CF	33	2	1	✓
Non-CF	75	5	1	✓
CF	32	2	1	✓
Non-CF	103	5	1	✓
CF	2	4	1	✓
Non-CF	78	5	1	✓
Non-CF	82	5	1	✓
Non-CF	83	5	1	✓
Non-CF	81	5	1	✓
CF	59	1	1	✓
Non-CF	106	5	1	✓
Environmental		Soil	-	x
CF	57	1	1	✓
CF	61	1	1	✓
CF	56	4	1	✓
CF	59	4	1	✓
CF	58	4	1	✓
Environmental		Soil	-	x
Non-CF	99	5	1	✓
CF	30	2	1	✓
Non-CF	94	5	1	✓
CF	35	2	1	✓
Non-CF	93	5	1	✓
CF	19	4	1	✓
Non-CF	68	4	1	✓
CF	8	4	1	✓
CF	9	4	1	✓
Non-CF	95	5	1	✓
CF	22	2	1	✓
Non-CF	96	5	1	✓
Non-CF	97	5	1	✓
Non-CF	98	5	1	✓
CF	60	1	1	✓
CF	24	2	1	✓
CF	42	2	1	✓
CF	40	2	1	✓
Non-CF	94	5	1	✓
CF	25	2	1	✓
CF	38	2	1	✓
CF	18	1	1	✓
CF	47	2	1	✓
CF	102	5	1	✓
CF	28	4	1	✓
CF	49	2	1	✓
CF	28	2	1	✓
CF	54	3	1	✓
CF	37	2	1	✓
Non-CF	107	5	1	✓
CF	46	2	1	✓
Non-CF	101	5	1	✓
CF	63	1	1	✓
CF	21	2	1	✓
CF	44	2	1	✓
CF	1	4	1	✓
CF	43	2	1	✓
Non-CF	66	4	1	✓
Non-CF	69	4	1	✓
CF	36	2	1	✓
Non-CF	92	5	1	✓

Figure 3.11: Microsatellite typing of CF and Non-CF *A. fumigatus* isolates from 5 Hospitals

Metadata associated with the dendrogram is colour coded based on CF (■) or Non-CF (□) or environmental (■) isolates. Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate. A larger scale of this dendrogram is available in appendix VI.

3.2.3.3 Epidemiology of multiple colonies per sample from CF and Non-CF patients

From a subset of CF and non-CF patients 4 to 5 *A. fumigatus* colonies were randomly picked from each individual patient's plated sputum sample. For a total of 12 patient samples the genotype of multiple colonies was determined. There was a total of 9 samples from 7 CF patients (two patients each had two consecutive samples) analysed and 3 samples from 3 non-CF patients. From the dendrogram (Figure 3.12) it can be seen that 4 of the 9 CF samples each had an indistinguishable genotype for the 5 random colonies selected from their sputum sample (p14 s1, p14 s2, p17 s1 and p19 s1). Although not all patient 15 isolates were indistinguishable, they were more closely related to each other than any of the other isolates (Figure 3.12).For the remaining 5 CF samples two or more distinguishable *A. fumigatus* genotypes were found in each sample. One CF patient (p14) had an indistinguishable genotype for the multiple colonies for their first and also the second sample; however all sample 1 genotypes were distinct from all sample 2 genotypes. The non-CF patient samples all showed at least two distinguishable genotypes per sample (Figure 3.12).



Patient type	Patient nº	Sample nº	Colony nº	Hospital nº	Azole Sensitive
CF	15	2	3	1	✓
CF	15	2	4	1	✓
CF	15	1	1	1	✓
CF	15	1	3	1	✓
CF	15	1	4	1	✓
CF	15	2	1	1	✓
CF	15	2	2	1	✓
CF	15	2	5	1	✓
CF	15	1	2	1	✓
CF	63	1	1	4	✓
CF	19	1	1	1	✓
CF	19	1	2	1	✓
CF	19	1	3	1	✓
CF	19	1	4	1	✓
CF	19	1	5	1	✓
Non-CF	67	1	5	1	✓
Non-CF	69	1	2	1	✓
Non-CF	69	1	5	1	✓
CF	63	1	2	4	✓
CF	63	1	3	4	✓
CF	63	1	4	4	✓
CF	16	1	5	1	✓
Non-CF	69	1	1	1	✓
Non-CF	69	1	3	1	✓
CF	14	1	1	1	✓
CF	14	1	2	1	✓
CF	14	1	3	1	✓
CF	14	1	4	1	✓
CF	14	1	5	1	✓
CF	15	1	5	1	✓
CF	17	1	1	1	✓
CF	17	1	2	1	✓
CF	17	1	3	1	✓
CF	17	1	4	1	✓
CF	17	1	5	1	✓
Non-CF	70	1	2	1	✓
CF	18	1	2	1	✓
CF	18	1	3	1	✓
CF	18	1	4	1	✓
CF	18	1	5	1	✓
CF	18	1	1	1	✓
CF	16	1	1	1	✓
Non-CF	69	1	4	1	✓
CF	16	1	2	1	✓
CF	16	1	4	1	✓
CF	16	1	3	1	✓
Non-CF	67	1	1	1	✓
Non-CF	67	1	4	1	✓
Non-CF	70	1	1	1	✓
Non-CF	70	1	3	1	✓
Non-CF	70	1	4	1	✓
Non-CF	70	1	5	1	✓
Non-CF	67	1	3	1	✓
CF	63	1	5	1	✓
CF	14	2	1	1	✓
CF	14	2	2	1	✓
CF	14	2	3	1	✓
CF	14	2	4	1	✓
CF	14	2	5	1	✓

Figure 3.12: Microsatellite typing of multiple colonies from CF and non-CF patient samples

Patient samples had 4 to 5 *A. fumigatus* colonies randomly picked from plated sputum samples and genotyped. Metadata associated with the dendrogram is colour coded by patient sample; p14 s1(■), p14 s2(■), p15 s1(■), p15 s2(■), p16 s1(■), p17 s1(■), p18 s1(■), p19 s1 (■), p63 s1 (■), p67 s1 (■),p69 s1 (■) and p70 s1 (□). Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate.

3.3 Discussion

A. fumigatus isolates from our CF and non-CF cohorts were tested to assess any antifungal drug resistance of the isolates to a panel of nine antifungal drugs. In order to test these strains first we needed to establish whether all isolates were in fact *A. fumigatus* as different antifungals have been shown to have varied responses to different species of *Aspergillus* [170, 201]. All isolates were confirmed as *A. fumigatus* by *ITS* PCR product sequencing of the culturable isolates (chapter 2 &3), by *ITS* qPCR of the unculturable isolates (chapter 2) and were distinguished from *A. lentulus* (which was not found) by *RodA* restriction digest (chapter 2).

Clinical breakpoints are not available for mould testing for the CLSI method [173]. While the European Committee of Antibiotic Susceptibility Testing (EUCAST) have proposed MIC breakpoints for the EUCAST reference method for posaconazole, itraconazole and voriconazole against *A. fumigatus* these breakpoints cannot be applied to any CLSI method due to differences in the test protocols (inoculum concentration and media supplement concentrations) [186]. Therefore in the absence of clinical breakpoints, ECVs have been suggested to help characterise the susceptibility of *A. fumigatus* isolates to itraconazole, posaconazole, voriconazole, amphotericin B and caspofungin to monitor the emergence of reduced antifungal susceptibility and azole resistance mutations. ECVs represent the MIC value identifying the upper limit of the wild type population, where the wild type (WT) is defined for a species by the absence of acquired mutation mechanisms of resistance to the antifungal drug [173, 186]. A MIC or MEC value exceeding the ECV would be suggestive of the presence of resistance. For this study we used ECVs from recent publications [173, 186, 239]. No antifungal drug resistance was found for any of the panel of antifungals tested with the exception of fluconazole and fluconazole has been reported to be inactive against *A. fumigatus* [170]. There was a small proportion of isolates that were equal to the ECV, 16% of caspofungin MECs, 2.7% of posaconazole and itraconazole MICs, 1.6% of voriconazole MICs and 11.8% of amphotericin MICs, however no isolates had MIC or MECs above the ECV value for that antifungal drug. While the echinocandins, amphotericin B and 5-flucytosine MICs were reported and no resistance was detected when using the available ECVs, these antifungal drugs are rarely used on CF patients. Triazoles are more commonly used to treat ABPA and *Aspergillus* colonisation in CF patients and therefore the antifungal drug susceptibility of *A. fumigatus* to the triazoles was the aim of our testing. The absence of triazole resistant *A. fumigatus* isolates within our cohort of CF patients that received itraconazole treatment for 6 weeks is different to findings made by Burgel *et al* showing evidence of itraconazole resistance following treatment [189]. Burgel *et al* screened 249 CF adults and found 52.6% to be colonised with *A. fumigatus* of which 4.6% of these isolates were less susceptible to itraconazole (MIC, ≥2 mg/litre) and with an overall triazole resistance prevalence of 20% [189].

The study presented in this thesis had less sample numbers with intraconazole treatment information (n=19) and the patterns of resistance reported by Burgel *et al* [189] may be more evident in larger scale studies over longer treatment exposures. Bader *et al* investigated the antifungal drug susceptibility of 527 *A. fumigatus* clinical isolates to the triazoles (voriconazole, itraconazole and posaconazole), where the isolates were collected in Germany over an 18 month period and 163 of the isolates came from CF patients [242]. The authors reported a prevalence of 3.2% triazole resistance within their cohorts of CF and non-CF patients, however in the study CF patients displayed the highest proportion of resistance with the prevalence of azole resistance of their CF patients being 5.5% [242]. The prevalence of triazole resistance in CF patients varies from study to study with 5.5% reported by Bader *et a*l [242], 8% reported by Howard *et al* [3] and 20% reported by *Burgel et at* [189]. Reports of successful treatment using itraconazole with little or no incidence of triazole resistance also exist in the literature [59, 241, 243]. Triazole resistance observed in isolates from triazole treated and also triazole naïve patients [198] demonstrate examples of resistance derived from exposure to triazoles as a result of prolonged triazole treatment while other resistance has been associated with environmental exposure to triazoles from fungicides used in agriculture [191, 193, 199, 205]. In Ireland CF patients are often treated with triazoles when antibiotic treatment for a pulmonary exacerbation is unsuccessful. Therefore CF patients may incur a high rate of exposure to azoles over their lifetime. Additionally, in Ireland triazoles are also used as fungicides in agriculture thus the emergence of azole resistance in Ireland remains a possibility [194, 197, 208, 231, 232]. The lack of antifungal susceptibility testing in Ireland leaves patients vulnerable to treatment with inappropriate antifungals which may incur a high financial cost to the health system. In order to assess whether triazole resistance is in fact present in *A. fumigatus* clinical isolates in Ireland larger studies are required. Ireland has the unique opportunity to monitor and survey triazole susceptibility or resistance in *A. fumigatus* before it becomes a problem similar to that in the UK and the Netherlands. We suggest antifungal drug susceptibility should be considered as part of the routine diagnostic services within hospital laboratories particularly for patients at risk of aspergillosis or those on triazole prophylaxis. A major problem with regard to MIC determinations of *Aspergillus* is the lack of approved/recognised breakpoints [173]. With limited clinical breakpoints assigned for *Aspergillus* [173], it is difficult to say what MIC value constitutes a resistant isolate versus a susceptible isolate. Additionally the existence of differences between CLSI and EUCAST protocols and breakpoints contributes to the lack of a single worldwide "gold standard" for antifungal drug susceptibility testing and reporting. Breakpoints for *Aspergillus* remain a hot topic of debate and require standardisation [170] [188].

The epidemiology of the *A. fumigatus* isolates from Irish CF patients colonised with *A. fumigatus* and other non-CF patients was investigated. The epidemiology of *A. fumigatus* in these patient

populations was monitored using the *A. fumigatus* specific STR*Af* assay[233]. Two patterns of colonisation were observed in our CF cohort; 1) persistent colonisation and 2) non-persistent colonisation and furthermore patients sharing an indistinguishable isolate was also observed. These findings correlate with other genotyping studies of CF isolates [7, 213, 214, 244, 245]. We found two examples of patients with persistent colonisation of indistinguishable genotypes over time and a further 3 patients with colonisation of very closely related genotypes over time (90% similarity). One patient (p61) showed evidence of persistent colonisation, where indistinguishable isolates were identified from the first two consecutive samples, followed by clearance, and the third consecutive sample a showed a distinguishable genotype (Figure 3.6). Two patients (p56 and p63) showed evidence of a possible recurrent or predominant isolate, where the genotype identified from their first sample and their last sample were indistinguishable however their intermittent samples were distinguishable from the first and last. This may be a case where the initial genotype was cleared and perhaps is a transmitted from a source within their home or natural environment thus permitting re-colonisation. From our results, half of the patients (8 out of 16) who had a number of samples over time, showed non-persistent colonisation. De Valk *et al* [7] found similar findings where they carried out a study on 204 isolates from three CF centres and found four different colonisation patterns; 1) patients chronically colonised with a single genotype (persistent colonisation), 2) patients colonised with only unique genotypes (non-persistent), 3) patients sharing an identical isolate however, they could not find a common source of aquisition for these patients and 4) re-colonisation with a **predominant genotype [7]**. Cimon *et al* carried out genotyping of 109 isolates from seven CF patients and found different results in the incidence of persistence, non-persistence and the diversity of genotypes between the patients, and suggested that persistent colonisation was associated with long-term *A. fumigatus* colonisation and a narrowing of diversity of genotypes [214]. In the study two of the seven patients were persistently colonised while the remaining five patients showed non-persistent colonisation [214]. In our study one *A. fumigatus* genotype was found to be persistent in patient 10 and the same genotype was non-persistent in patient 6. This suggests that the ability for *A. fumigatus* to persist in the CF airways may be a host trait. Patients non-persistently colonised appear to be able to clear one genotype only to become re-colonised by a different genotype. Persistently colonised patients seem to harbour a unique genotype, even despite antifungal therapy as was the case of patient 10 who had an indistinguishable genotype both pre- and post itraconazole therapy, despite the reduction *A. fumigatus* bioburden post-itraconazole treatment (Figure2.5). Whether these examples of persistent colonisation are in fact cases where the patient fails to clear the isolate over time or whether they are continually re-colonised from the same source remains unclear. The correlation between patient health status and persistent versus non-persistent colonisation patterns remains unclear but warrants further investigation. Furthermore, our genotyping results showed a number of patients sharing indistinguishable genotypes and this was observed in our CF cohorts,

non-CF cohorts and also between CF and non-CF patients. De Valk *et al* found examples of CF patients sharing indistinguishable isolates however they could not find a common source of acquisition for these patients [7]. Patients sharing indistinguishable genotypes without any common factors have been reported in a number of genotyping studies [7, 209, 213, 214].

Only one example of an indistinguishable isolate shared between two different hospitals was observed in our study. These two hospitals are both located in Dublin but any other common factors between the patients are unknown. There did not appear to be any clustering based on CF centre or hospital suggesting that hospital acquired acquisition of a common genotype cannot be considered. De Valk *et al* found a single example of a genotype shared between two CF centres in their multi-centre study which fits with our finding [7]. Additionally, these authors found no relation between colonisation patterns and ABPA. However there were only small numbers with ABPA diagnosed within their study [7]. Examples of genotypes shared between centres or hospitals have been reported in a number of publications [7, 209, 213, 214, 246]. There appeared to be no correlation between CF versus non-CF genotypes with CF genotypes dispersed among the non-CF genotypes. We found no one genotype was associated with CF which consolidates previous findings [7]. This suggests that no one genotype is associated with one patient group.

Analysis of multiple colonies per sample showed examples of some CF patient samples (n=4) being colonised with a unique genotype, while others were colonised with a number of genotypes. The diversity of genotypes within a patient sample has been associated with colonisation patterns, with numerous genotypes found in recently colonised patients [214]. Cimon *et al* found that a narrowing of diversity of genotypes correlated to long-term colonisation where long-term colonised patients often have only a few or a single dominant genotype [214] and similar findings have also been reported in other publications [244, 245]. Although our numbers were small we only found indistinguishable genotypes within our CF samples. Also while four of the nine CF samples had indistinguishable genotypes for all colonies from the same sample, it is worth noting that although not all patient 15 isolates were indistinguishable, they are more closely related to each other than any of the other isolates. This was noted for patient 18 also. This raises the question whether these similar isolates in the one patient have evolved from each other. The ability of *A. fumigatus* to evolve resistance mechanisms against antifungals such as the azoles [189, 190, 194, 196, 202, 203] supports the theory that *A. fumigatus* may also evolve within the patient to aid survival and persistence.

Considering the fact that any patient sample whether CF or non-CF may harbour multiple different genotypes this calls into question whether multiple colonies should be selected not only for genotyping but also for antifungal susceptibility as a resistant strain may be missed based on

whether or not it is selected for testing. With no clear association between *A. fumigatus* isolates and patient group or between *A. fumigatus* isolate and persistence, studies into host factors conveying genetic susceptibility to *A. fumigatus* may provide some insight into why some patients can clear a particular *A. fumigatus* genotype and others become persistently colonised by the same genotype. Whether the narrowing of the diversity of genotypes during prolonged colonisation is a host or strain factor also requires investigation. While patterns of colonisation were observed it should be noted that numbers of isolates studied was low for the current study. Finally, although the STR*Af* assay shows a high discriminatory power [211], one can only determine whether isolates are indistinguishable based on the 9 STRs assessed and only whole genome sequencing would allow complete comparison with 100% identity, however the high cost associated with whole genome sequencing would likely make it unsuitable for high throughput studies for the foreseeable future.

Chapter 4

Virulence of *A. fumigatus* in the *Galleria mellonella* insect model

Chapter 4

Virulence of *A. fumigatus* in the *Galleria mellonella* insect model

4.0 Introduction

Insect models have been exploited for evaluating virulence of fungal and bacterial pathogens due to the strong structural and functional similarities between the innate immune system of higher mammals and that of insects [247-250]. There is a strong correlation between results obtained using insects and mammals [247]. The use of insect models has a number of advantages over using mammals; low cost, faster results and no specialist housing or ethical approval requirements. The larvae of the Greater wax moth *Galleria mellonella* provide an excellent speedy and cost effective *in vivo* model for studying virulence of fungal pathogens [247-249, 251, 252]. In chapter 3, *A. fumigatus* isolates were genotyped and different genotypes and patterns of colonisation in CF patients were observed, namely persistent and non-persistent colonisation. Furthermore we found no one genotype associated with CF and no clustering based on hospital or CF centre. It remains unclear whether different genotypes of *A. fumigatus* have a different impact on the individual patient. We sought to carry out a preliminary study investigating: (1) the virulence of persistent *versus* non-persistent *A. fumigatus* genotypes, (2) whether this virulence was inactivated by shock heat treatment of *A. fumigatus* conidia and (3) the efficacy of antifungal treatment following inoculation but before infection became established.

4.1 Materials and Methods

4.1.1 Preparation of *A. fumigatus* conidia

A. fumigatus isolates were cultured and harvested as previously described in section 2.1.1.

A serial dilution of the harvested conidia was performed from 10^0 to 10^{-3}. The 10^{-3} dilution was counted on a Neubauer haemocytometer as per section 2.1.2. Considering the results of the count, dilutions were performed to achieve the required number of conidia for future experiments. Triplicate ten-fold dilutions were also made for plating to confirm conidia concentration for each experiment.

4.1.2 Heat inactivation of *A. fumigatus* conidia

Heat inactivation of conidia was performed by heating conidia to 96°C for 1 hr [253]. Following heat inactivation conidia were centrifuged at 3,000 *x* g for 5 min and supernatant removed. Heat inactivated conidia were re-suspended in sterile PBS. Inactivation was confirmed by plating 20µl of heat inactivated sample onto an MEA plate in triplicate and incubated at 37°C for 1 to 7 days. No growth confirmed heat inactivation. Microscopic examination of heat inactivated conidia revealed intact conidia.

4.1.3 Preparation of culture supernatants (CSN) of *A. fumigatus*

A culture containing 10^6 conidia in 3ml Malt Extract Broth (MEB) (Sigma) was incubated at 37°C with shaking for 8, 24, 48 and 72 hr. Cultures were then sterile filtered using a 0.2µm syringe filter (Sarstedt) to remove all *A. fumigatus* conidia and hyphae. Culture supernatants were confirmed as containing no viable conidia or hyphae by plating 20µl culture filtrate onto an MEA plate in triplicate and incubated at 37°C for 1 to 7 days. No growth confirmed sterile filtration of culture supernatants.

4.1.4 Preparation and inoculation of *G. mellonella*

G. mellonella were supplied in boxes of wood shavings (Live Foods Ltd.). After visual inspection and removal of dead larvae, larvae were stored at 15°C for up to 10 days. For experimental use, each larva that was absent of any grey marking or signs of melanisation and 10 larvae of approximately 0.3g were dispensed into a Petri dish lined with whatman filter paper (food source for *G. mellonella*). A volume of 20µl containing a specified concentration of conidia suspended in sterile PBS was injected into the right hind pro- leg of each larva using a 26 gauge syringe [248]. Controls used for every experiment included; 10 larvae injected with 10^6 conidia from reference isolate AF26933, 10 un-injected larvae and 10 larvae injected with sterile PBS (to confirm that the injection process produced no ill effect). Larvae were incubated at 37°C in the dark and mortality was monitored daily over 7 days. Results were recorded as percent survival.

Mortality was assessed by lack of movement following physical stimulation and complete discolouration (melanisation) of the cuticle. Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.5 Optimisation of *A. fumigatus* conidia concentration for use in the *G. mellonella* insect model

A range of conidia concentrations was used in order to assess the optimal inoculum for use in all further *G. mellonella* survival experiments. Conidia were isolated from two separate *A. fumigatus* reference isolates ATCC 293 (AF293) and ATCC 26933 (AF26933) as per section 4.1.1. A series of conidia concentrations were prepared in 20µl volumes (10^3, 10^4, 10^5, 10^6 and 10^7). Larvae were injected with each concentration for AF293 or AF26933. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.6 Comparison of the virulence of fourteen *A. fumigatus* isolates in the *G. mellonella* insect model

Larvae were injected with 10^6 conidia from fourteen different *A. fumigatus* clinical isolates (p1 s1, p2 s1, p3 s1, p4 s1, p5 s1, p6 s1, p7 s3, p8 s1, pp9 s1, p10 s4, p62 s1, p57 s1, p56 s2 and p58 s1; Tables 2.1 and 3.1). These isolates were randomly chosen and ensuring that each isolate was from different CF patients in each case. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.7 Lethal dose to 50% of the population (LD_{50}) for four *A. fumigatus* isolates

Larvae were injected with a ten-fold serial dilution 10^9 to 10^0 of conidia suspended in 20µl sterile PBS from four *A. fumigatus* clinical isolates (p7 s3, p10 s4, p59 s1 and p62 s1). These fours isolates were chosen because isolate p7 s3 was found to be a non-persistent isolate (N-Af), whereas p10 s4 was a persistent isolate (P-Af) as defined in chapter 3, and the other two isolates were chosen at random ensuring they were isolated from different patients. LD_{50} for each isolate was determined from a plot of percentage survival against conidia concentration, where the LD_{50} was the concentration at which there was 50% survival and this was performed at 4 and 7 days post-inoculation. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.8 Comparison of the virulence of a persistent and non-persistent colonising *A. fumigatus* isolate in the *G. mellonella* insect model

Larvae were injected with 10^6 conidia from a persistent (p10 s3) and a non-persistent (p7 s3) colonising *A. fumigatus* clinical isolate as defined in chapter 3. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.9 Comparison of the virulence of four consecutive indistinguishable *A. fumigatus* isolates from patient 10, in the *G. mellonella* insect model

Patient 10 showed an indistinguishable genotype in all the isolates collected over a 6 month period. We defined this isolate as a persistent coloniser in chapter 3. In order to assess if this genotype changed its level of virulence overtime in the host, each of the isolates from patient 10 were investigated in the *G. mellonella* model. Larvae were injected with 10^6 conidia from each of patient 10's *A. fumigatus* clinical isolates (p10 s1, p10 s2, p10 s3 and p10 s4). Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.10 Effect of heat inactivated *A. fumigatus* conidia on *G. mellonella* mortality

Larvae were injected with 10^6 *A. fumigatus* conidia or heat inactivated conidia, prepared as per sections 4.1.1 and 4.1.2, respectively. The persistent (p10 s4) (P-Af) and non-persistent (p7 s3) (NP-Af) colonising isolates and the reference isolates AF293 and AF26933 were used. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.11 Effect of *A. fumigatus* culture supernatants (CSN) on *G. mellonella* survival

Larvae were injected with 20µl of culture supernatants (CSN) from persistent (p10 s4) (P-Af) and non-persistent (p7 s3) (NP-Af) *A. fumigatus* clinical isolates and reference isolates (AF293 and AF26933) as per section 4.1.3. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus* CSNs.

4.1.12 Effect of administration of itraconazole post *A. fumigatus* inoculation on *G. mellonella* survival

Larvae were injected with 10^6 conidia from the persistent (p10 s4) and non-persistent (p7 s3) clinical isolates and the reference isolate AF293 into the right hind pro leg. A stock solution of itraconazole (Sigma) was suspended in dimethyl sulfoxide (DMSO). Further dilutions were made in sterile PBS. All itraconazole concentrations contained no more than 1% DMSO (v/v). Four hours post *A. fumigatus* inoculation each larva was injected with itraconazole into the left hind pro-leg. Itraconazole concentrations equivalent to the MIC (0.03μg/ml = 0.0006 μg/20μl), one concentration above MIC and one concentration below MIC for each respective isolate were injected (Table 4.1). MICs were previously determined in chapter 3 and adjusted to the concentration per 20μl volume.

Table 4.1: Itraconazole concentrations used in *G. mellonella* experiment

Isolate	MIC*	One concentration below MIC	One concentration above MIC
AF293	0.0006 μg/20μl	0.0003 μg/20μl	0.0012 μg/20μl
p10 s4	0.0006 μg/20μl	0.0003 μg/20μl	0.0012 μg/20μl
p7 s3	0.0006 μg/20μl	0.0003 μg/20μl	0.0012 μg/20μl

*0.0006 μg/20μl = MIC of 0.03μg/ml

Ten larvae were injected with 10^6 conidia of each isolate but with no itraconazole administered. Furthermore drug controls were also included; 10 larvae injected with 1% DMSO per 20μl PBS and 10 larvae injected with the highest concentration of antifungal (0.0012μg/20μl) used with no *Aspergillus*. Controls were included and all plates incubated at 37°C. Mortality was monitored daily over 7 days and results recorded as previously described (section 4.1.4). Each scenario was performed on three separate days with fresh preparations of *Aspergillus*.

4.1.13 Statistical analysis

All statistical analysis throughout this chapter was performed on GraphPad Prism version 5. Statistical significance was tested using the two-way analysis of variance (ANOVA) and bonferroni post-test in the optimisation of *A. fumigatus* conidia concentration for use in the *G. mellonella* insect model (section 4.1.5), comparison of the virulence of a persistent and non-persistent colonising *A. fumigatus* isolate in the *G. mellonella* insect model (section 4.1.8), comparison of the virulence of four consecutive indistinguishable *A. fumigatus* isolates from patient 10, in the *G. mellonella* insect model (section 4.1.9), comparison of the virulence of live and heat inactivated *A. fumigatus* conidia in the *G. mellonella* insect model (section 4.1.10) and

effect of administration of itraconazole post *A. fumigatus* inoculation on *G. mellonella* mortality (section 4.1.12). Statistical significance was tested using one-way ANOVA and Tukey's multiple comparison post-test in the comparison of the virulence of fourteen *A. fumigatus* isolates in the *G. mellonella* insect model (section 4.1.6) and in the lethal dose to 50% of the population (LD_{50}) for four *A. fumigatus* isolates (section 4.1.7).

4.2 Results

4.2.1 Optimisation of *A. fumigatus* conidia concentration for use in *G. mellonella* insect model

G. mellonella were prepared, inoculated by injection and appearance and mortality monitored over 7 days. Following inoculation, larvae displayed melanisation as demonstrated in figure 4.1 and mortality was assessed by complete melanisation and lack of movement even following stimulation (Figure 4.2). For both AF293 and AF26933 lower concentrations (10^3 and 10^4) of conidia caused no mortality with 100% of larvae surviving at 7 days (Figure 4.2A and 4.2B). AF293 at the 10^5 concentration of conidia caused 6.7% mortality by day 7. The 10^6 concentration caused a steady increase in mortality, with 76.7% mortality by day 7 (Figure 4.2A). The 10^7 concentration caused a marked increase in mortality with 83.3% mortality on day 2 and 100% mortality by day 4 (Figure 4.2A). The PBS and uninjected controls had no ill effect on survival over time. Only the 10^6 and the 10^7 concentrations of AF293 showed a significant increase in mortality compared to the PBS and un-injected controls at all time-points after day 1 post inoculation ($p<0.0001$, Two-way ANOVA and Bonferroni post-test).

AF26933 at 10^5 caused a significant increase in mortality compared to the PBS and un-injected controls on days 4, 5 ($p<0.05$, Two-way ANOVA and Bonferroni post-test), 6 and 7 ($p<0.0001$, Two-way ANOVA and Bonferroni post-test), with 23.3% mortality by day 7. The 10^6 concentration caused a steady increase in mortality, with 96.7% mortality by day 7 ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.2B). The 10^7 concentration caused a marked increase in mortality over 7 days with 93.3% mortality on day 2 and 100% mortality by day 3 ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.2B).

Since the 10^7 concentration for both AF293 and AF26933 caused a marked increase in mortality within the first two days whereas the 10^6 concentration showed a more steady increase in mortality over 7 days, the 10^6 concentration was chosen for all further survival experiments (Figure 4.2A and 4.2B). Comparison of the two reference strains showed a significant difference between the isolates from day 2 onward ($p<0.0001$, Two-way ANOVA and Bonferroni post-test), with the AF26933 causing greater mortality (Figure 4.2C). The PBS and uninjected controls had no ill effect on survival over time.

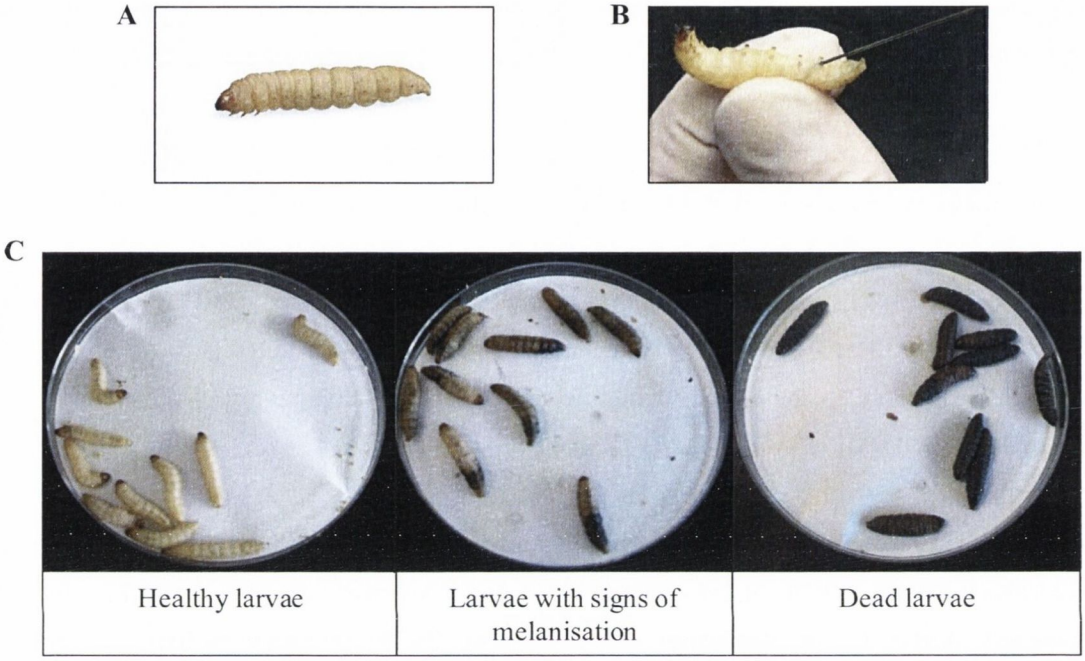


Figure 4.1: ***Galleria mellonella***

A) *G. mellonella* larva. **B)** *G. mellonella* larva receiving inoculation of *A. fumigatus* by injection into the right hind pro-leg. **C)** Plates of larvae for experimental use illustrating healthy larvae used at the beginning of an experiment, larvae with signs of melanisation in response to *A. fumigatus* infection and dead larvae showing complete melanisation.

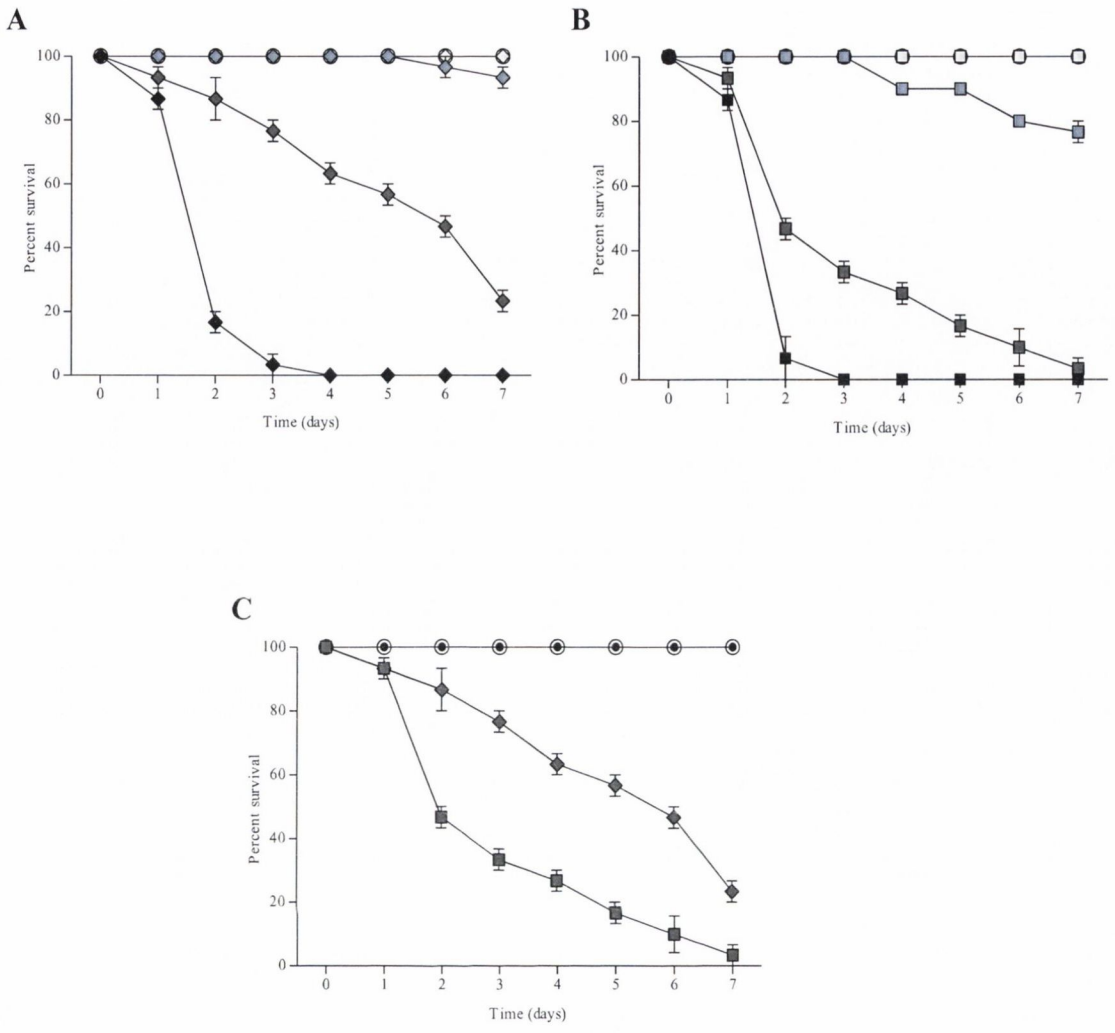


Figure 4.2: Determination of optimal concentration of *A. fumigatus* to use in the *G. mellonella* model.

A) Larvae un-injected (-o-) or, injected with PBS (-•-) and *A. fumigatus* AF293 conidia at 10^3(-◊-), 10^4(-♦-), 10^5(-♦-), 10^6(-♦-) and 10^7(-♦-) concentrations. **B)** Larvae un-injected (-o-) or, injected with PBS (-•-) and *A. fumigatus* AF26933 conidia at 10^3(-□-), 10^4(-■-), 10^5(-■-), 10^6(-■-) and 10^7(-■-) concentrations. **C)** Comparison of AF293 at 10^6(-♦-), AF26933 at 10^6 (-■-), PBS (-•-) and un-injected controls (-o-). The y axis represents percentage survival and the x-axis represents days post inoculation. Error bars represent standard error of at least three independent replicates.

4.2.2 Comparison of the virulence of fourteen *A. fumigatus* isolates in the *G. mellonella* insect model

Comparison of virulence of fourteen different *A. fumigatus* clinical isolates showed different mortality rates daily over 7 days. Two isolates (p1 s1 and p3 s1) showed a significant increase in mortality compared to PBS and un-injected controls from day 1 onward ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.3). Seven isolates (p2 s1, p4 s1, p5 s1, p7 s3, p10 s4, p62 s1 and p56 s2) showed a significant increase in mortality compared to PBS and un-injected controls from day 2 onward ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.3). Two isolates (p8 s1 and p9 s1) showed a significant increase in mortality compared to PBS and un-injected controls from day 3 onward ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.3). Two isolates (p6 s1 and p57 s1) showed a significant increase in mortality compared to PBS and un-injected controls from day 4 onward ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.3). One isolate (p58 s1) showed a significant increase in mortality compared to PBS and un-injected controls from day 5 onward ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.3). However, a comparison of the fourteen isolates overall, not taking into account the time post inoculation that death occurred found no statistical significance between isolates (One-way ANOVA and Tukey's multiple comparison post-test). The PBS and uninjected controls had no ill effect on survival over time.

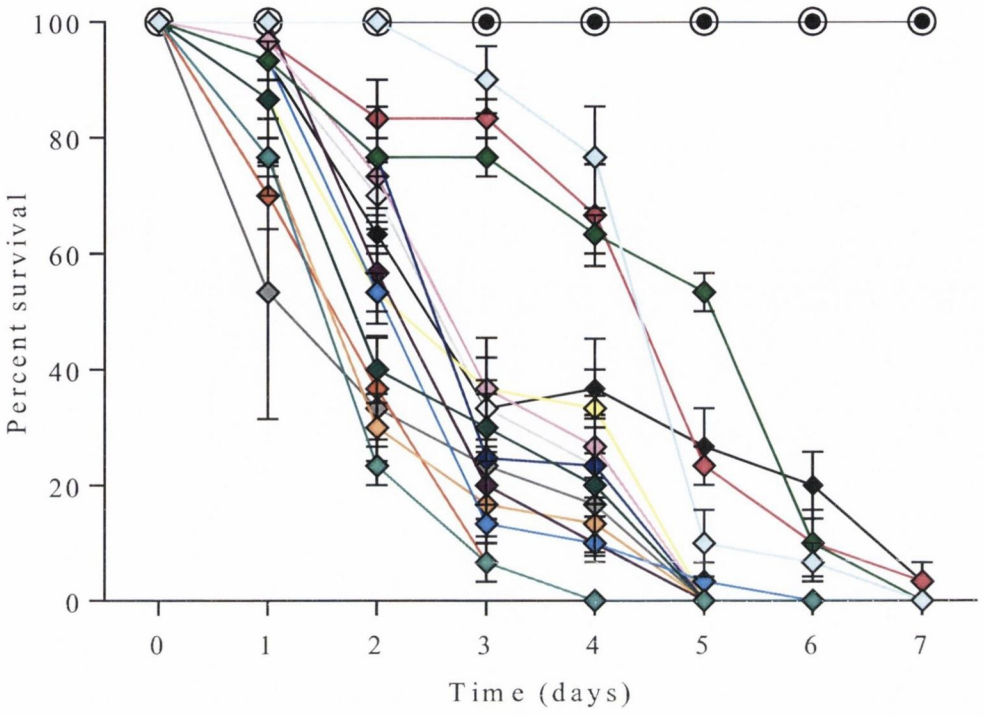


Figure 4.3: Comparison of the virulence of fourteen *A. fumigatus* isolates in the *G. mellonella* insect model.

Larvae injected with 10^6 conidia from isolates; p1 s1 (-♦-), p2 s1 (-♦-), p3 s1 (-♦-), p4 s1 (-♦-), p5 s1 (-♦-), p6 s1 (-♦-), p7 s3 (-♦-), p8 s1 (-♦-), p9 s1 (-♦-), p10 s4 (-♦-), p62 s1 (-♦-), p57 s1 (-♦-), p56 s2 (-♦-) and p58 s1 (-♦-)). Controls; PBS (-•-), un-injected (-o-) and AF26933 (-♦-). The y axis represents percentage survival and the x-axis represents days post inoculation. Error bars represent standard error of at least three independent replicates.

4.2.3 Lethal dose to 50% of the population (LD_{50}) for four *A. fumigatus* isolates

LD_{50} calculations showed a significant difference between, the reference isolate AF26933 compared to the four clinical isolates(p7 s3, p10 s4, 59 s1 and p62 s1) on day 4 (*$p<0.0001$, One-way ANOVA and Tukey's multiple comparison test) (Figure 4.4A). The LD50 of the reference isolate was 1.8×10^6/20ul on day 4 compared to an LD 50 of 2×10^5 for p10 s4, 2.8×10^5 for p7 s3, 4.1×10^5 for p59 s1 and 2.2×10^5 for p62 s1. However, no significant difference in LD50 was found between the reference strain and any clinical isolate by day 7 (One-way ANOVA and Tukey's multiple comparison test) (Figure 4.4B). No statistical significance was observed between any of the clinical isolates (p7 s3, p10 s4, 59 s1 and p62 s1) on day 4 or day 7 (One-way ANOVA and Tukey's multiple comparison test) (Figure 4.4A and 4.4B).

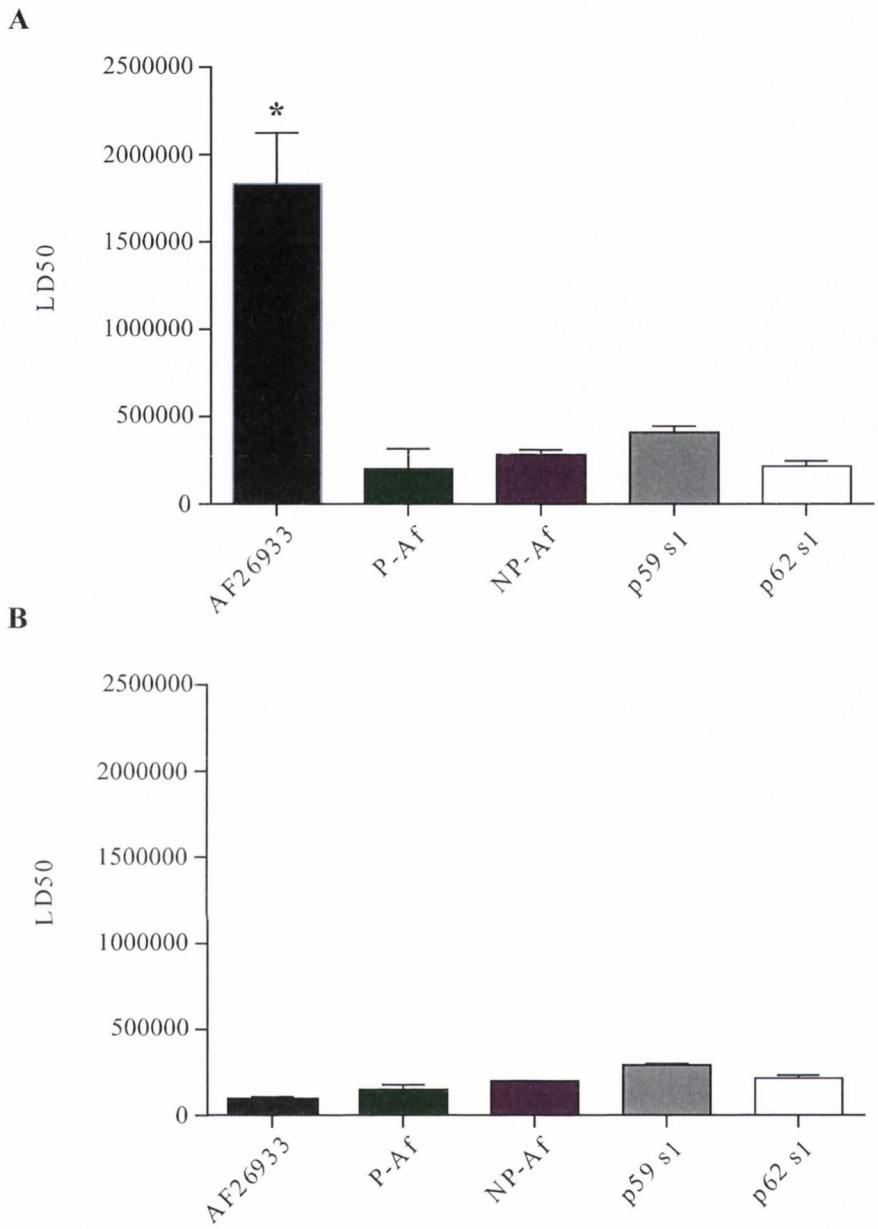


Figure 4.4: LD_{50} of AF 26933 and four clinical isolates in the *G. mellonella* insect model.
A) Day 4 and **B)** Day 7 LD_{50} for reference isolate AF26933 (■), the persistent isolate (p10 s4) (P-Af) (■), non-persistent isolate (p7 s3) (NP-Af) (■), two other CF clinical isolates p59 s1 (■), and isolate p62 s1 (□). The y axis represents the lethal dose to 50% of the population and the x-axis represents the different isolates. Error bars represent standard error of at least three independent replicates, *($p<0.0001$).

4.2.4 Comparison of the virulence of a persistent and non-persistent colonising *A. fumigatus* isolate in the *G. mellonella* insect model

The reference AF26933, and the persistent (p10 s4) and non-persistent (p7 s3) *A. fumigatus* isolates, all caused a steady increase in mortality over time (Figure 4.5). Comparison of the persistent (p10 s4) and non-persistent (p7 s3) isolates showed very similar trends in mortality in the *G. mellonella* model and overall no significant difference was seen between them (One-way ANOVA and Bonferroni post-test) (Figure 4.5). By comparing the isolates on a day by day basis, the only significant difference was observed on day 2 ($p<0.0001$, Two-way ANOVA and Bonferroni post-test). Overall no significant difference was seen between the clinical isolates or the reference isolate but by comparing the isolates on a day by day basis, a significant difference was observed on day 3 and 4 between the clinical isolates versus the reference isolate ($p<0.001$, Two-way ANOVA and Bonferroni post-test). However by day 7 there was no statistical difference between any of the isolates (Figure 4.5). The PBS and uninjected controls had no ill effect on survival over time.

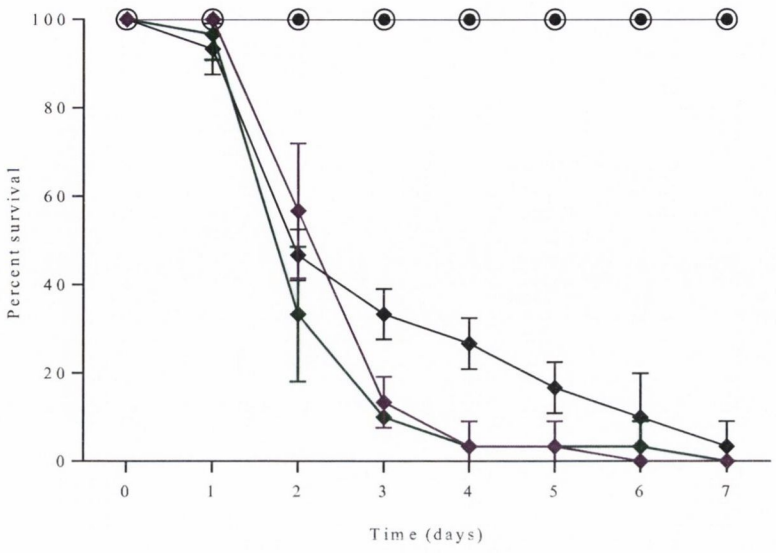


Figure 4.5: Comparison of the virulence of a persistent and non-persistent *A. fumigatus* in the *G. mellonella* infection model.

Larvae injected with 10^6 conidia from: persistent colonising isolate (p10 s4) (-♦-), non- persistent colonising isolate (p7 s3) (-♦-) and reference isolate AF26933 (-♦-). Controls: PBS (-•-) and un-injected larvae (-o-)). The y axis represents percent survival and the x-axis represents days post inoculation. Error bars represent standard error of at least three independent replicates.

4.2.5 Comparison of the virulence of four consecutive indistinguishable *A. fumigatus* clinical isolates from patient 10, in the *G. mellonella* insect model

Four consecutive isolates from patient 10 who was persistently colonised with an indistinguishable genotype over a 6 month period were compared. On day 1 post inoculation the first (p10 s1), second (p10 s2) and third isolates (p10 s3) caused significantly less mortality compared to the last isolate (p10 s4) ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 4.6). The two later isolates (p10 s3 and p10 s4) caused a significant increase in mortality on day 2 when compared to the two earlier isolates (p10 s1 and p10 s2) ($p<0.00001$, Two-way ANOVA and Bonferroni post-test), although the fourth isolate (p10 s4) also caused a significant increase in mortality on day 2 compared to the third isolate (p10 s3) ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 4.6). However by day 3 there was 100% mortality with all four of patient 10's isolates (Figure 4.6). The PBS and uninjected controls had no ill effect on survival over time.

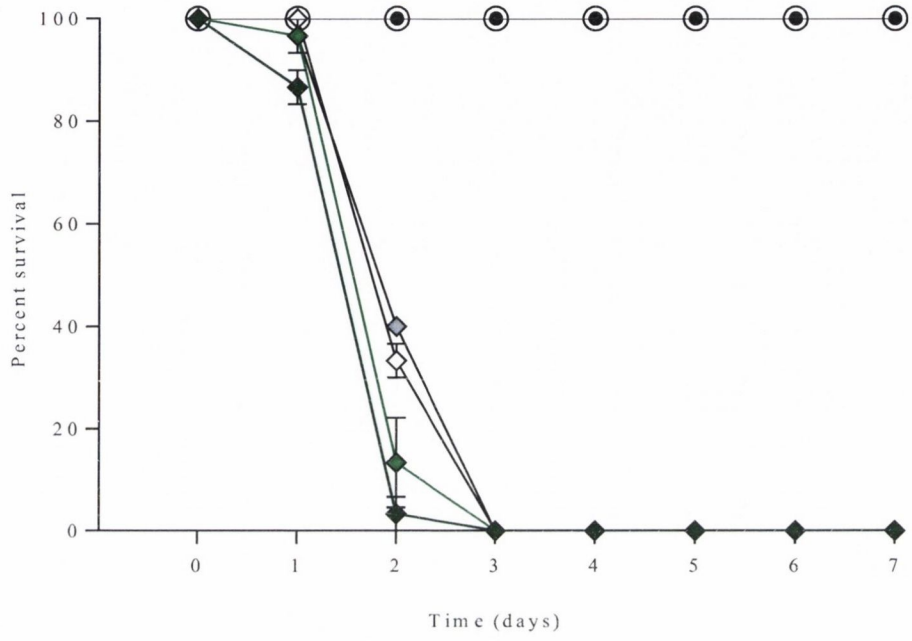


Figure 4.6: Differential virulence of identical *A. fumigatus* genotypes isolated from the same patient over 6 months. Larvae were injected with conidia from isolates p10s1 (-◊-), isolate 2 (-♦-), isolate 3 (-♦-) and isolate 4 (-♦-). The y axis represents percent survival and the x-axis represents days post inoculation. Error bars represent standard error of at least three independent replicates.

4.2.6 Effect of heat inactivation of *A. fumigatus* conidia on *G. mellonella* survival

Live *A. fumigatus* conidia from AF293, AF26933, persistent (p7 s3) and non-persistent genotypes (p10 s4) caused a significant steady increase in mortality over time compared to the PBS and uninfected controls ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.6). Heat inactivated *A. fumigatus* from all isolates (AF293, AF26933, p7 s3 and p10 s4) caused no increase in mortality over 7 days, with 100% survival (Figure 4.7). There was a significant increase in mortality of the live compared to the heat inactivated conidia for AF293 on day 2 ($p<0.001$), 3, 4, 5, 6, 7 ($p<0.0001$, Two-way ANOVA and Bonferroni post-test). Likewise there was a significant increase in mortality when comparing the live to the heat inactivated conidia for each of the other isolates (AF26933, p7 s3 and p10 s4) on day 2, 3, 4, 5, 6, 7 ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.7). The PBS and uninjected controls had no ill effect on survival over time (Figure 4.7).

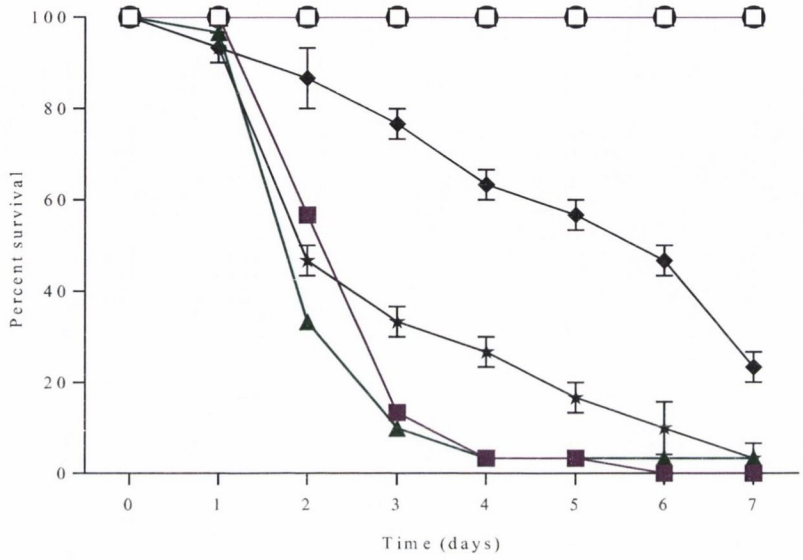


Figure 4.7: Comparison of survival for heat inactivated versus live *A. fumigatus* conidia from clinical isolates and reference isolates in the *G. mellonella* infection model.

Larvae were injected with: PBS (circle), live AF293 10^6 (-♦-), AF26933 10^6 (-*-), p7 s3 (-■-), p10 s4 (-▲-). Live inocula are denoted by closed symbols and heat inactivated inocula are denoted by open symbols. A control of un-injected larvae (-o-) was also included. The y axis represents percent survival and the x-axis represents days post inoculation. Error bars represent standard error of at least three replicates.

4.2.7 Effect of *A. fumigatus* culture supernatants on *G. mellonella* survival

Culture supernatants caused no ill effect on mortality, with 100% survival for all culture supernatant scenarios.

4.2.8 Effect of administration of itraconazole post *A. fumigatus* inoculation

Administration of itraconazole to larvae inoculated with AF293 resulted in a significant reduction in mortality on days 3, 4, 5, 6 and 7 compared to larvae inoculated with AF293 but no itraconazole was administered ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.8). Itraconazole at a concentration equivalent to the MIC or one concentration above the MIC reduced mortality by an average of 53% by day 7. Treatment with one concentration below the MIC resulted in a 43% reduction in mortality by day 7(Figure 4.8).

Administration of itraconazole to larvae inoculated with the persistent (p10 s4) or the non-persistent (p7 s3) isolate, showed in each case a significant reduction in mortality from day 2 compared to larvae inoculated with persistent or non-persistent isolates but no itraconazole was administered ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 4.8). Itraconazole treatment with the MIC or one concentration above the MIC reduced mortality by an average of 47% in larvae inoculated with the persistent isolate (p10 s4) and of 58% in those inoculated with the non-persistent isolate (p7 s3) by day 7 (Figure 4.8). Treatment with one concentration below the MIC resulted in a 33% reduction in mortality for the persistent isolate (p10 s4) and a 40% reduction for the non-persistent isolate (p7 s3) by day 7 (Figure 4.8). Larvae treated post inoculation with the MIC concentration or one concentration above the MIC showed a significant increase in survival on day 7 compared to those treated with a concentration lower than the MIC ($p<0.05$, Two-way ANOVA and Bonferroni post-test). Larvae inoculated with either 1% DMSO, antifungal alone, or sterile PBS showed 100% survival (Figure 4.8).

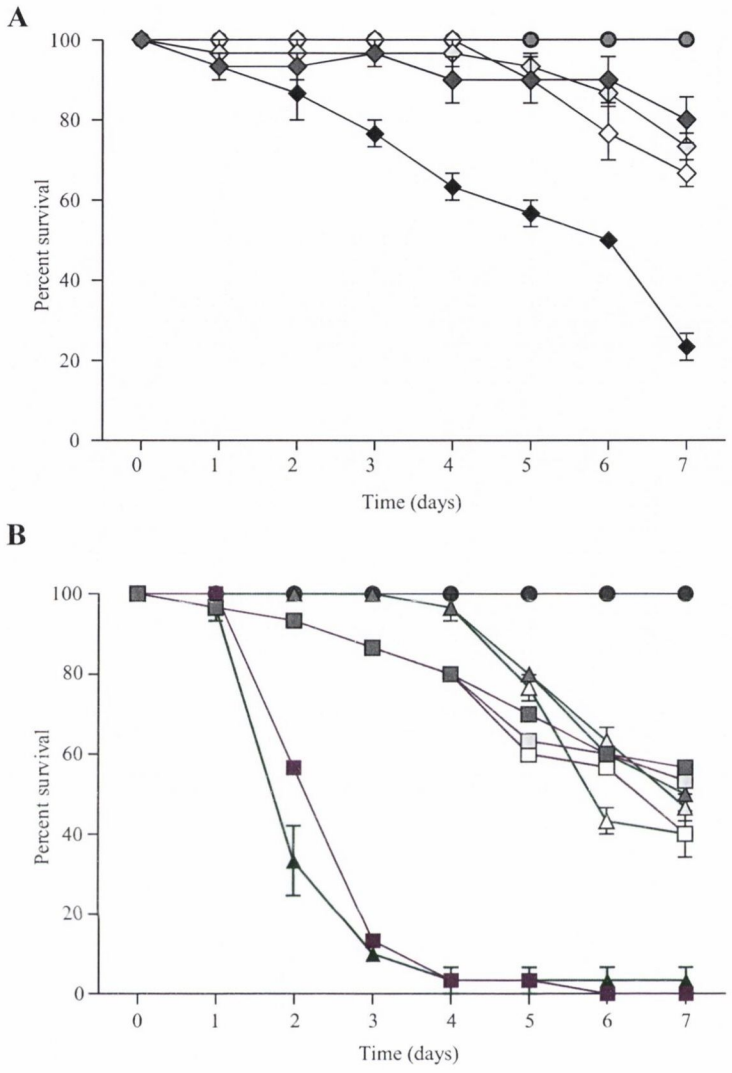


Figure 4.8: Effect of administration of Itraconazole on survival of larvae.

Larvae were injected with **A)** *A. fumigatus* AF293 10^6 (-♦-)and following 4 hr at 37°C were inoculated with itraconazole at 0.0006µg/20µl (-♦-), 0.0003µg/20µl (-◊-) or 0.0012µg/20µl (-♦-). Additionally, larvae were injected with **B)** persistent (p10 s4) (-▲-) or non-persistent (p7 s3) (-■-)and following 4h at 37°C were inoculated with itraconazole at 0.0003µg/20µl (-▲-,-■-), 0.00015µg/20µl (-Δ-, -□-) or 0.0006µg/20µl (-▲-, -■-) Controls of un-injected larvae (-o-), itraconazole only (0.4µg/20µl) (-●-) and 1% DMSO (-●-) were also included. The y axis represents percent survival and the x-axis represents days post inoculation. Error bars represent standard error of at least three independent replicates.

4.3 Discussion

We sought to carry out a preliminary study, investigating the virulence of different clinical isolates of *A. fumigatus* collected from different CF patients, and also consecutive isolates of the same genotype persistently colonising a single CF patient over 6 months. Although oral inoculation by feeding has been described as an infection method for *G. mellonella*, the larvae might not eat the full inoculum and therefore may lead to inoculation with an unknown quantity [248]. Considering this, we chose injection for inoculation in all experiments as this allows inoculation with a known quantity. A conidia inoculum concentration of $1x10^6$/20ul was chosen to compare *A. fumigatus* virulence as this concentration of conidia showed a steady increase in mortality over the seven days for both the control organisms AF293 and AF26933. The 10^7 concentration resulted in more rapid increase in mortality (Figure 4.2A and 4.2B) so slight differences in isolate virulence would not be as easily detected using this more lethal dose. The control AF26933 was significantly more virulent than the control AF293 ($p<0.0001$). Therefore AF26933 was chosen as the control for comparison of virulence of the clinical isolates (Figure 4.2C).

Comparison of fourteen different clinical *A. fumigatus* isolates revealed different patterns of mortality in the *G. mellonella* infection model (Figure 4.3). Some isolates caused a statistically significant increase in mortality after only one day, while others took between two and five days to produce a significant increase in mortality compared to un-injected or PBS controls (section 4.1.1). This suggests that different isolates of *A. fumigatus* may have different levels of virulence. This may be important when considering *A. fumigatus* in the clinical setting as different colonising genotypes may have different levels of virulence in the airways and so different clinical courses may result.

The LD_{50} results for four clinical isolates and the control AF26933 showed that mortality was concentration- and time-dependent. The LD_{50} was calculated for four clinical isolates at 4 and 7 days post inoculation (Figure 4.4). On day four post inoculation there was a statistically significant difference between LD_{50}'s of the AF26933 control and each of the clinical isolates (Figure 4.4A). However there was no significant difference between the LD_{50} of any of the clinical isolates. On day seven post inoculation there was no significant difference found between any of the clinical isolates or between the control AF26933 and any of the clinical isolates. The concentration required to cause a 50% mortality by day seven was lower when compared to the concentration required to cause 50% mortality by day 4. Day 4 LD_{50}'s required 95% greater concentration of conidia to cause a 50% mortality compared to day 7 concentrations for the AF26933 control, 25% more conidia than that required on day 7 for the persistent isolate (p10 s4), 29% more conidia than that required on day 7 for the non-persistent isolate (p7 s3), 41% more

conidia than that required on day 7 for the p59 s1 isolate and 5% more conidia than that required on day 7 for the p62 s1 isolate. This demonstrated that the effect of *A. fumigatus* on survival in the *G. mellonella* host was concentration- and time-dependent, where higher concentrations produced a greater decrease in survival of the larvae over time compared to lower concentrations. This supports the possibility that in CF patients speed of diagnosis and treatment may correlate with patient outcome [4, 41, 260] .

The use of the term "persistently colonised" by *A. fumigatus* in publications may have a number of meanings: many groups refer to a patient being chronically or persistently colonised with *A. fumigatus* when a patient is found to be *A. fumigatus* culture positive over time [24, 59, 177, 261, 262]. However, few groups define a patient as being persistently colonised with *A. fumigatus* only when the patient is colonised with an indistinguishable isolate over time as we have done [7, 213]. We defined those patients who are persistently *A. fumigatus* positive but demonstrate different genotypes over time to be non-persistently colonised as they appear to be capable of clearing one genotype only to become colonised with another. What impact these two different states of colonisation have on the patient over time are unclear and warrant further investigation. Additionally an individual may be colonised by multiple genotypes at any one time or a single genotype as was reported in our results from chapter 3 along with other publications [214, 244, 245]. However whether a patient is colonised by a single or multiple genotypes at any one time can only be determined by genotyping each colony of *A. fumigatus* from the patient sample but the presence of an indistinguishable genotype in consecutive samples does demonstrate that, that genotype is persisting in the patient regardless of whether other genotypes are also present. The presence of a mixture of genotypes can easily be identified during genotyping using the STR*Af* assay as mixed genotypes will result in more than one STR number when analysed, samples demonstrating this cannot be used in results as it is impossible to identify which repeat number belongs to which isolate. In these circumstances culturing of the isolate and separate selection and genotyping of individual colonies can allow differentiation once the two isolates are separated.

From genotyping our CF clinical isolates we found examples of persistent and non-persistent colonisation as defined in chapter 3. Persistence of an indistinguishable isolate over time has been associated with patients who demonstrate long-term colonisation [7, 214]. However whether these persistent isolates are more or less virulent than the non-persistent isolates remains unclear. We compared the virulence of a persistent colonising isolate (p10 s4) and a non-persistent colonising isolate (p7 s3) in the *G. mellonella* model (Figure 4.5). We observed no overall significant difference between these, suggesting that whether an isolate is a persistent coloniser or a non-persistent coloniser may not have a direct effect on virulence (Figure 4.5). However, it would be interesting to investigate specific virulence factors such as catalases [34, 263], superoxide

dismutases [18, 20, 34], toxins such as gliotoxin [59, 63, 104], fumigaclavin C, helvolic acid [20, 30, 34], fumagilin [20, 30, 34], Asp-hemolysin [20, 30, 34], and ribotoxin [20, 30, 34] and allergens (Asp f1 to Asp f23) [31] [32] [33] [34] and their levels of production between the persistent and non-persistent genotypes. Although the persistent and non-persistent colonising isolates did not affect mortality differently, it is possible that the isolates may produce different levels of inflammation and damage to local tissues and this requires further attention.

Consecutive persistent clinical isolates of other CF pathogens such as *Pseudomonas aeruginosa* taken from the CF airway have exhibited different expression patterns of multi-drug efflux pumps which play a role in antibiotic drug susceptibility, virulence factors such as protease sectretion, and biofilm formation illustrating the adaption of microorganisms to their environment in the host [259, 264]. In this study, patient 10 showed persistent colonisation where 4 isolates collected bi-monthly over 6 months (month 0, 2, 4 and 6) showed an indistinguishable genotype (Figure 3.3). We sought to investigate whether the virulence of the isolates differed from each other. From our results we found that the fourth isolate (p10 s4) caused a significant increase in mortality in the *G. mellonella* compared to the three earlier isolates (P10 s1, 2, 3) ($p<0.01$) on days 1 and 2 post inoculation (Figure 4.6). Furthermore, the two earlier isolates (p10 s1 and p10 s2) caused significantly less mortality compared to the two later isolates (p10 s3 and p10 s4) on day two, however all four genetically indistinguishable isolates resulted in 100% mortality by day 3 (Figure 4.6). The differing virulence patterns between these four indistinguishable isolates raises a number of questions as to whether *A. fumigatus* persistently or chronically colonising the CF airways may evolve and adapt to its hostile environment. These preliminary results suggest that *A. fumigatus* isolates, and appearing to represent the same isolate by genotyping may behave differently in the same host. Furthermore, these results indicate that persistently colonising isolates of *A. fumigatus* may modify their behaviour over time in the host.

When *A. fumigatus* is inhibited either by antifungal treatment or the immune system, inactivated conidia may remain for some time in the host. Therefore the effect of inactivated conidia may be of relevance in the host. We challenged the *G. mellonella* with live or heat inactivated conidia to assess whether inactivated conidia retained the ability to kill the larvae. Heat inactivation of *A. fumigatus* conidia completely eradicated their ability to kill the larvae with 100% survival by day seven (Figure 4.7). This suggests that the physical presence of *A. fumigatus* conidia does not have a negative effect on survival of the *G. mellonella* and that conidia must be viable in order to cause disease. Gomez-Lopez *et al* made similar observations in their study when investigating the virulence of *A. fumigatus* isolates with and without a *cyp51A* mutation [249]. They found the patterns of virulence were similar for clinical isolates with the *cyp51A* mutation and clinical isolates that showed no *cyp51A* mutation, while heat inactivated *A. fumigatus* conidia caused no

mortality [249]. Rivera *et al.* studied the immune response to live and heat inactivated *A. fumigatus* conidia in a murine model and found the CD4+ T cell response of immunologically intact mice to live or heat inactivated *A. fumigatus* conidia differed [265]. The live conidia stimulated IFN-ϒ producing *A. fumigatus*-specific CD4+ T cells while heat inactivated conidia only primed CD4+ T cells in draining lymph nodes [265]. They concluded that Th1 T-cell and humoral responses were limited to challenge with conidia that had the potential to germinate and cause infection. How the adaptive immune system distinguishes between metabolically active or inactive fungal spores remains unclear. Morgenstern *et al* investigated the immune response to sterile hyphae from *A. fumigatus* in an X-linked chronic granulomatous disease murine model (X-CGD mice) that was absent of a NADPH respiratory burst oxidase complex [266]. They reported an increased neutrophil response and expression of IL-1β and TNF-α in X-CGD mice compared to wild type mice (with a functional NADPH respiratory burst oxidase complex) suggesting the absence of respiratory oxidants can lead to an enhanced inflammatory response even to inactivated hyphe and therefore the NADPH respiratory burst may play a role in the different immune response to live versus inactivated *A. fumigatus*, however this study only looked at inactivated hyphae but not conidia [266]. The authors speculated that defects in phagocyte oxidant production in their murine model were associated with a prolonged inflammatory response that was independent of active fungal infection [266].

Antifungal treatment showed a significant reduction in mortality for the reference AF26933, and also for the persistent (P10 s4) and non-persistent (p7 s3) isolates (Figure 4.8). This confirms the efficacy of itraconazole as a treatment for *A. fumigatus* colonisation or infection and further supports the conclusions of Coughlan *et al* that itraconazole treatment correlates with improved CF patient status [59]. Considering the fact that itraconazole treatment in the *G. mellonella* model below the MIC concentration for the clinical isolates resulted in a greater mortality compared to those treated with a dose that achieved either the MIC or a concentration higher than the MIC, highlights the importance of antifungal sensitivity testing of clinical isolates and therapeutic drug monitoring to avoid sub-optimal treatment.

Chapter 5

The interaction of *A. fumigatus* with Respiratory Epithelial cell tight junctions

Chapter 5

The interaction of *A. fumigatus* with Respiratory Epithelial cell tight junctions

5.0 Introduction

The upper airway acts as the first physical barrier that protects against inhaled pathogens. Epithelial cells of the airways are tightly bound together providing a highly regulated and impermeable barrier formed by tight junctions [106, 267, 268]. These tight junctions regulate ions, water and immune cell transport between epithelial cells of the airways and aim to prevent microbial translocation [106]. Recent evidence suggests that tight junctions also play a part in signal transduction pathways that regulate epithelial cell proliferation, gene expression, differentiation, and morphogenesis [267]. A limited amount of research has been published on the interaction of *A. fumigatus* and the lung epithelium and there are only a handful of publications investigating the effect of *A. fumigatus* on the tight junctions of the lung epithelium. Considering *A. fumigatus* come into contact with the tight junctions of the lung epithelium on a regular basis, this area requires further investigation as to what signal mechanisms may be triggered and how this may contribute to inflammation in the CF airway. Some microorganisms such as *Burkholderia cepacia* complex, have the ability to open these tight junctions by direct interaction [269]. *Burkholderia cepacia* complex has been shown to open the tight junctions of epithelial cell tight junctions and then translocate from the apical side to the basolateral side of intact epithelial monolayers *in vitro* [269]. Other organisms have been shown to release compounds such as proteases from the house dust mite in order to gain access to blood vessels [270, 271] . Wan *et al.* demonstrated that the proteases, Der p1 (a cysteine protease) from the house dust mite *Dermatophagoides pteronyssinus* allowed transepithelial migration of allergens by disrupting the epithelial tight junctions [270, 271]. This disruption and transepithelial delivery of allergens led to an enhanced release of proinflammatory cytokines from immortalised and primary bronchial epithelial cells [270, 271]. In light of the fact that several hundred conidia are inhaled on a daily basis [4, 25, 272] and *A. fumigatus* is found in 6-80% of CF patient respiratory samples at one time or another [59, 124-126], an understanding of the effect of *A. fumigatus* and/or its secretions on the tight junction of the lung epithelium may provide more insight into allergic *Aspergillus* disease in CF.

5.1 Materials and Methods

5.1.1 An Investigation of the Ability of *A. fumigatus* to open Human Bronchial Epithelial (HBE) and Cystic Fibrosis Bronchial Epithelial cell (CFBE) Tight Junctions

5.1.1.1 Preparation of *A. fumigatus* conidia

A. fumigatus isolates were cultured and harvested as previously described in section 2.1.1.

A serial dilution of the harvested conidia was performed from 10^0 to 10^{-3}. The 10^{-3} dilution was counted on a Neubauer's haemocytometer as per section 2.1.2. Considering the results of the count, dilutions were performed to achieve the required number of conidia (2×10^5 and 5×10^5) for future experiments. Conidia were prepared for the following *A. fumigatus* isolates; AF293, a *gliG* gliotoxin mutant of AF293 which has been shown not to produce gliotoxin (*ΔgliG*) [67], the persistent (p10 s4) (P-Af), NP-Af (p7 s3) (NP-Af). Triplicate ten-fold dilutions were also made for plating to confirm conidia concentration for each experiment.

5.1.1.2 Preparation of heat inactivated *A. fumigatus* conidia

A concentration of 2×10^5 conidia from *A. fumigatus* isolates; AF293, *ΔgliG*, P-Af and NP-Af were heat inactivated as previously described in chapter 4 section 4.1.2.

5.1.1.3 Preparation of latex beads

Sterile latex beads (Sigma) 3μm in diameter were counted using a haemocytometer as previously in section 2.1.2. Considering the results of the count, dilutions were performed to achieve the required number of beads for use in experiments (2×10^5).

5.1.1.4 Preparation of *E. coli* at MOI of 50:1

The non-invasive *Escherichia coli* isolate NCIB 9485 was included as a control for each experiment [273]. The *E.coli* was cryopreserved at -80°C in 30% glycerol (v/v). Prior to any assay, bacteria were streaked from frozen stock preparations onto Luria Burtani (LB) Agar (Fannin) plates and incubated at 30 °C for 24 to 48 hr. For each experiment *E. coli* was grown in LB broth to OD_{600} 0.6 and was applied apically to the monolayer at an multiplicity of infection (MOI) of 50:1 [273].

5.1.1.5 Preparation of culture supernatants of *A. fumigatus*

A culture containing 2×10^5 conidia in 3ml minimal essential medium (MEM) (Sigma) was incubated at 37°C for 0, 4, 8, 12, 24, 48 and 72 hr. Culture supernatants (CSN) were prepared for AF293, *ΔgliG*, P-Af and NP-Af isolates. Cultures were passed through a sterile cell sieve and then sterile filtered using a 0.2μm syringe filter (Sarstedt) to remove all *A. fumigatus* conidia and

hyphae. Culture supernatants were confirmed as containing no conidia or hyphae by plating 20µl culture supernatant onto an MEA plate in triplicate and incubated at 37°C for 1 to 7 days. No growth confirmed sterile filtration of CSNs.

5.1.1.6 Preparation of Gliotoxin

Gliotoxin (Sigma) was suspended in DMSO at a stock concentration of 0.1M and was diluted in MEM to 0.8, 8 and 80µM. All final gliotoxin concentrations contained no more than 0.08% DMSO for tissue culture assays.

5.1.1.7 Tissue culture conditions

Two bronchial epithelial cell lines 16HBE14o$^-$ (HBE) and a CF line, CFBE41o$^-$ (CFBE) collected from a CF patient homozygous for the cystic fibrosis transmembrane regulator (*CFTR*) $delF_{508}$ mutation [269, 274, 275]. The HBEs (passage P2.77 to P2.97) were grown in fibronectin coated flasks in minimal essential medium supplemented (S-MEM) with 1% L-glutamine (v/v), 1% penicillin/streptomycin (v/v) and 10% foetal bovine serum (v/v) at 37°C with 5% CO_2 [269, 273, 276]. The CFBEs (passage P3.103 to P3.123) were also maintained at 37°C with 5% CO_2, in the same medium supplemented (S-MEM) with 1% non-essential amino acids [269]. Cells were split by trypsin digestion when growth had reached 60-80% confluence. Old medium was decanted from the flask and flask was rinsed well with PBS to remove serum which may inactivate trypsin. Trypsin was added to the flask and incubated at 37°C for 5 to 10 min until all cells moved freely from the flask. An equal volume of S-MEM was added to the flask to deactivate the trypsin. Flask contents were transferred to a sterile 20ml universal and centrifuged at 2,500 *x* g for 5 min to pellet cells. Supernatant was discarded and the pellet re-suspended in fresh S-MEM and sub-cultured into sterile fibronectin coated T-75 flasks (Sigma) and incubated at 37°C with 5% CO_2.

5.1.1.8 The ability of *A. fumigatus* isolates to open HBE and CFBE tight junctions

Cells were harvested, ensuring all cells had been removed from the flask, counted using a haemocytometer as previously described (Section 5.1.1.12) and re-suspended at a dilution of 1 x 10^5/0.5ml. A volume of 0.5 ml of cells were, applied to each 0.4µm transwell filter (Figure 5.1) (Sigma Aldrich) with 1.5ml of media applied to the underside of the transwell support (well) (Figure 5.1). A blank well containing only S-MEM [269] and a control of cells only was also included. Following 18 to 24 hr incubation, the mucosal (apical) medium is removed creating an air-liquid interface [269, 273]. Cells were fed with S-MEM and allowed to grow over a 5 day period to enable the formation of tight junctions. On day 6, Trans-Epithelial Resistance (TER) measurements were taken in triplicate for all wells using an EVOM meter (Figure 5.1) (World Precision Instruments) and only those exceeding TER measurement of 150 (above blank) were used for the experiment. Cells were inoculated with *A. fumigatus* conidia, latex beads, *E. coli*, *A.*

fumigatus culture supernatant or gliotoxin. Measurements were taken at two hour intervals from 0 hr to 12 hr and again at the 24 hr time point and results recorded.

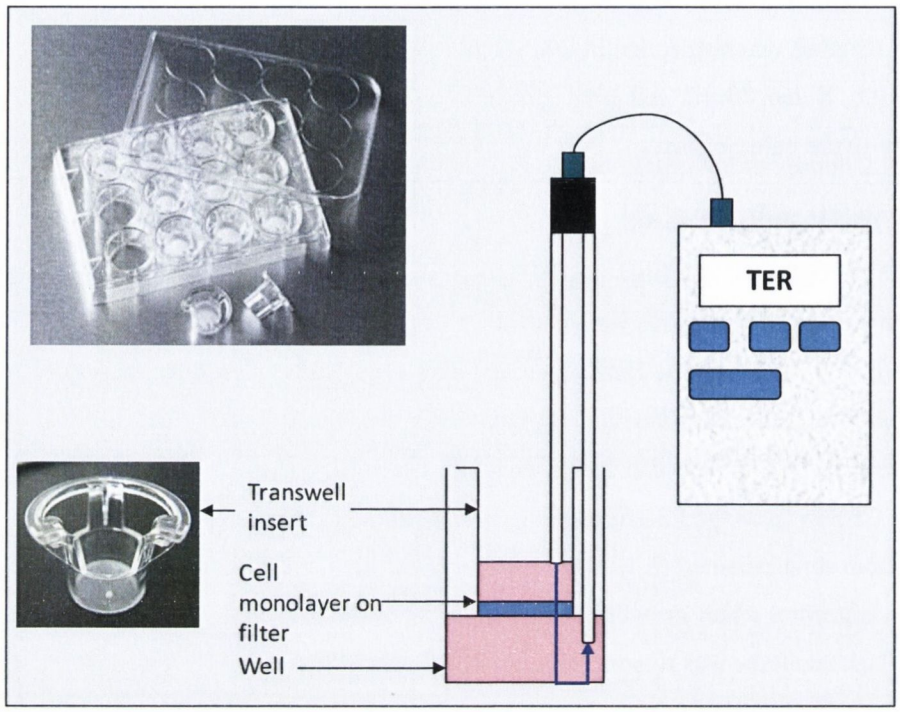


Figure 5.1: Measurement of Trans epithelial Resistance (TER)

Twelve well plate containing Transwell inserts. TER measurements are taken by inserting the probe of the EVOM meter into the inset which measures the transepithelial resistance of an electrical current (blue line) as it passes from the shorter end of the probe above the cells to the longer end of the probe submerged in media at the bottom of the well.

5.1.1.9 Calculations of final TER values

TER calculations were performed using the following equation;

$$\frac{\text{Resistance of cells on filter} - \text{resistance of blank filter (with no cells)}}{1.12\ \text{cm}^2\ \text{(area of filter)}} = \text{TER}\ \Omega/\text{cm}^2$$

From the TER calculations, results were recorded as percentage of the control for each time point.

5.1.1.10 The ability of *A. fumigatus* isolates to open HBE and CFBE tight junctions

Cells were prepared as per section 5.1.1.8. The isolates AF293, *ΔgliG*, P-Af and NP-Af isolates were prepared as per section 5.1.1.1 and applied to an insert containing either HBEs or CFBEs at a multiplicity of infection (MOI) of 2:1 [110, 277]. A number of MOI's have been used in *A. fumigatus* cell interaction studies ranging from 2 to 10 [110, 277-280], we felt that investigation using the MOI of 2:1 would provide insight into interactions which would only be matched or heightened by higher MOI's. Controls of; cells only, sterile latex beads at a MOI of 2:1 (section 5.1.1.3), non-invasive *E.coli* at a MOI of 50:1 [273](section 5.1.1.4) were included for each experiment. TERs were monitored every two hours from 0 to 12 hr and again at 24 hr post infection.

5.1.1.11 The ability of *A. fumigatus* heat inactivated conidia to open CFBE tight junction

Cells were prepared as per section 5.1.1.8. The AF293, *ΔgliG*, P-Af and NP-Af isolates were prepared as per section 5.1.1.1 and heat inactivation of each isolate was performed as per section 5.1.1.2 and applied to an insert containing CFBEs at an MOI of 2:1. Controls of; cells only, sterile latex beads at a MOI of 2:1 (section 5.1.1.3), non-invasive *E.coli* at a MOI of 50:1 [273](section 5.1.1.4) were included for each experiment. TERs were monitored every two hours from 0 to 12 hr and again at 24 hr post infection.

5.1.1.12 The ability of *A. fumigatus* culture supernatants to open HBE and CFBE tight junctions

Cells were prepared as per section 5.1.1.8. Culture supernatants were prepared as previously described in section 5.1.1.5 and 500µl culture supernatant was applied to an insert containing either HBEs or CFBEs. Controls of; cells only were included for each experiment. TERs were monitored every two hours from 0 to 12 hr and again at 24 hr post infection.

5.1.1.13 The ability of gliotoxin to effect the tight junctions of HBEs and CFBEs

Cells were prepared as per section 5.1.1.8. Gliotoxin was prepared as per section 5.1.1.6 and was applied to an insert containing either HBEs or CFBEs at a concentration of 0.8, 8 or 80µM. Controls of; cells only or 0.08% DMSO were included. TERs were monitored every two hours from 0 to 12 hr and again at 24 hr post infection.

5.1.2 The effect of AF293 and the gliotoxin mutant *ΔgliG* on tight junction proteins junctional adhesion molecule (JAM-A) and zonula occludens -1 (ZO-1)

Cells were prepared as previously described in section 5.1.1.8. Culture supernatants of wild type AF293 and *ΔgliG* cultured for 4, 8 or 72 hr prepared as per section 5.1.1.5 were applied to Transwell inserts containing either HBEs or CFBEs and a control of cells alone included. TERs were monitored over 24 hr.

5.1.2.1 Protein harvest and storage

Twenty-four hours after exposure to culture supernatants or medium alone; epithelial cell monolayers were washed three times with ice cold PBS. Protein was harvested by adding 100µl radioimmunoprecipitation assay buffer (RIPA) plus protease inhibitors to each Transwell filter. Cells were gently scraped off using a cell scraper and lysed with repeated pipetting. The suspension was transferred to a tube on ice and incubated for at least 15 min. Protein samples were then sonicated for 1min to aid in cell lysis and transferred immediately to -80°C for later use.

5.1.2.2 Western Blot: Protein separation and immunoblotting

Prior to western blot analysis, proteins were quantified using the Qubit fluorometer and protein quantification assay kit (Life Technologies). The protein quantification assay working solution was prepared by mixing protein reagent and protein buffer in a ratio of 1:200. Protein concentration was assessed for each sample by mixing 1µl of sample with 196µl of working solution in a thin walled 0.5ml PCR tube and incubating at room temperature for 15min. Following incubation concentration was read on the Qubit fluorometer and final concentration calculated using the following equation;

Concentration = Qubit reading x 200

Proteins were applied at a concentration of 30µg to 4-12% Bis-Tris SDS gels (NU-PAGE, Life Technologies) and separated by electrophoresis alongside a 4-250kDa protein standard (Life technologies) at 200V for 50 min. Following electrophoresis, proteins were transferred to nitrocellulose membranes at 30V for 1 hr. Membranes were blocked for 1hr with Tris-buffered saline with 5% non-fat dried milk and 0.1% Tween 20 (MTBST) for glyceraldehydes 3-phosphate dehydrogenase protein (GAPDH) or with bovine serum albumin (BSA) and 0.1% Tween 20 (BSA-TBST) for zonula occludens-1 (ZO-1) and junctional adhesion molecule-A (JAM-A). Blots were incubated with primary antibody (1:5000 for GAPDH (Calbiochem)) and (1:500 for ZO-1 (Life Technologies) and JAM-A (Santa Cruz Biotechnology)) over night at 4°C. Membranes

were washed and then incubated with either HRP-conjugated anti-mouse (1:50000 for GAPDH) or HRP- conjugated anti-rabbit (1:50000 for ZO-1 and JAM-A) (Thermoscientific) for 1 hr at room temperature. Following immunoblotting proteins were detected by chemiluminescence and visualised using a Fuji film ImageQuant LAS 4000 analyser. The density of each individual band was compared with the corresponding control band and normalised against GAPDH by densitometry using Quantity One version 4.6.6 software. Results were presented as percentage of the untreated control.

5.1.3 Immuno-fluorescence and confocal microscopy

Monolayers of cells for HBEs and CFBEs were prepared on Transwell inserts as previously described in section 5.1.1.8. After 24 hr exposure to medium alone or culture supernatants of AF293 or *ΔgliG* as per section 5.1.2 monolayers were prepared for immuno-fluorescence. Monolayers on transwell inserts were washed with PBS, then permeablised with ice cold (-20°C) methanol (Sigma) for 30 min and blocked with PBS containing 1% BSA (w/v) for 10 min. Cells were then incubated with 10μg/ml primary antibody (rabbit anti-ZO-1 (Life Technologies) or mouse anti-JAM-A(Santa Cruz Biotechnology)) for 1 hr at room temperature. Cells were then washed four times with PBS containing 1% BSA (w/v) and subsequently incubated with FITC-conjugated anti-rabbit (ZO-1) or anti-mouse (JAM-A) (Jackson Immunoresearch) antibody for 1 hr at room temperature protected from the light. Monolayers were washed five times with PBS containing 1% BSA (w/v) and then post-fixed in PBS containing 4% paraformaldehyde (w/v) for 10 min. Following fixation filters were removed from the inserts and mounted on slides with vectashield containing DAPI and examined by confocal microscopy. Confocal microscopy was performed using the Olympus FLOUVIEW FV1000 microscope utilising the FITC and DAPI channels and FV10-ASW software version 03.01. Images were captured at x600 magnification.

5.1.4 Statistical analysis

All statistical analysis throughout this chapter was performed on GraphPad Prism version 5. Statistical analysis using the Two-way Analysis of Variance (ANOVA) and Bonferroni post-test was carried out on each experiment investigating the ability of *A. fumigatus* conidia or sterile latex beads or non-invasive *E. coli* (section 5.1.1.8, section 5.1.1.10 and section 5.1.1.11) or culture supernatants (section 5.1.1.12) or gliotoxin (section 5.1.1.13) to affect the tight junctions of HBEs and CFBEs. Comparison of densitometry of western blots (section 5.1.2.2) was performed using One-way ANOVA and Bonferroni post-test. Comparison of densitometry of HBEs versus CFBEs (section 5.1.2.2) was performed using Two-way ANOVA and Bonferroni post-test. P values of <0.05 were considered significant.

5.2 Results

5.2.1 The ability of *A. fumigatus* conidia isolates to open HBE and CFBE tight junctions

Control cells where no conidia were added remained in tight monolayers with TER values remaining between 90 and 120% of the initial TER for the duration of the experiment (Figure 5.2). Sterile latex beads produced no ill effect on CFBE monolayer tight junction integrity with TER values remaining between 93 and 106% of the initial TER for the duration of the experiment (Figure 5.2). Likewise the non-invasive *E. coli* control had no significant effect on CFBE tight junction integrity with TER values remaining between 96 and 100% of the initial TER for the duration of the experiment (Figure 5.2).

A. fumigatus conidia were capable of opening CFBE tight junctions. A statistically significant decrease in TER values was observed between infected versus non-infected (control) CFBE monolayers from 10 hr for AF293 ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 5.2A), and at 24 hr for both the persistent (P-Af) and non-persistent (NP-Af) isolates ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.2A). There was no significant difference in TER values between the P-Af and the NP-Af isolates. While the AF293 conidia were capable of reducing tight junction integrity from 10 hr ($p<0.05$) the P-Af and NP-Af did not open tight junctions until 24 hr ($p<0.0001$). However there was no statistical difference found between the AF293, P-Af and NP-Af.

Interestingly heat inactivated *A. fumigatus* conidia were able to open tight junctions of CFBEs (Figure 5.2B, 5.2C and 5.2D). A statistically significant decrease in TER values was observed between infected versus non-infected (control) CFBE monolayers from 10 hr for AF293 live conidia ($p<0.01$) and heat inactivated conidia ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.2B). For P-Af TERs were significantly decreased compared to non-infected controls at 24 hr for live and heat inactivated conidia ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.2C). For NP-Af TERs were significantly decreased compared to non-infected controls at 24 hr for live ($p<0.0001$) and heat inactivated conidia ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.2D). The only significant difference found between the live versus heat inactivated conidia for each isolate was at 24 hr, where the live conidia caused a greater reduction in TER compared to the heat inactivated conidia and this was seen for AF293 ($p<0.05$), P-Af ($p<0.001$) and NP-Af ($p<0.05$, Two- way AVOVA and Bonferroni post-test) (Figure 5.2B, 5.2C and 5.2D). This illustrates that heat inactivated conidia were capable of opening tight junctions. It should be noted that all heat inactivated conidia preparations were

plated on MEA for 7 days and no growth of heat inactivated conidia was observed. Furthermore the ability of heat inactivated conidia to open the tight junctions cannot be explained simply by the physical presence of the conidia as sterile latex beads of similar size to conidia caused no significant decrease in TERs compared to non-infected controls (Figure 5.2B, 5.2C and 5.2D).

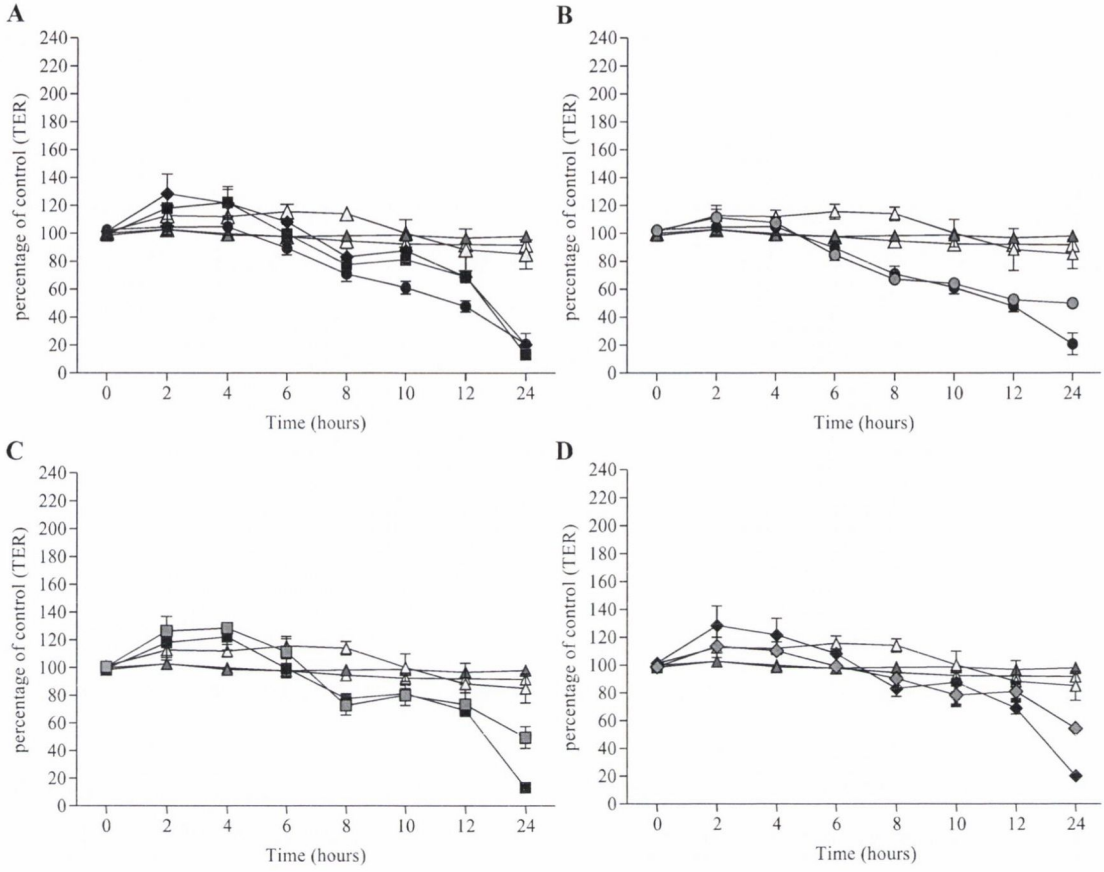


Figure 5.2: Effect of live and heat inactivated *A. fumigatus* isolates on TER of CFBEs.

The TER of CFBEs infected with **A)** live conidia from AF293 (-●-), P-Af isolate (-■-) and the NP-Af isolate (-♦-), **B)** live conidia of AF293 (-●-) and heat inactivated conidia of AF293 (-●-), **C)** live P-Af isolate (-■-) and heat inactivated conidia of P-Af isolate (-■-), **D)** live conidia of NP-Af isolate (-♦-) and heat inactivated conidia of NP-Af isolate (-♦-). For all experiments controls of cells only (-△-), sterile latex beads (-▲-) and non-invasive *E. coli* (-▲-) were included. The y-axis represents percentage of control (TER) and the x-axis represents hours post inoculation. Error bars represent standard error of at least three independent replicates.

5.2.2 The ability of *A. fumigatus* culture supernatants to open the tight junctions of CFBEs

Culture supernatants of AF293, P-Af and NP-Af isolates were capable of opening the tight junctions of CFBEs in a concentration dependent manner (Figure 5.3). The 0 hr CSN for AF293, P-Af and NP-Af isolates caused no negative effect on CFBE monolayer tight junction integrity with TER values remaining between 92 and 117% of the initial TER for the duration of the experiment (Figure 5.3). The 4 hr CSNs of AF293, P-Af and NP-Af isolates caused different rates of disruption of tight junction integrity of CFBEs (Figure 5.3). An increase in tight junction integrity was seen in the initial 2 to 6 hr of the experiment for the early CSNs of AF293 and P-Af (Figure 5.3). The AF293 4 hr CSN caused a significant increase in TER compared to the non-infected control at 4 hr ($p<0.05$, Two-way ANOVA and Bonferroni post-test). The P-Af caused the greatest tightening of tight junctions where the P-Af 4 hr CSN caused a significant increase at 2 hr, 4 hr, and 6 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) and additionally the P-Af 8 hr CSN caused a significant increase in TER at 2 hr ($p<0.01$, Two-way ANOVA and Bonferroni post-test). For the NP-Af an increase in TER was observed however it did not reach statistical significance compared to the non-infected control. Early CSNs also caused a disruption of tight junction integrity measured by TER when exposed to tight junctions for 12 hr or more. The AF293 4 hr CSN caused a significant decrease in TER after 24 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3 A and 5.3D) but the P-Af 4 hr CSN and the NP-Af 4 hr CSN caused no significant decrease in TER compared to the control (Two-way ANOVA and Bonferroni post-test) (Figure 5.3C and 5.3D). The AF293 8 hr CSN caused a significant decrease in TER at 12 hr ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3A and 5.3D). The P-Af 8 hr CSN caused a significant decrease in TER at 24 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test), (Figure 5.3B and 5.3D). However, the NP-Af 8 hr CSN caused no significant decrease in TER (Two-way ANOVA and Bonferroni post-test) (Figure 5.3C and 5.3D).

The AF293 12 hr CSN caused a significant decrease in TER at 12 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3A). The P-Af and NP-Af 12 hr CSNs caused a significant decrease in TER at 24 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3B and 5.3C). The AF293 24 hr CSN caused a significant decrease in TER compared to the control from 6 hr ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3A and 5.3D). The P-Af and NP-Af 24 hr CSNs caused a significant decrease in TER at 24 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3B and 5.3C). The AF293 48 hr CSN caused a significant decrease in TER compared to the control from 6 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3A and 5.3D). The P-Af 48 hr CSN caused a significant decrease

in TER compared to the control from 8 hr ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3B and 5.3D) and the NP-Af 48 hr CSN caused a significant decrease in TER compared to the control from 24 hr ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3C and 5.3D). The 72 hr CSNs resulted in the most marked decrease in TER compared to the control. The AF293 72 hr CSN caused a significant decrease in TER from as early as 2 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3A and 5.3D). The P-Af 72 hr CSN caused a significant decrease in TER from 8 hr ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3B and 5.3D) and the NP-Af 72 hr CSN caused a significant decrease in TER from 12 hr ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3C and 5.3D). Comparison of the 72 hr CSN of all three *A. fumigatus* strains revealed that AF293 72 hr CSN caused the greatest decrease in TER compared to non-infected controls (Figure 5.3D). The AF293 72 hr CSN caused a significantly greater decrease in TER compared to the P-Af 72 hr CSN from 2 hr onward ($p<0.05$, Two-way ANOVA and Bonferroni post-test) (Figure 5.3D). The AF293 72 hr CSN also caused a significantly greater decrease in TER compared to the NP-Af 72 hr CSN from 2 hr to 8 hr ($p<0.01$, Two-way ANOVA and Bonferroni post-test); however by 10 hr both 72 hr CSNs from AF293 and NP-Af caused similar decreases in TER with no statistical difference between them for 10 hr, 12 hr or 24 hr (Figure 5.3D).

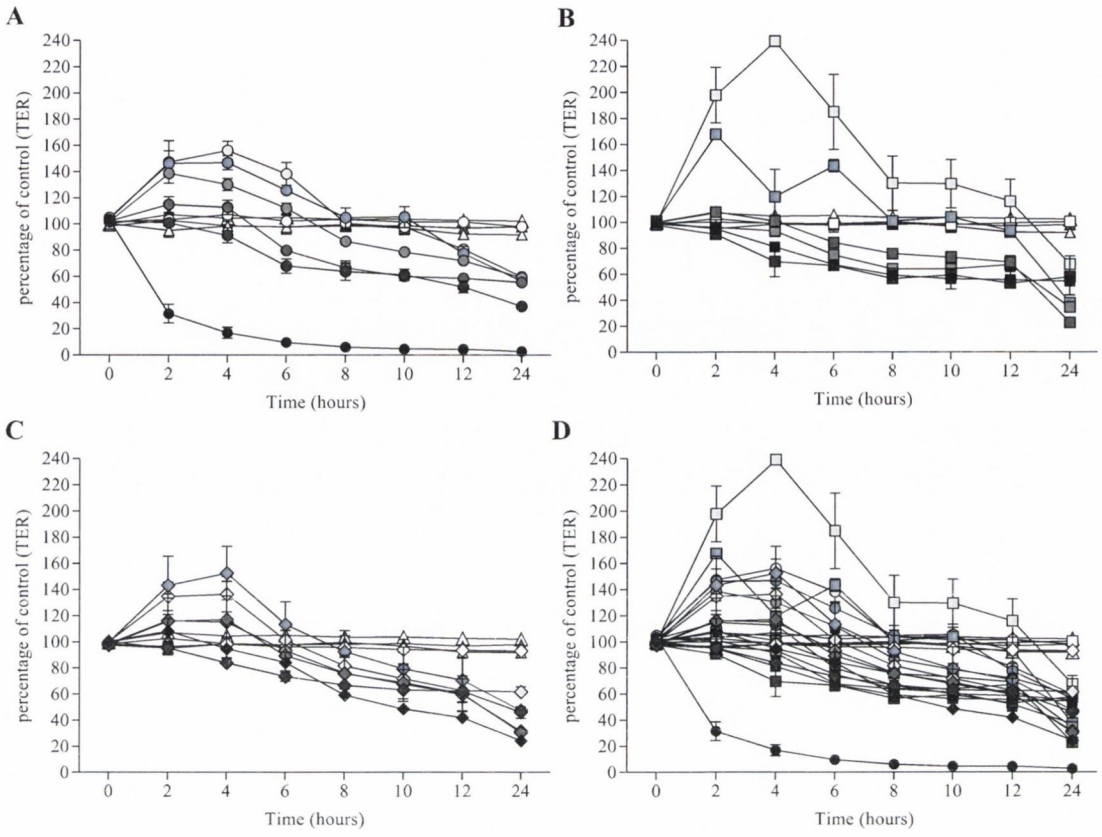


Figure 5.3: Effect of *A. fumigatus* culture supernatants on TER of CFBEs

The TER of CFBEs infected with, **A)** AF293 CSNs; 0 hr CSN (-○-), 4 hr CSN (-●-), 8 hr CSN (-●-), 12 hr CSN (-●-), 24 hr CSN (-●-), 48 hr CSN (-●-), 72 hr CSN (-●-), **B)** P-Af isolates CSNs; 0 hr CSN (-□-), 4 hr CSN (-■-), 8 hr CSN (-■-), 12 hr CSN (-■-), 24 hr CSN (-■-), 48 hr CSN (-■-) and 72 hr CSN (-■-)e, **C)** NP-Af isolate CSNs; 0 hr CSN (-◊-), 4 hr CSN (-♦-), 8 hr CSN (-♦-), 12 hr CSN (-♦-), 24 hr CSN (-♦-), 48 hr CSN (-♦-) and 72 hr CSN (-♦-), **D)** AF293 CSNs; (0 hr CSN (-○-), 4 hr CSN (-●-), 8 hr CSN (-●-), 12 hr CSN (-●-), 24 hr CSN (-●-), 48 hr CSN (-●-), 72 hr CSN (-●-)), P-Af isolates CSNs; (0 hr CSN (-□-), 4 hr CSN (-■-), 8 hr CSN (-■-), 12 hr CSN (-■-), 24 hr CSN (-■-), 48 hr CSN (-■-) and 72 hr CSN (-■-)) andNP-Af isolate CSNs; (0 hr CSN (-◊-), 4 hr CSN (-♦-), 8 hr CSN (-♦-), 12 hr CSN (-♦-), 24 hr CSN (-♦-), 48 hr CSN (-♦-) and 72 hr CSN (-♦-)).For all experiments controls of cells only (-△-), sterile latex beads (-▲-) and non-invasive *E. coli* (-▲-) were included. The y-axis represents percentage of control (TER) and the x-axis represents hours post inoculation. Error bars represent standard error of at least three independent replicates.

5.2.3 The ability of the wild type gliotoxin producing AF293 and the gliotoxin mutant *ΔgliG* conidia to effect the tight junctions of HBEs and CFBEs

In CFBEs the conidia of wild type AF293 and the gliotoxin mutant *ΔgliG* both resulted in a significant decrease in TER compared to the control by 24 hr. The AF293 conidia caused a significant decrease in TER of CFBEs compared to non-infected controls from 10 hr for AF293 ($p<0.01$, Two-way ANOVA and Bonferroni post-test). The *ΔgliG* conidia caused a significant decrease in TER of CFBEs compared to non-infected controls at 24 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.4 A). There was a significant difference between TER values for AF293 conidia versus *ΔgliG* conidia at 6 hr ($p<0.05$), 8 hr ($p<0.0001$) and 10 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test), however at 12 hr and 24 hr both caused a similar decrease in TER with no statistical difference (Figure 5.4A).

In the HBEs the conidia of AF293 and *ΔgliG* both resulted in a significant decrease in TER compared to the control by 24 hr. The AF293 conidia caused a significant decrease in TER of HBEs compared to non-infected controls from 10 hr for AF293 ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 5.4B). The *ΔgliG* conidia caused a significant decrease in TER of HBEs compared to non-infected controls from 12 hr ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 5.4 B). The AF293 and *ΔgliG* conidia followed a similar pattern of decrease in TER in HBEs and the only significant difference between then was at 24 hr where the AF293 caused a significantly greater decrease in TER compared to non-infected control ($p<0.05$, Two-way ANOVA and Bonferroni post-test), however at 12 hr and 24 hr both caused a similar decrease in TER with no statistical difference (Figure 5.4B).

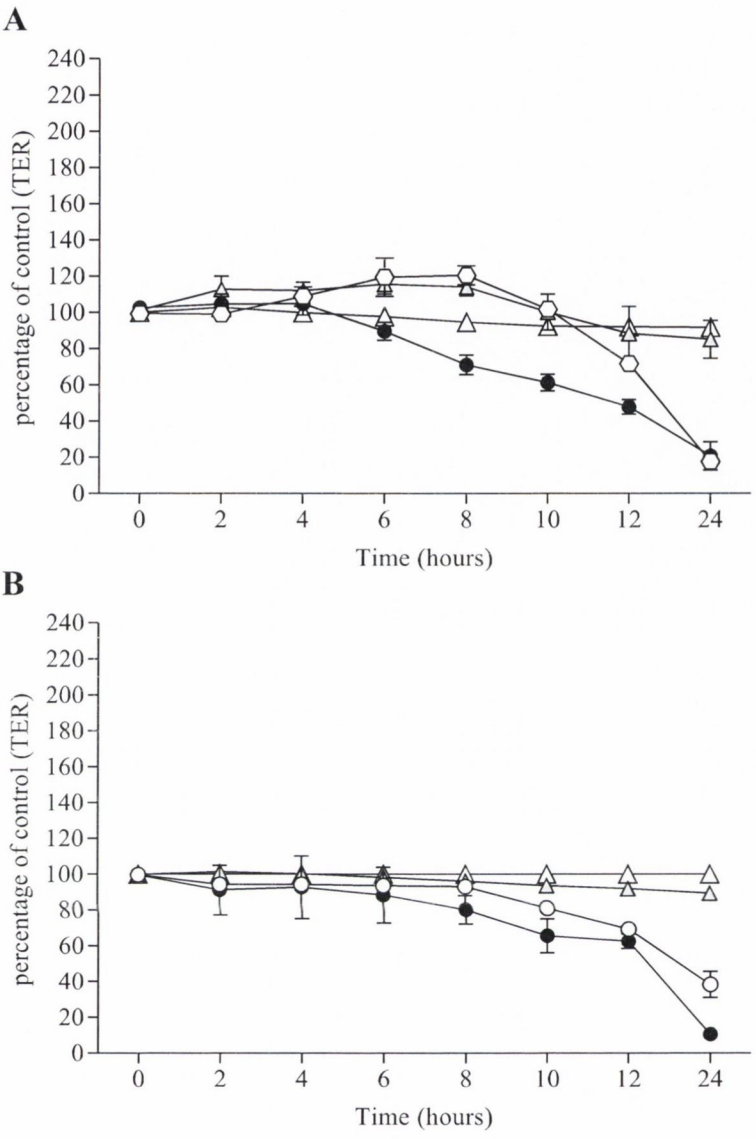


Figure 5.4: Effect of AF293 and *ΔgliG* on TER of CFBEs and HBEs.

The TER of **A)** CFBEs infected with AF293 (conidia (-●-) and *ΔgliG* (conidia (-○-). and **B)** HBEs infected with AF293 (conidia (-●-) and *ΔgliG* (conidia (-○-). For all experiments controls of cells only (-△-) and non-invasive *E. coli* (-▲-) were included. The y-axis represents percentage of control (TER) and the x-axis represents hours post inoculation. Error bars represent standard error of at least three independent replicates.

5.2.4 The ability of the wild type gliotoxin producing AF293 and the gliotoxin mutant *ΔgliG* CSNs to effect the tight junctions of HBEs and CFBEs

The 4 hr CSNs of AF293 caused a significant decrease in TER at 24 hr in CFBEs ($p<0.01$, Two-way ANOVA and Bonferroni post-test) but the 4 hr CSN of *ΔgliG* caused no decrease (Figure 5.5A). However in the first 2 to 6 hr of the experiment in CFBEs the 4 hr CSNs of AF293 (at 2 hr and 4 hr) and *ΔgliG* (at 2, 4 and 6 hr) caused a significant tightening (increase in TER). In HBEs, the 4 hr CSNs of AF293 and *ΔgliG* showed similar decreases in TER at 24 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) but no significant tightening in the early hours of exposure was observed (Figure 5.5B). The 8 hr CSNs of AF293 and *ΔgliG* caused a significant decrease in TER at 24 hr in HBEs and also CFBEs ($p<0.01$, Two-way ANOVA and Bonferroni post-test) (Figure 5.5A and 5.5B). By comparing the CSNs at 24 hr post exposure there was no significant difference in TER between the 4 hr CSN for AF293 and *ΔgliG* or between the 8 hr CSN for AF293 and *ΔgliG* in CFBEs or HBEs (Two-way ANOVA and Bonferroni post-test) (Figure 5.5C).

The 72 hr CSN displayed the greatest difference between the AF293 and *ΔgliG* in both the CFBEs and the HBEs (Figure 5.5). In CFBEs the AF293 72 hr CSN caused a significant decrease in TER after only 2 hr ($p<0.0001$, Two-way ANOVA and Bonferroni post-test), however for the *ΔgliG* 72 hr CSN there was no significant decrease in TER (Two-way ANOVA and Bonferroni post-test) (Figure 5.5A). In HBEs the AF293 72 hr CSN caused a significant decrease in TER after only 2 hr ($p<0.01$, Two-way ANOVA and Bonferroni post-test). For the *ΔgliG* 72 hr CSN there was a significant decrease in TER compared to the control at 8 hr ($p<0.01$) and 10 hr ($p<0.05$,Two-way ANOVA and Bonferroni post-test) however at 12 hr and 24 hr no statistical difference was found between the *ΔgliG* 72 hr CSN and the control (Figure 5.5B).

For the 72 hr CSN there was a significant difference observed between the AF293 and *ΔgliG* from ($p<0.0001$, Two-way ANOVA and Bonferroni post-test) in both CFBEs and HBEs (Figure 5.5C). In CFBEs the AF293 72 hr CSN caused 70% greater decrease in TER compared to the *ΔgliG* 72 hr CSN. In HBEs the AF293 72 hr CSN caused 82% greater decrease in TER compared to the *ΔgliG* 72 hr CSN (Figure 5.5C).

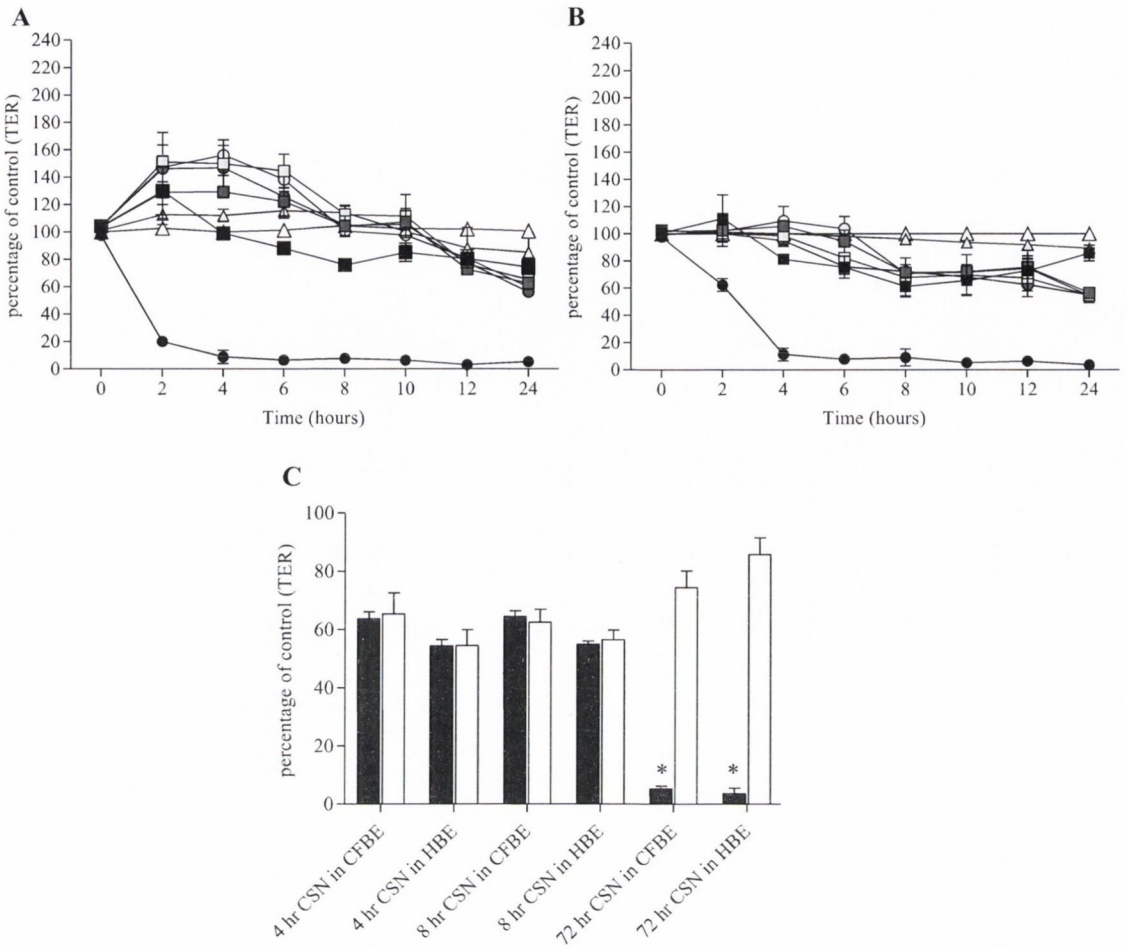


Figure 5.5: Effect of CSNs from AF293 and *ΔgliG* on TER of CFBEs and HBEs.

The TER of **A)** CFBEs infected with AF293 4 hr CSN (-▲-), 8 hr CSN (-■-) and 72 hr CSN (-●-) and *ΔgliG* CSNs 4 hr CSN (-Δ-), 8 hr CSN (-□-) and 72 hr CSN (-○-)) and **B)** HBEs infected with; AF293 4 hr CSN (-▲-), 8 hr CSN (-■-) and 72 hr CSN (-●-) and *ΔgliG* CSNs 4 hr CSN (-Δ-), 8 hr CSN (-□-) and 72 hr CSN (-○-). For all experiments controls of cells only (-△-) and non-invasive *E. coli* (-▲-) were included. The y axis represents percentage of control (TER) and the x-axis represents hours post inoculation. Error bars represent standard error of at least three independent replicates. **C)** Histogram illustrating the percentage decrease in tight Junction integrity compared to control at 24 hr for CSNs from AF293 (■) and *ΔgliG* (□)in HBEs and CFBEs. The y axis represents percentage of control (TER) and the x-axis represents CSNs in CFBEs or HBEs and *($p < 0.0001$, Two-way ANOVA and Bonferroni post-test).

5.2.5 The ability of gliotoxin to effect the tight junctions of HBEs and CFBEs

When gliotoxin was applied to the CFBE and HBE monolayers a decrease in TER compared to the control was observed. In CFBEs all gliotoxin concentration caused a significant decrease TER compared to the control and this was observed in a concentration dependent manner with higher concentrations causing a faster decrease in TER (Figure 5.6A). For the lowest concentration of gliotoxin (0.8μM) a significant decrease in TER was found at 24 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.6A). For the (8μM) a significant decrease was seen from 12 hr ($p<0.01$, Two-way ANOVA and Bonferroni post-test) and for the (80μM) a significant decrease compared to the control was observed after only 8 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.6A). Interestingly only the highest concentration of gliotoxin (80μM) caused a significant decrease in HBEs and this was observed from 8 hr ($p<0.001$, Two-way ANOVA and Bonferroni post-test) (Figure 5.6B). After 24 hr exposure to 80μM gliotoxin an 83% decrease in TER compared to the DMSO control was seen in CFBEs and a 51% decrease in HBEs (Figure 5.6C). The DMSO control caused no significant decrease in TER compared to the control (Figure 5.6).

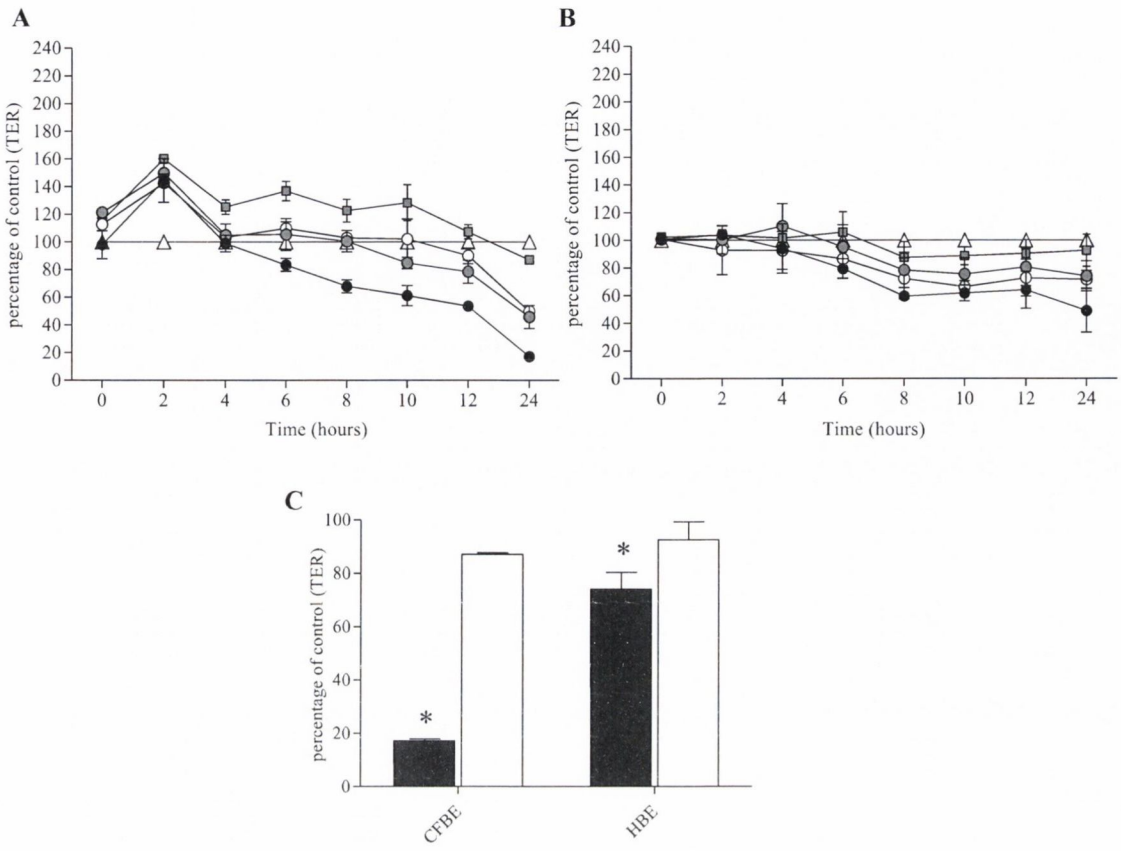


Figure 5.6: Effect of gliotoxin on TER of CFBEs and HBEs.

The TER of **A)** CFBEs exposed to gliotoxin at 0.8μM (-○-), 8μM (-●-) and 80μM (-●-) and **B)** HBEs exposed to gliotoxin at 0.8μM (-○-), 8μM (-●-) and 80μM (-●-). For all experiments controls of cells only (-△-) and 0.08% DMSO (-■-) were included. The y axis represents percentage of control (TER) and the x-axis represents hours post inoculation. Error bars represent standard error of at least three independent replicates. **C)** Histogram illustrating the percentage decrease in tight Junction integrity compared to control at 24 hr for 80 μM gliotoxin (■) and 0.08% DMSO control (□) in HBEs and CFBEs. The y axis represents percentage of control (TER) and the x-axis represents CFBEs or HBEs and *($p < 0.0001$, Two-way ANOVA and Bonferroni post-test).

5.2.6 Western blot Analysis of tight junction proteins ZO-1 and JAM-A following exposure to culture supernatants of AF293 and *Δ*gliG

GAPDH expression was observed in both CFBEs and HBEs (Figure 5.7A) and was used to normalise ZO-1 and JAM-A expression for densitometric analysis (Figure 5.7B and 5.7C).

Western blot analysis of ZO-1 and JAM-A demonstrated a clearly weaker band after 24 hr exposure to the 72 hr CSN of AF293 compared to the control or to the 72 hr CSN *ΔgliG* for ZO-1 (Figure 5.7A) and this was found to be significantly reduced in both CFBEs and HBEs when bands were analysed by densitometry ($p<0.0001$, One-way ANOVA and Bonferroni post-test) (Figure 5.7B). Likewise the AF293 72 hr CSN produced a weaker band for JAM-A in western blot analysis compared to the control or the 72 hr CSN *ΔgliG* (Figure 5.7A) and a significant difference between AF293 72 hr CSN and the control or *ΔgliG* 72 hr CSN was observed in both CFBEs and HBEs ($p<0.0001$, One-way ANOVA and Bonferroni post-test). No statistical difference was seen between the CFBEs and HBEs for any infection scenario (Figure 5.7). GAPDH levels at 72 hr CSN AF293 also very slightly reduced. This was not significantly reduced from control furthermore the concentrations of JAM-A and ZO-1 levels were normalised to GAPDH in this sample.

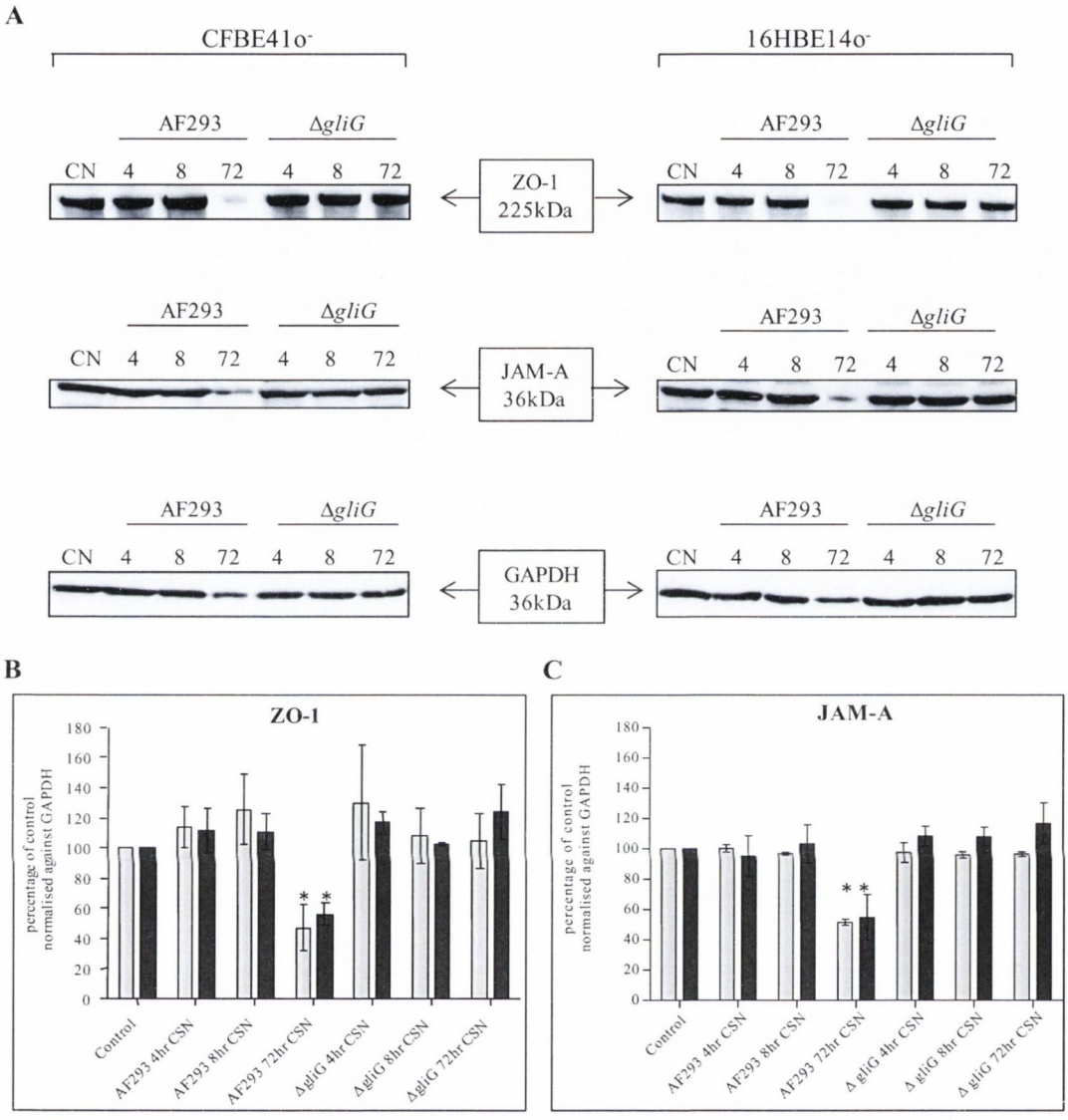


Figure 5.7: Expression of ZO-1, JAM-A and GAPDH in CFBEs and HBEs.

A) Western blot analysis of ZO-1, JAM-A and GAPDH in CFBEs and HBEs. Cells were treated with medium alone (control (CN)), 4 hr CSN (4), 8 hr CSN (8) or 72 hr CSN (72) from AF293 or *ΔgliG*. **B)** Densitometric analysis of ZO-1 in HBEs (▪) and CFBEs (■). **C)** Densitometric analysis of JAM-A in HBEs (▪) and CFBEs (■). Band intensities from three independent experiments were normalised against GAPDH values and are expressed as the mean. Error bars represent standard error of at least three independent replicates, *($p < 0.0001$, One-way ANOVA and Bonferroni post-test).

5.2.7 Immuno-fluorescence and Confocal microscopy of ZO-1 and JAM-A following exposure to 72 hr culture supernatants from AF293 and *ΔgliG*

FITC staining of ZO-1 and counterstaining using DAPI, showed ZO-1 demonstrating a distinct outline of each cell for the control of both CFBEs (Figure 5.8A) and HBEs (Figure 5.8B), illustrating the presence of tight junctions between cells. It can be seen that the AF293 72 hr CSN has caused complete disruption and/or internalisation of the ZO-1 tight junction protein and this was evident in both CFBEs (Figure 5.8A) and HBEs (Figure 5.8B). In contrast, the *ΔgliG* 72 hr CSN demonstrated distinct outline of ZO-1 around the cells of CFBEs (Figure 5.8A) and HBEs (Figure 5.8A) in a similar manner to the control.

FITC staining of JAM-A and counterstaining using DAPI, showed a similar result to ZO-1. JAM-A staining demonstrated a distinct outline of each cell and also evidence of small amounts of cystolic JAM-A within the cells for the control of both CFBEs (Figure 5.9A) and HBEs (Figure 5.9B), illustrating the presence of tight junctions between cells. Like ZO-1, for JAM-A it can be seen that the AF293 72 hr CSN has caused complete disruption and/or internalisation of the JAM-A tight junction protein and this was evident in both CFBEs (Figure 5.9A) and HBEs (Figure 5.9B). The JAM-A demonstrated distinct outlines around the cells of CFBEs (Figure 5.9A) and HBEs (Figure 5.9A) following exposure to the *ΔgliG* 72 hr CSN as in the control.

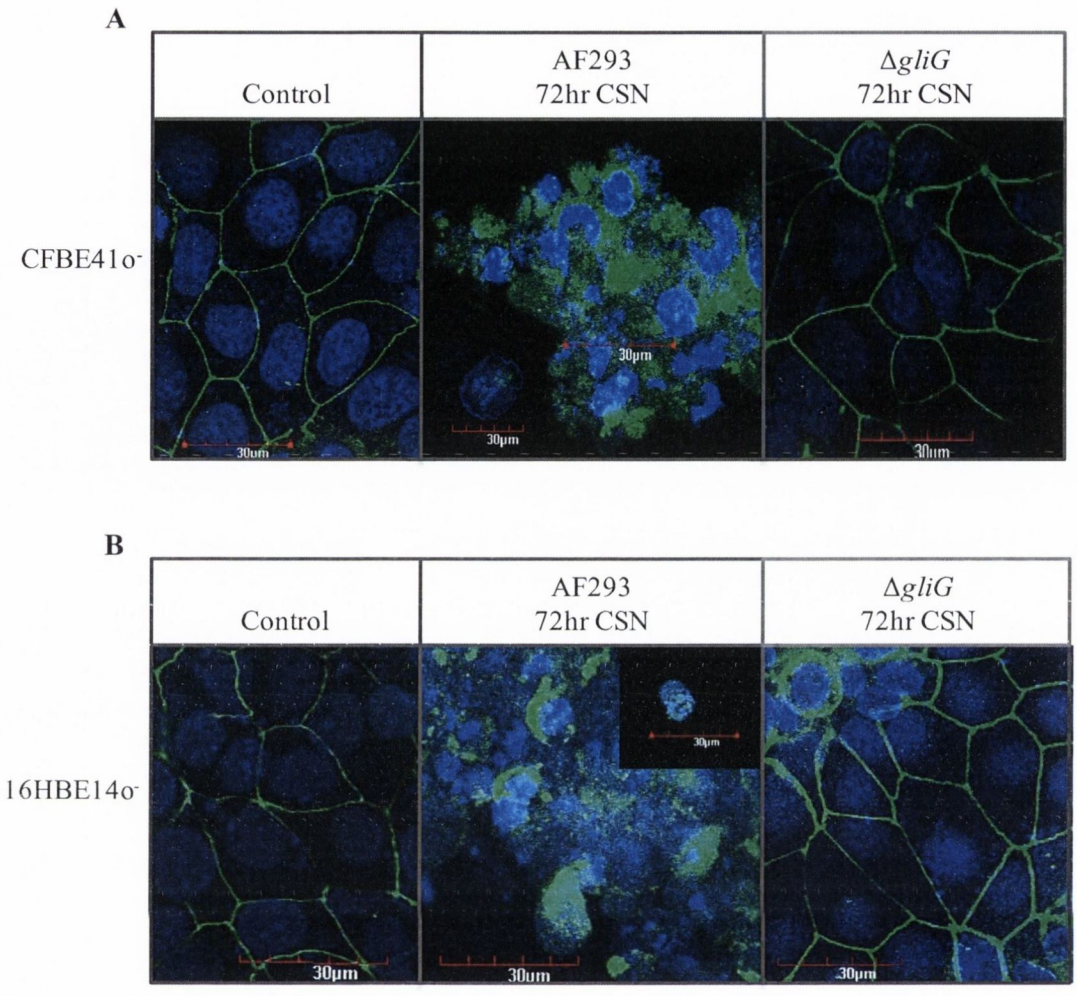


Figure 5.8: Effect of exposure to 72 hr culture supernatants on expression and distribution of ZO-1.

Effect of exposure to 72 hr CSN of AF293 and *ΔgliG* on expression and distribution of ZO-1 in **A)** CFBEs and **B)** HBEs, following 24 hr exposure. The cells were immunnostained with FITC conjugated anti-ZO-1 antibody (green), counterstained with DAPI (blue) and examined by confocal microscopy. Magnification x600, scale bar 30µm.

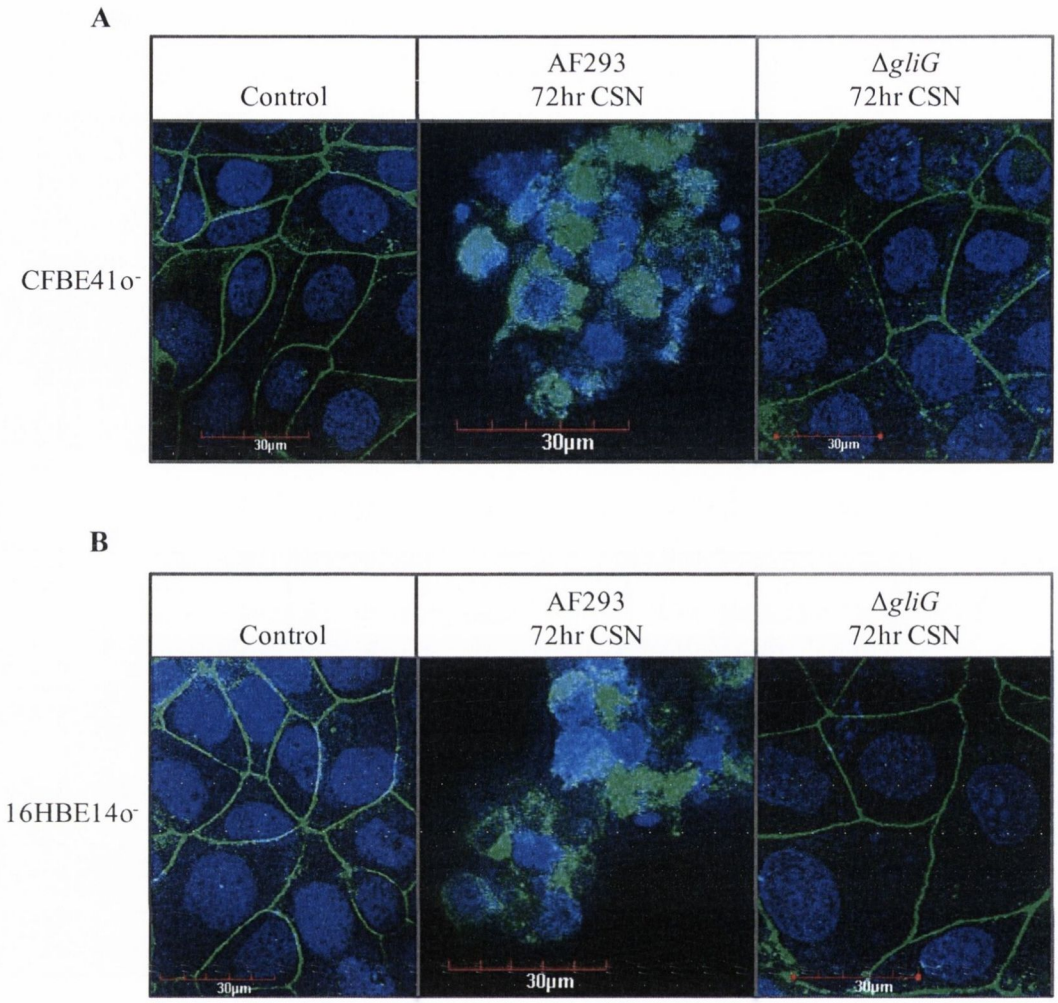


Figure 5.9: Effect of exposure to 72 hr culture supernatants on expression and distribution of JAM-A.

Effect of exposure to 72 hr CSN of AF293 and *ΔgliG* on expression and distribution of JAM-A in **A)** CFBEs and **B)** HBEs, following 24 hr exposure. The cells were immunnostained with FITC conjugated anti-JAM-A antibody (green), counterstained with DAPI (blue) and examined by confocal microscopy. Magnification x600, scale bar 30µm.

5.3 Discussion

The lung epithelium integrity relies on the individual epithelial cells to form tight junctions between all neighbouring cells [268]. With the daily exposure of *A. fumigatus* to the lung epithelium, the potential for *A. fumigatus* to affect the tight junctions exists and furthermore could contribute to inflammation in the CF lung. Here the ability of different *A. fumigatus* isolates to open epithelial cell tight junctions was explored.

The control CFBE and HBE monolayers where no conidia were added to CFBE and HBE cells maintained tight junction integrity for the duration of the experiment. This indicates that the lung epithelial cells maintained tight monolayer *in vitro* in the absence of infection and this has been observed in other studies focusing on bacteria [269, 273]. Controls of sterile latex beads that were 3μm in diameter (a size similar to that of *A. fumigatus* conidia) produced no significant reduction in tight junction integrity for the duration of the experiment (Figure 5.2), demonstrating that the physical presence of a structure of similar size to *A. fumigatus* conidia produced no disruption of tight junction integrity. Likewise the non-invasive *E. coli* caused no disruption of tight junction integrity showing that all microorganisms do not cause disruption in tight junction integrity within the experimental model (Figure 5.2). This *E. coli* strain has been previously used as a non-invasive comparative strain against *Burkholderia* [269, 273]. Tight junctions of HBE and CFBE cells infected with *A. fumigatus* conidia began to open tight junctions at 10 hours post infection and continued to open up to 24 hour post infection (Figure 5.2 and 5.4). Conidia of AF293 initiated tight junction opening of CFBEs earlier (10 hr) than the conidia of the persistent (P-Af) and non-persistent (NP-Af) clinical isolates which both did not open tight junctions until 24 hr (Figure 5.2A). A significant decrease in CFBE tight junction integrity was observed between infected versus non-infected (control) monolayers after 24 hr. A small number of other groups have looked at the effect of *A. fumigatus* conidia on the integrity of the epithelium [64, 281-283]. Amitani *et al* found conidia within the tight junctions of cells after 6 hr in an organ culture of human bronchial mucosal tissue (where human bronchial mucosal tissues were collected from the proximal bronchi of the lungs of patients with lung cancer and were then cultured and maintained in minimal essential medium) [64]. They also observed hyphae penetrating into the epithelial cells and through the tight junctions (at 12 hr) when examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) [64]. The authors noted that conidia within the intercellular spaces (tight junctions) were associated with dilation of the intercellular space [64]. These findings support our results that conidia cause a disruption of the epithelium integrity however, our findings provide a quantifiable description of the disruption of tight junctions.

Heat inactivated conidia caused a significant disruption of tight junction integrity in a similar pattern to that of live conidia. The same amount of time was required for the live or heat inactivated conidia to cause a significant decrease in tight junction integrity compared to non-infected controls. However at 24 hr post infection live conidia caused a significantly greater disruption of tight junction integrity compared to heat inactivated conidia. This may be due to the ability of live conidia to germinate and form hyphae at this later time point of the assay. These results show that inactivated conidia that cannot germinate are able to open tight junctions. This suggests that physical disruption of tight junctions by hyphae is not the only means by which *A. fumigatus* can disrupt tight junctions. The negative effect that heat inactivated conidia had on tight junctions cannot be explained simply by their physical presence as sterile latex beads of a similar size caused no reduction in tight junction integrity. In the *G. mellonella* model heat inactivated conidia caused no mortality (Section 4.2.6, Figure 4.7) however here we have shown that despite inactivation, *A. fumigatus* conidia can open CF and normal bronchial epithelial tight junctions. This illustrates the shortcomings of employing animal model survival as the sole measurement of virulence assessment. In CF, lung damage is accumulated over time by recurrent and chronic airway infections. The ability of a microorganism to cause lung damage and induce inflammatory responses is of great significance. Therefore conidia inactivated for example by antifungal drugs may still ellict a damaging effect and clearance of the organism would be as important as killing it by antifungal drug therapy. Differences in immune response in a murine model, reported that CD4+ T cell response of immunologically intact mice to live or heat inactivated *A. fumigatus* conidia differed [265]. They found that heat inactivated conidia were capable of priming CD4+ T cells in draining lymph nodes, in comparison to live conidia which stimulated IFN-Ɣ producing *A. fumigatus*-specific CD4+ T cells [265]. How the adaptive immune system distinguishes between live or inactive fungal spores remains unclear but it suggests that even if an organism is dead it may still ellicit a negative response during direct cell-to-cell interactions. This is significant in CF as the allergic form of *A. fumigatus* infections, ABPA, is a hypersensitivity response to allergens on the surface of conidia and hyphae. Heat inactivated conidia are still likely to contain these allergens and so could potentially elicit an allergic response. What is causing the heat inactivated *A. fumigatus* conidia to open tight junctions warrants further investigation (Section 6.1). Heat inactivated preparations of other microorganisms does not always open tight junctions. Hering *et al* found that *Yersinia enterocolitica* opened the tight junctions of colonic cell epithelial monolayers *in vitro* however heat inactivated organisms were not capable of opening tight junctions [284]. To our knowledge, there are no reports in the literature of a heat inactivated microorganism opening tight junction of the respiratory epithelium but the investigation of heat inactivated microorganisms on tight junctions does not appear to be common practice [64, 93, 269-271, 273, 281, 285]. Our results provide evidence that heat inactivated *A. fumigatus* conidia maintain the ability to open tight junctions.

There was no significant difference in the ability of conidia from the P-Af and NP-Af isolates to open the tight junctions (Figure 5.2). This corraborates our results in the *G. mellonella* infection model in chapter 4, where both strains caused a similar level of mortality (Figure 4.5). These findings, collectively provide preliminary evidence that these differentially colonising isolates are not more or less virulent than each other and that susceptibility to long term colonisation is perhaps more likely determined by host factors. In additional support of this argument, the P-Af isolate used in these experiments (p10 s4) was also shown to be non-persistent in another patient in the study (p6 s1). The genetic susceptibility of patients to *A. fumigatus* infection could provide further insight to conditions that may aid persistent colonisation {Mezger, 2010 #63;Ok, 2011 #64} [26]. Single nucleotide polymorphisms (SNPs) are frequent genetic variations within the human genome [26]. A number of SNPs have been associated with differences in susceptibility to *A. fumigatus* [286] [26]. This includes genes encoding for cytokines, chemokines, receptors, toll-like receptor genes, and other genes involved in innate immunity [286] [26]. Different SNPs have been associated with different types of *Aspergillus* disease including ABPA and IA and also with immune responses to *A. fumigatus* [26, 286]. Mannose-binding lectin (MBL) functions in fungal detection that activates innate immune mechanisms by binding to *A. fumigatus* and a defined SNP in the MBL gene has been associated with the development of ABPA [287]. Carvalho *et al.*, recently demonstrated a significant association between the presence of a SNP in *TLR4* and fungal colonisation [288] and SNPs identified in IL-10 (an anti-inflammatory cytokine) have been shown to be associated with *A. fumigatus* colonisation or ABPA in CF [289]. There are numerous SNPs identified to date and new SNPs are continuing to be described. With SNPs being associated with susceptibility to types of aspergillosis perhaps there may be a SNP yet to be described that may make a CF patient more likely to be persistently colonised by *A. fumigatus*. A study by Gomez-Lopez *et al* which looked at the virulence of *A. fumigatus* clinical isolates with and without the *cyp*51A mutations (which causes triazole resistance) in the *G. mellonell*a model found the patterns of virulence were similar for all isolates [249]. The comparison of epithelial cell interactions with different strains in a single study appears to be an uncommon practice where in general studies use a single reference strain or the comparison of a wild type and genetically mutated strain [59, 122, 123, 274, 281]. However bacterial studies have investigated the virulence of different clinical isolates or clinical versus environmental isolates in tissue culture studies. Bevivino *et al* who investigated the interaction of two environmental and a clinical *Burkholderia cepacia complex* isolate with CFBEs and found no difference in environmental versus clinical isolates for effect on tight junction integrity however clinical isolates were able to invade into the epithelial cells more readily that environmental isolates [269]. There are a limited number of studies investigating the difference in virulence between clinical isolates or genotypes of *A. fumigatus* within tissue culture studies [249, 290] and further studies in this area would be valuable to our understanding of *A. fumigatus* infections in CF. Despite reports of *A. fumi*gatus

genotypes persisting in a CF patient over time [7, 213, 214, 245], the virulence of these persistent isolates has not been characterised to our knowledge.

The lung epithelium integrity relies on the individual epithelial cells to form tight junctions between all neighbouring cells [268]. It is estimated that an individual inhales several hundred conidia on a daily basis [4] and furthermore conidia are 2-3μm in diameter and are therefore small enough to reach the alveoli of the lungs. In light of this the tight junctions of the respiratory epithelium incurs a lot of exposure to *A. fumigatus* and its diffusible components and secretions. We have shown that heat inactivated conidia can open tight junctions and therefore cell-to-cell contact is important for *A. fumigatus* to open tight junctions. Whether *A. fumigatus* produces diffusible components that affect the tight junctions is unclear. In our study we investigated the effects of *A. fumigatus* CSNs on the integrity of the tight junctions of HBEs and CFBEs. Interestingly, in response to early *A. fumigatus* CSNs the tight junctions of CFBEs significantly tightened in the first 6 hours of exposure. AF293 4 hr CSNs, P-Af 4 hr and 8 hr CSNs and *ΔgliG* 4 hr and 8 hr CSNs all elicited this affect. This tight junction tightening was only statistically significant for 2 to 6 hours. The same trend was observed in the NP-Af however no statistical significance was reached (Figure5.3). No significant tightening of HBE tight junctions was found for any *A. fumigatus* CSNs (Figure 5.5). Differing responses of CFBEs and HBEs to *A. fumigatus* has been previously reported [291]. Reihill *et al* reported CFBE produced greater levels of pro-inflammatory cytokines IL-6 and IL-8 than HBEs in response to *A. fumigatus* conidia [291]. Whether this is beneficial to the CF host or the isolate is unclear, especially considering the tightening of the epithelial barrier aids in the prevention of translocation of conidia out of the lungs. It might be possible that this considerable tightening could limit immune cell transport between the epithelial cells. This could be beneficial to the P-Af isolate which elicited the most dramatic tightening effect on CFBE tight junctions, allowing it to persist unhindered by immune cells in the CF airways. This tightening effect was only observed in CFBEs, suggesting that CF respiratory epithelial tight junctions may respond differently to HBEs in the initial response to *A. fumigatus* secretions. The tightening of tight junctions has been reported in tight junction repair [292] however we are not aware of any publications studying this tightening effect in response to a microorganism. As tightening caused by these early CSNs is transient we can hypothesise that the molecule causing tightening breaks down or is used up after 4 to 6 hr. The molecule or molecules causing this tightening in earlier *A. fumigatus* CSNs was not identified as part of this thesis however this would be an interesting avenue for future work (Section 6.2) as identification of a compound that can tighten the epithelial tight junctions could have therapeutic potential.

Although early CSNs caused a tightening of tight junctions this was transient and CSNs caused an opening of tight junctions over longer exsposure time points. The reference strain AF293 CSNs

caused the greatest disruption of tight junctions in CFBEs with the 4, 8, 12, 24, 48 and 72 hr CSNs all causing a significant reduction in tight junction integrity within 24 hr post infection (Figure 5.3A and 5.3B). The 8, 12, 24, 48 and 72 hr CSNs from P-Af caused a disruption in tight junction integrity within 24 hr post infection (Figure 5.3C). The 12, 24, 48 and 72 hr CSNs from NP-Af caused a disruption in tight junction integrity within 24 hr post infection (Figure 5.3D). From our results it can clearly be seen that CSNs derived from longer culturing times produced the greatest and fastest disruption of tight junction integrity. This was best illustrated by the CSNs of AF293 (Fig 5.3); the 4 hr CSN caused a significant reduction in tight junction integrity after 24 hr exposure, the 8 hr and 12 hr CSNs caused a significant reduction after 12 hr exposure, the 24 hr and 48 hr CSNs caused a significant decrease after 6 hr exposure while the 72 hr CSN caused a significant reduction in tight junction integrity after only 2 hr exposure (Figure 5.5.3B). This trend was also observed for CSNs from the P-Af and NP-Af isolates but to a lesser extent (Figure 5.1B & C). Comparison of the 72 hr CSN of AF293, P-Af and NP-Af revealed that AF293 72 hr CSN caused the greatest and fastest disruption of tight junction integrity compared to non-infected controls (Figure 5.3A). Importantly, even though CSNs cultured for a longer time caused a greater and more rapid disruption of tight junction integrity. Some of the earlier CSNs, namely the AF293 4 hr CSNs and P-Af 4 hr CSNs caused a significant decrease in tight junction integrity after 24 hr exposure, despite the initial tightening effect these CSNs elicited. This is important, particularly when considering conidia that are colonising the CF lung which may only be in the airways a short time. If 4 hr CSNs are capable of decreasing tight junction integrity after 24 hr, we hypothesise that conidia within the airways even for a short period of time may have adverse effects on the airway epithelium. Furthermore therapeutic and/ or host immune response eradication of the conidia will not eliminate the diffusible component's produced by the fungus and these may still remain in the airways. This demonstrates that even though the conidia of different isolates disrupt tight junction integrity to a similar extent after 24 hr, the CSNs of these isolates cause tight junctions to open at different rates. In CF *A. fumigatus* conidia are able to persistent despite antifungal therapy (chapter 3). Therefore conidia may remain in the airways and be exposed to the respiratory epithelium over time and diffuse componets into its surroundings. A handful of publications have begun to investigate *A. fumigatus* CSNs and their effect on the respiratory epithelium [25, 93, 281, 283, 285, 293]. Amitani *et al* investigated the effect of *A. fumigatus* CSNs on human nasal epithelial cells (HNCEs) from 14 different *A. fumigatus* clinical isolates [281]. They reported that *A. fumigatus* CSNs caused a significant damage to respiratory epithelium and slowing of ciliary beat frequency and found differences in the extent of damage and speed at which damage was caused [281]. Tomee *et al* reported that an *A. fumigatus* serine proteases not only induced IL-6 and IL-8 production but also caused epithelial barrier damage by cell detachment of A549 cells [93]. Khoufache *et al* investigated the role of an *A. fumigatus* myctoxin called verruculogen (isolated from 72 hr CSNs) on the respiratory

epithelium of HNECs [283]. They found that when verrculogen was isolated from cultures it reproduced similar results to the CSNs causing reduced TERs of HNECs [283].

A. fumigatus produces several secondary metabolites, proteases, elastases, allergens and mycotoxins including gliotoxin and how these effect tight junctions is not fully understood [4, 20, 34]. The 72 hr CSN caused the fastest and greatest reduction in tight junction integrity of all the CSNs tested. We hypothesised that gliotoxin, a secondary metabolite of *A. fumigatus* produced in highest quantities at 72-96 hr [63, 72, 294], may be contributing to the disruption in tight junction integrity observed here. In order to investigate whether gliotoxin played a role in reducing tight junction integrity we investigated whether live conidia and CSNs from AF293 (gliotoxin producing *A. fumigatus* [67]) and from *ΔgliG* (a mutant of AF293 that does not produce gliotoxin [67]) were equally capable of disrupting tight junctions [67]. Comparison of the live conidia of AF293 and *ΔgliG* showed that both opened CFBE and HBE tight junctions after 24 hr (Figure 5.4A and 5.4B). However the AF293 opened tight junctions earlier (10 hr) in both CFBEs and HBE compared to the *ΔgliG* which took 24 hr to begin to open tight junctions in CFBEs and 12 hr in HBEs (Figure 5.4). This may be attributed to stage of growth and hyphae production, which usually do not begin to produce hyphae until after12 hr. Gliotoxin production can start as early as 15 hr of culture [295]. The AF293 and *ΔgliG* have been shown to have the same rate of growth and both grew at the same rate in the presence of exogenous gliotoxin [67]. There was no significant difference in the virulence of the two isolates in the *G. mellonella* model [67] which corroborates our results. Following 48 hr of culture AF293 gliotoxin production was detected however no gliotoxin was detected in the 48 hr, 72 hr and 96 hr cultures of *ΔgliG* by liquid chromatography mass spectrometry (LC-MC) and reversed phase high performance liquid chromatography (RP-HPLC) [67]. The AF293 and *ΔgliG* culture filtrates were compared by LC-MS and RP-HPLC and the only difference other than gliotoxin production, was that *ΔgliG* produced a gliotoxin related metabolite shunt called M12.3. This M12.3 was found to have no thiols or di-sulphide bridge, the di-sulphide bridge has been shown to be crucial for the damaging activity of gliotoxin [56]. In our study, 4 hr and 8 hr CSNs of AF293 and *ΔgliG* caused similar reductions in tight junction integrity at 24 hr in HBEs and caused no disruption in CFBEs (Figure 5.5A and 5.5B). However the 72 hr CSN of AF293 and *ΔgliG* had different effects on the integrity of tight junctions of CFBEs and HBEs from 2 hr onward (Figure 5.5). After 24 hr exposure of CFBEs; 72 hr CSNs of AF293 resulted in a 70% greater decrease in tight junction integrity compared to the *ΔgliG* 72 hr CSN. Similarly after 24 hr exposure of HBEs to the 72 hr CSNs of AF293caused, an 82% greater decrease in tight junction integrity compared to the *ΔgliG* 72 hr CSN (Figure 5.5). Davis *et al.* demonstrated that AF293 produced gliotoxin after 72 hr culture but the *ΔgliG* did not [67]. This suggests that gliotoxin is likely contributing to the reduction in tight junction integrity of CFBEs and HBEs observed in our study. To further investigate whether

gliotoxin contributed to a reduction in tight junction integrity an experiment exposing CFBEs or HBEs to gliotoxin alone was performed. Results demonstrated that gliotoxin was capable of causing a reduction of tight junction integrity compared to the untreated control (Figure 5.6). In CFBEs all gliotoxin concentrations caused a significant reduction in tight junction integrity compared to the control and this was observed in a concentration dependent manner with higher concentrations causing a faster decrease (Figure 5.6A). Interestingly only the highest concentration of gliotoxin (80μM) caused a significant reduction compared to the control in HBEs. After 24 hr exposure to 80μM gliotoxin an 83% reduction in TER was seen in CFBEs and a 51% reduction in HBEs (Figure 5.6C). This suggests that the CFBEs were more susceptible to disruption of tight junction integrity in response to gliotoxin. Botterel *et al* investigated the effect of 24 hour *A. fumigatus* CSNs on human nasal epithelial cells (HNECs) by TER, optical microscopy and TEM when CSNs were exposed for 3 hr [282]. The authors found that after 3 hours exposure of *A. fumigatus* 24 hr CSNs to HNECs a significant decrease in TER was recorded however 24 hours after the CSNs were removed the epithelium was able to repair with TER readings reverting back to that of values recorded pre-exposure [282]. The authors stated that TEM analysis of HNECs showed that exposure of HNECs to 24 hr CSNs caused an altered morphological appearance with cytoplasmic vacuolization and increased intercellular spaces, however they noted that tight-junctions were present. Our study differed from the Botterel *et al* study in a number of points firstly, the above mentioned study [282] investigated the effects of 24 hr CSNs where our study looked at TER measurements of 24 hr CSNs but also, 4 hr, 8 hr, 12 hr, 48 hr and 72 hr CSNs of *A. fumigatus* from which we then studied the 72 hr CSN in more detail and furthermore the above study used HNECs which differed to the HBE and CFBEs we used. Where the above study measured changes in the epithelium by TER measurements and TEM, we used TER measurements, western blot analysis of tight junction proteins and immuno-staining of the tight junction proteins and viewing by confocal microscopy. Additionally the authors found that while 3 hr exposure of 24 hr CSNs which lead to TER measurements decreasing, the tight junctions were visibly present. We did demonstrate that after 24 hr exposure to AF293 72 hr CSNs that tight junction structure was completely lost but 24 hr CSNs were not inspected by confocal microscopy.

Although the Botterel *et al* study differed from ours considerably they also found results which support ours, namely that CSNs from *A. fumigatus* have a negative effect on the epithelium barrier. Studies investigating effects of gliotoxin on the respiratory epithelium have been reported, Amitani *et al* reported that gliotoxin slowed ciliary beat frequency (CBF) in an organ culture of human bronchial mucosal and the slowing in CBF was associated with epithelial cell damage and disruption [64]. Coughlan *et al.* found that *in vivo* gliotoxin was increased in CF patients positive for *A. fumigatus* and a reduction in bioburden (Figure 2.5 B and 2.5C) correlated

with a decrease in gliotoxin expression [59]. There is an ever increasing amount of evidence demonstrating the ill effect that gliotoxin has on the respiratory epithelium *in vitro* and these findings highlight the level of damage colonising *A. fumigatus* conidia may be causing in the CF airway.

We then sought to determine if the reduction in tight junction integrity by AF293 CSNs was caused by direct effects on the tight junction proteins. We investigated the quantitative presence and distribution of two tight junction proteins, ZO-1 and JAM-A in the monolayers of CFBEs and HBEs exposed to AF293 and *ΔgliG* CSNs. The tight junctions are formed by a complex of different proteins including ZO-1, JAM-A, occludins and claudins [267, 296-298]. The zonaula occludens proteins (ZO-1, ZO-2 and ZO-3) are a family of tight junction associated proteins [267, 296-298]. ZO-1 is a peripheral membrane protein found on the cytoplasmic surfaces of epithelial cell membranes such as the airways mucosa where it contributes to the intercellular seal that makes a tight junction. ZO-1 interacts with ZO-2 and ZO-3 and has been suggested to have both structural and signalling roles in the tight junction [267, 296-298]. Additionally ZO-1, ZO-2 and ZO-3 can associate with other tight junction proteins including claudin and occludin at tight junctions, where they provide a linkage between the actin cytoskeleton and the tight junction [267, 296-298]. JAM-A is a member of the immunoglobulin super family and is expressed in the tight junctions of epithelial cells and also endothelial cells and has been reported to play a role in leukocyte migration [267, 296, 299]. JAM-A localises with F-actin in epithelial cell tight junctions and is responsible for cell to cell adhesion and homophilic interactions [267, 296, 299]. Furthermore JAM-A dimerisation has been reported to be necessary for tight junction barrier regulation [300]. The levels of ZO-1 and JAM-A protein in CFBE and HBE monolayers exposed to 4, 8 and 72 hr CSNs of AF293 and *ΔgliG* was determined by western blot analysis and subsequent densitometric analysis of western blot bands. CFBEs exposed to 72 hr CSN of AF293 had markedly reduced levels of ZO-1 and JAM-A. In contrast CFBEs exposed to the *ΔgliG* 72 hr CSN had no reduction in ZO-1 and JAM-A proteins with levels being comparable to control untreated cells. This reduction of ZO-1 and JAM-A levels in response to 72 hr CSNs of AF293 was observed in CFBEs and HBEs. The gliotoxin producing AF293 72 hr CSN caused a 44% reduction in the levels of ZO-1 in CFBEs and a 53% reduction in HBEs compared to the control. Similarly, the AF293 72 hr CSN caused a 45% reduced expression of JAM-A in CFBEs and a 48% reduction in HBEs compared to the control. Our results further supports our hypothesis that gliotoxin is contributing to the loss of tight junction integrity observed when the respiratory epithelium are exposed to *A. fumigatus* CSNs. It should be noted that GAPDH levels also diminished slightly following 24 hr exposure to AF293 72 hr CSNs however the bar chart representing densitometric analysis is of ZO-1 and JAM-A was normalised to GAPDH for each exposure time and treatment scenario (Figure 5.7).

To confirm western blot analysis, confocal imaging of the tight junction proteins ZO-1 and JAM-A in CFBEs or HBEs following 24 hr exposure to 72 hr CSNs of both AF293 and *ΔgliG* was performed. HBEs and CFBEs exposed to *ΔgliG* 72 hr CSNs showed ZO-1 and JAM-A distribution similar to that of control untreated cells (Fig 5.8 & 5.9). In contrast exposure to 72 hr CSN of AF293 caused a complete breakdown of the structure of the tight junction (Fig 5.8 & 5.9). These results further demonstrate that *A. fumigatus* CSN disrupt the tight junctions of CFBE and HBE monolayers and that this disruptive ability is completely absent from the gliotoxin deficient *A. fumigatus* mutant. It may be more informative to look at earlier time points in the assay as perhaps too much damage has been caused to the epithelial monlayers by 72 hr CSNs after 24 hr exposure. This warrants further investigation (Section 6.2).

The results from this study suggest that exposure to *A. fumigatus* conidia or CSNs of *A. fumigatus* has a disruptive effect on tight junction integrity. In particular 72 hr CSNs were distructive to tight junction integrity although earlier CSNs also opened tight junctions. Wan *et al.* demonstrated that a breakdown in tight junction integrity in response to the house dust mite allowed migration of Der p allergens outside of the respiratory epithelium which could initiate an inflammatory response [270, 271]. It has been proposed by a number of studies [293, 297, 301] that disruption of airway epithelial tight junction integrity would permit allergens access to the underlying capillaries. Therefore opening of tight junctions could allow translocation of *A. fumigatus* conidia, hyphae and potentially allergens into the blood stream which may exacerbate or initiate an allergic reaction or ABPA.

While we have shown that gliotoxin is capable of contributing to the disruption of tight junction integrity other allergens and proteases have been reported to cause damage to the epithelial barrier and elicit inflammatory response [64, 93, 285, 302] and should not be ruled out as contributing factors to the effects on tight junctions observed in this study. Borger *et al.* [285] and Tomee *et al* both [93] reported that *A. fumigatus* proteases contribute to epithelial cell desquamation which caused an up-regulation in IL-6 and IL-8 in A549 cells. Tai *et al.* demonstrated that Pen ch 13, an alkaline serine protease damaged the epithelial barrier of A549 cells and primary HBEs by cleaving the tight junction protein occludin which produced an increase in the pro-inflammatory cytokine IL-8 [302]. The authors suggested that the allergen Pen ch 13 may contribute to asthma by damaging the airway epithelium and stimulating the release of mediators that could initiate an inflammatory response [302]. Khoufache *et al.* demonstrated that an *A. fumigatus* mycotoxin, named verruculogen isolated form 72 hr culture supernatants, damaged the epithelium of nasal epithelial cells and they suggest investigation of this effect in the context of colonisation and invasion of the respiratory epithelium [283]. The authors also stated that gliotoxin isolated from the CSNs did not cause a decrease in TER [283] which contradicts our findings.

Elastase is an enzyme which is produced by *Aspergillus* and also many other fungi and bacteria, it breaks down elastin which has an essential role in the structure of the lung [303]. Considering elastase is not detectable in CSNs of AF293 until at least day 4 of culture [304, 305], we are assured that it is not contributing to tight junction opening in this study. Additionally the differences observed between the AF293 and *gliG* mutant (which can also produce elastase) [67] provide validation for this. Our results combined with other publications provide in *vitro* evidence that molecules produced by *A. fumigatus* are capable of disrupting tight junction integrity which may play a role in initiating or exacerbating inflammatory responses. We report here for the first time that *A. fumigatus* CSNs disrupt CFBE and HBE tight junction integrity and we have provided evidence that gliotoxin contributes to this effect. These findings have significance in the clinical setting as *A. fumigatus* conidia colonising the CF airways have the potential to disrupt the epithelial barrier and potentially contribute to inflammatory responses which ultimately damages the CF airways irreversibly.

Chapter 6
Discussion

Chapter 6

Discussion

6.0 Main discussion

6.1 Detection and bioburden of *A. fumigatus* in an Irish CF cohort

Aspergillus fumigatus is the most commonly isolated fungal pathogen in cystic fibrosis (CF) patients, being isolated from 6-80% of patients at one time or another [59, 124-126]. Lack of standardisation of CF sputum processing has likely contributed to the large variation in reports of the frequency of recovery of *A. fumigatus* in CF samples. Although a high proportion of CF patients are culture positive for *A. fumigatus*, it is interesting that the incidence of APBA in CF is relatively low, at only 7-9% [22, 27, 95, 169]. This is because many CF patients are colonised with *A. fumigatus* in the respiratory tract without displaying any symptoms of ABPA. It is not currently known what effect this colonisation, in the absence of ABPA, may have on the patient's health. While the direct impact of asymptomatic colonisation remains unclear, it has been linked to increased hospitalisations due to respiratory exacerbations not associated with ABPA [24, 166] and worsen radiological appearances despite minimal effect on lung function [166]. Microbial infections and the associated inflammation and subsequent lung damage are responsible for 95% of morbidity and mortality in CF patients [306]. Therefore any element contributing to the accumulation of lung damage in individuals with CF is of clinical significance. The treatment of CF patients who are asymptomatically or silently colonised with *A. fumigatus* remains at the discretion of the attending physician in most Irish CF centres as there is no standard for treatment of this group of CF patients. Additionally this choice of treatment is also observed internationally where CF patients who are culture positive for *A. fumigatus* are not treated if they do not have ABPA [177, 307].

The efficiency of antifungal drug therapy at reducing *A. fumigatus* bioburden in the CF airways is not fully understood. In CF patients dosage of antifungal drug (s) administered to the patient is often inadequate to achieve therapeutic levels in the blood or airways [177, 228] and poor absorption, as a complication of CF, presents further challenges in reaching therapeutic levels of drugs in the patient. Sub-optimal therapeutic levels of antifungal drugs in CF patients have been reported: Bentley *et al* reported that only 4 out of 8 children with CF reached therapeutic levels of itraconazole and 2 out of 8 reached therapeutic levels of voriconazole [228]. Likewise, Aaron *et al* reported only 57% of CF children and adults given oral itraconazole reached therapeutic

blood levels [177]. Despite these inadequacies, itraconazole remains a widely used treatment of ABPA without the use of corticosteroids for a number of reasons; available in oral formulation, distributes widely to tissues such as the respiratory tract and is suitable when corticosteroid toxicity in the patient is reported [21, 189, 243]. Additionally the long-term use of corticosteroids is associated with an increased incidence of the isolation of *A. fumigatus* from CF patient samples [308].

6.1.1 Optimisation of DNA extraction protocol

A key issue in determination of *A. fumigatus* colonisation in CF is identification of *A. fumigatus* from CF sputum or bronchoalveolar lavage fluid (BAL). Due to a lack of standardisation in the processing of CF sputum samples, some centres use culture techniques while other use DNA based techniques. With the vast number of DNA extraction kits available in the market, it can be difficult to choose which to use and which additional steps should be undertaken in order to produce the greatest DNA yield to allow accurate quantification. The optimisation of the extraction of *A. fumigatus* DNA from CF sputum has been studied by a number of groups with comparison of DNA extraction methods and kits [11, 130, 131, 134, 137, 142, 223, 226, 309, 310], however there remains no "gold standard" protocol for the detection and quantification of *Aspergillus* from clinical samples [130].

6.1.2 Determination of *Aspergillus* Bioburden in an Irish CF patient group undergoing itraconazole therapy for asymptomatic *Aspergillus* colonisation

In order to measure the bioburden of *A. fumigatus* we first sought to optimise a method for reliably recovering *A. fumigatus* DNA from CF sputum. The optimal method for extraction of *A. fumigatus* DNA from CF sputum and BAL included bead beating with 1mm beads for 180sec at 2000rpm followed by extraction using the Roche High Pure PCR template kit [136, 141, 223-225]. Although both kits tested gave mainly comparable results the Roche High Pure PCR template kit gave more reliable results at the lower conidia concentrations (Figure 2.3). The Roche kit produced expected results on par with the conidia concentration from which they were extracted from (Figure 2.3). Employing these optimised methods and culture techniques *A. fumigatus* bioburden was monitored in asymptomatically colonised CF patients pre- and post-itraconazole treatment. Determination of *Aspergillus* bioburden in an Irish CF patient group undergoing itraconazole therapy for asymptomatic *Aspergillus* colonisation

While a number of studies have looked at the benefit of treating with an antifungal by looking at patient symptoms and therapeutic drug levels, we took a different approach to monitoring the efficacy of itraconazole treatment. We assessed the treatment effectiveness of itraconazole by

monitoring the bioburden of *A. fumigatus* in the airways of asymptomatically colonised adults with CF pre- and post-itraconazole treatment. *Aspergillus* bioburden, as measured by CFU counts (CFU/g) and qPCR (equivalent conidia/g), was significantly reduced following itraconazole treatment (Figure 2.5). To our knowledge this is the first report of examining itraconazole treatment effectiveness with reducing fungal bioburden in the airways [59, 177]. Other studies have monitored the therapeutic levels of itraconazole in blood to indirectly interpret efficiency of antifungal therapy [177, 228]. However no study has directly tested the efficiency of antifungal therapy at reducing airway bioburden [59]. It is a limitation of our study that therapeutic levels reached in the airways and circulatory system were not also monitored alongside fungal bioburden however the reduction in airway bioburden of *A. fumigatus* is direct evidence of the efficacy of treatment in this study.

In this study we employed both culture-based and culture-independent techniques for determination of *A. fumi*gatus bioburden. Our results showed that CFU counts produced higher readings overall compared to qPCR results (Figure 2.5), a finding which has also been observed in other studies [130]. We hypothesised that this discrepancy may be attributed to the small portion of a sputum sample used for CFU count sample processing in which clumping of *A. fumigatus* conidia may occur, this could lead to an over-representation for CFU sampling or conversely a negative culture result depending on whether clumping of *A. fumigatus* conidia has occurred in the aliquot taken. In contrast, the DNA extraction process homogenises the sample very thoroughly and may give a more representative result. Discrepancies between culture-based and nonculture-based techniques have been reported not only in relation to *A. fumigatus* in CF [130] but also in diagnosis of; invasive aspergillosis [311], bacteria including *Escherichia coli*, *Klebsiella pneumonia* and *Enterococcus spp.* in pancreatic infections [312] and noscomial transmission of methicilin-resistant *Staphylococcus aureus* (MRSA) [313]. Culture based-methods are effective at identifying viable microorganisms such as *A. fumigatus* from patient samples however some bodily fluids can inhibit fungal growth such as blood [311]. Nonculture-based techniques such as β-D-glucan or DNA by qPCR are useful tests for the detection of *A. fumigatus* in patients, when culture-based techniques are unsuitable [311]. Additionally culture-based techniques in general test a small proportion of a sample which may lead to inaccurately high or low quantification of burden if the sample if not fully homogenised. While culture-based methods have the advantage of detecting only viable microorganisms, negative results may be recorded when incorrect nutritional and/or oxygen requirement are not met for the microorganism in question. Nonculture-based techniques such as DNA by qPCR detection can process the whole sample providing a more complete analysis of the sample however this method may also provide opportunity for loss of DNA due to numerous processing steps. Furthermore qPCR will detect DNA from not only viable microorganisms but additionally from any microorganisms which are

dead.As a result of the differences between the two methods, discrepancies between results is often noted; where a patient sample may be culture negative but qPCR positive [130, 311-313].

At a 12 month follow-up time point taken as part of this study, CFU counts remained low whereas qPCR results showed a minor increase when compared to the 6 month follow-up (Figure 2.5). This suggests that qPCR may be a more sensitive method for detecting small increases in *Aspergillus* bioburden in CF sputum. This increase in *A. fumigatus* bioburden at the 12 month follow-up time point also raises the question of whether these CF patients chronically colonised with *A. fumigatus* should be continually monitored especially considering a proportion of these patients will go on to develop ABPA [21, 24]. Our results, suggest that standard culture alone may miss, *A. fumigatus* present in the sample which has also been reported in other studies [130]. However, it should be kept in mind that while molecular methods are very sensitive they do not distinguish between viable and dead organisms. DNA can be very stable and is able to persist for extended periods of time in bacteria [314] and fungi including *Aspergillus* [315]. Propidium monoazide (PMA) is a dye which selectively penetrates cells with a compromised membrane (which are considered dead) [316]. Once inside the cell PMA binds to DNA, which in turn strongly inhibits PCR amplification of that DNA [316]. By using PCR and PMA treatment, the analysis of only viable organisms can be performed. The use of PCR and PMA has been used in bacterial [316, 317] and *Aspergillus* [315] studies. However the use of PCR and PMA with *Aspergillus* has so far been limited to ecological studies. In order to use this technique with *Aspergillus* and patient samples, an investigation of protocol technique specific to a type of patient sample would be required. Optimisation of PMA concentration, incubation time and length of light exposure which activates PMA would need to be performed before it should be applied to patient samples. Therefore we propose that both standard culture and molecular methods or the addition of pre-treatments such as PMA to molecular methods should be used for the optimal detection of *Aspergillus* in CF sputum.

While other studies have linked itraconazole treatment to improved patient health or reduced lung exacerbations, no studies have directly linked this to a reduction in *A. fumigatus* bioburden in the airways [28]. Therefore, these studies cannot draw conclusions on the antifungal properties of itraconazole being responsible for reduced lung exacerbations. The study presented here has clearly shown that treating asymptomatic *A. fumigatus* colonised CF patients reduced *Aspergillus* bioburden in the airways and this correlated with improved lung structure (a significant reduction in mosaic pattern following itraconazole treatment), decreased infective exacerbations with an overall improvement in respiratory symptoms, as measured by CFQR scoring system [59]. Studies showing that colonising *A. fumigatus* conidia are not innocent bystanders in the CF airways are increasing [24, 166]. McMahon *et al,* reported that patients with *A. fumi*gatus

colonisation and no ABPA showed greater radiological abnormalities (more severe bronchiectasis) compared to patients not colonised by *A. fumigatus* [166]. Our study further supports this finding as the reduction of *A. fumigatus* bioburden due to itraconazole treatment was associated with an improvement in radiological findings [59]. CF patients asymptomatically colonised with *A. fumigatus* may be acquiring lung damage which could contribute to the overall decline in lung capacity over their lifetime. Physicians should consider treating asymptomatic *Aspergillus* colonised CF patients.

6.2 Antifungal Susceptibility and Epidemiological Analysis of *A. fumigatus*

6.2.1 Antifungal Susceptibility

Although we have demonstrated benefit with the use of itraconazole to treat asymptomatically colonised CF patients this approach requires careful consideration as over use of any triazole comes with the risk of the development of resistance to the triazole antifungal drugs. There have been numerous publications of triazole resistance from around the world [189-197]. In the context of CF, *A. fumigatus* triazole resistance has been reported from triazole treated [189] and triazole naïve patients [318]. Triazole resistance has been associated with prolonged exposure to the triazoles and has also been separately attributed to the pressure of triazole fungicides used in agriculture. CF patients may incur a high rate of exposure to triazoles over their lifetime. Furthermore in Ireland triazoles are also used as fungicides in agriculture. Considering this, *A. fumigatus* isolates from our CF patient isolates (n=126) and a subset of non-CF patient isolates (n=61) were tested to assess any triazole antifungal drug susceptibility of the isolates. Clinical breakpoints are not available for mould testing by the CLSI method [173] therefore we used ECVs from recent publications [173, 186, 239]. ECVs have been suggested to help characterise the susceptibility of *Aspergillus spp.* isolates to itraconazole, posaconazole, voriconazole, amphotericin B and caspofungin to monitor the emergence of reduced antifungal sensitivity and triazole resistance mutations. We observed no antifungal drug resistance for any of the 187 isolates tested (Figure 3.3, Figure 3.4, Figure 3.5 and appendix II), with the exception of fluconazole which is inactive against *A. fumigatus*. While the echinocandins, amphotericin B and 5-flucytosine MICs were included in this report and no resistance was observed according to EVCs, these antifungal drugs are rarely used systemically in CF patients. The main aim of our testing was to determine the susceptibility of *A. fumigatus* isolates to triazoles which are commonly used to treat ABPA and *Aspergillus* colonisation in CF patients. No *A. fumigatus* isolates collected during this study were resistant to any of the triazoles tested (posaconazole, itraconazole, voriconazole and fluconazole), with the exception of fluconazole (Figure 3.4 and

appendix II). Even isolates collected from CF patients pre- and post-itraconazole treatment did not show any resistance to the triazoles tested. Amorim *et al,* found no triazole resistance (posaconazole, itraconazole and voriconazole) in 86 *A. fumigatus* isolates collected from 4 CF patients receiving triazole antifungal therapy (itraconazole (n=2) or voriconazole (n=2)) [241]. However the emergence of triazole resistance following treatment in CF patients has been reported [189]. Burgel *et al* screened 249 CF adults and found 52.6% to be colonised with *A. fumigatus* of which 4.6% of these isolates were less susceptible to itraconazole (MIC, ≥2 mg/litre) following itraconazole treatment, with an overall triazole resistance prevalence of 20% [189]. Considering the high rate of exposure to triazoles which CF patients may incur over their lifetime and additionally considering the use of triazoles as fungicides in agriculture the emergence of triazole resistance in Ireland remains a possibility [194, 197, 208, 231, 232]. Furthermore the lack of antifungal susceptibility testing in Ireland would make it difficult to identify the emergence of triazole resistance. Larger scale studies are required in order to assess whether triazole resistance is in fact present in *A. fumigatus* clinical isolates in Ireland and we suggest antifungal susceptibility should be considered as part of the routine diagnostic services within hospital laboratories particularly for patients at risk of aspergillosis or those on triazole prophylaxis.

6.2.2 Epidemiological Analysis of *A. fumigatus* in an Irish CF and non-CF cohort

After collecting CF and non-CF *A. fumigatus* isolates we sought to investigate the epidemiology of these isolates. The epidemiology of *A. fumigatus* isolates from Irish CF patients colonised with *A. fumigatus* and other non-CF patients revealed two patterns of colonisation; 1) persistent colonisation, 2) non-persistent colonisation and furthermore patients sharing an indistinguishable isolate was also found (Figure 3.7, 3.8, 3.10 and 3.11). Persistent colonisation was defined by us as colonisation with an indistinguishable genotype from two or more consecutive samples. Non-persistent colonisation was defined by us as colonisation with distinguishable isolates over time (no occurrence of the same isolate in consecutive samples). These definitions of persistent colonisation have been used in a similar context in other genotyping studies of CF isolates [209, 214, 245]. The findings from our results correlate with other genotyping studies of CF isolates [7, 213, 214, 244, 245]. De Valk *et al* found four different colonisation patterns from 204 isolates from three CF centres; 1) patients chronically colonised with a single genotype (persistent colonisation), 2) patients colonised with only unique genotypes (non- persistent), 3) patients sharing an identical isolate and 4) re-colonisation with a predominant genotype [7]. Cimon *et al* found examples of persistence, non-persistence and diversity of genotypes between patients, in 109 *A. fumigatus* isolates from seven CF patients. They suggested that persistent colonisation was associated with long-term (culture positive for *A. fumigatus* long-term) colonisation and a narrowing of diversity of genotypes [214]. In our study, one CF patient demonstrated colonisation

with an indistinguishable genotype over 6 months despite itraconazole treatment (patient 10) (Figure 3.4 and 3.5). We demonstrated that these isolates were susceptible to itraconazole (appendix II) and although itraconazole treatment caused a significant decrease in *Aspergillus* burden, this genotype persisted in the patient. How these persistent genotypes remain in the airways despite antifungal therapy is unclear and raises questions about whether these isolates adapt in order to persist. *A. fumigatus* has been shown to be able to alter its own environment and subsequently change expression profiles such as development of resistance which has been observed in CF patients [187, 191, 242, 320]. While we called indistinguishable isolates which persisted in a patient over time to be "persistent colonisation", one of the persistent *A. fumigatus* genotypes (patient 10) was indistinguishable from an isolate demonstrating non-persistent colonisation in patient 6. This suggests that the ability of *A. fumigatus* to persist in the CF airways is possibly a host trait. We found no one genotype was associated with CF which was illustrated by a large diversity of genotypes found in our CF isolates (Figure 3.6) and consolidates previous findings [7].

With no clear association between *A. fumigatus* isolates and a patient group or between *A. fumigatus* isolates and persistence, studies into host factors conveying genetic susceptibility to *A. fumigatus* colonisation could provide further insight into conditions that may aid persistent colonisation [26, 286]. Single nucleotide polymorphisms (SNPs) are frequent genetic variations within the human genome [26]. A number of SNPs have been described that genetically determine susceptibility to *A. fumigatus* infection [26, 286]. This includes SNPs in genes encoding for cytokines, chemokines, toll-like receptor (TLR) and other genes involved in innate immunity [26, 286]. Different SNPs have been associated with different types of *Aspergillus* disease including ABPA and IA and also with impaired immune responses to *A. fumigatus* [26, 286]. Mannose-binding lectin (MBL) plays a role in fungal detection that activates innate immune mechanisms by binding to *A. fumigatus*. A defined SNP in the MBL gene has been associated with the development of ABPA [287]. Carvalho *et al* recently demonstrated a significant association between the presence of a SNP in *TLR4* and fungal colonisation [288], A SNP identified in IL-10 (an anti-inflammatory cytokine) has been shown to be associated with *A. fumigatus* colonisation or ABPA in CF patients [289]. CF patients have been previously shown to have an altered production of IL-10 in the lung [321, 322]. There are numerous SNPs identified to date and new SNPs are continuing to be described. With SNPs being associated with susceptibility to particular types of aspergillosis perhaps there may be a SNP yet to be described that makes a CF patient more likely to be persistently colonised by a unique genotype of *A. fumigatus*.

Our analysis of multiple colonies per sputum sample showed examples of some CF patients being colonised with a unique genotype, while other were colonised with a number of genotypes (Figure

3.9). Although the number of samples from which we picked multiple colonies was small (n=9 for CF and n=3 for non-CF) we only found indistinguishable genotypes within our CF samples. We found 44% of CF samples had a single genotype present and 100% of the non-CF samples had multiple genotypes present. The diversity of genotypes within a patient sample has been associated with colonisation patterns, with numerous genotypes found in recently colonised patients and a single genotype being associated with long-term colonisation [214, 244, 245]. What causes the diversity of genotypes in the CF airways to narrow over time is unclear [70]. However the CF airway is a hostile environment for an organism to survive in and may impose many selective pressures. Exposure of *A. fumigatus* to exogenous gliotoxin has been reported to cause significant changes in *A. fumigatus* protein expression including up-regulation of the allergen Asp f3 [72]. Therefore multiple genotypes in the airways may cause interactions between the different genotypes which could result in an up-regulation in the expression of allergens.

Although patient 18's isolates had distinguishable genotypes, they were more closely related to each other than any of the other isolates (Figure 3.9). This was seen in patient 15's sample also, with the exception of one colony (p15 s1 c5) (Figure 3.9). This raises the possibility that these closely related genotypes in the one patient sample may evolve from each other. The ability of *A. fumigatus* to develop resistance against antifungals drugs such as the triazoles [189, 190, 194, 196, 202, 203] supports the theory that *A. fumigatus* may also evolve within the patient to aid its survival and persistence. Studies monitoring the whole genomes of multiple colonies per sample over longer periods of time in larger patient cohorts will be useful in answering these questions. Considering the fact that any patient sample may harbour multiple different genotypes, multiple colonies should be selected not only for genotyping but also for antifungal susceptibility as a resistant strain may be missed based on whether or not it is selected for testing.

The STR*Af* assay used in our study for epidemiological analysis of *A. fumigatus* isolates has been shown to be a highly discriminatory and reproducible typing method. De Valk *et al.* carried out a study comparing all techniques available for molecular typing of *A. fumigatus* and the performance of each method was evaluated with regard to feasibility, ease of interpretation and discriminatory power. The authors concluded that microsatellites/STRs was the method of choice for typing *A. fumigatus* isolates [17]. Vanhee *et al.* carried out a study comparing RAPD, SSPD, MLST, MLEE and STR; they found the STR method (STR*Af* assay method) produced the highest resolution, with the greatest reproducibility and discriminatory power [16]. Although the STR*Af* assay shows a high discriminatory power [211], one can only determine whether isolates are indistinguishable based on the 9 STRs assessed and only whole genome sequencing would allow complete comparison of the whole genome with 100% identity. In order to assess any and every difference in isolates and to see how *A. fumigatus* is evolving over time in the airways whole

genome sequencing would be required. Whole genome methods are complex and costly but ultimately provide more information. However the high cost associated with whole genome sequencing would likely make it unsuitable for large scale studies for the foreseeable future.

6.3 Virulence of *A. fumigatus* genotypes in the *G. mellonella* infection model

After finding a number of different genotypes and different patterns of colonisation we sought to investigate the virulence of different *A. fumigatus* genotypes. The variable pathogenicity of persistent and non-persistent *A. fumigatus* isolates has to date not been studied and remains unclear. We hypothesised that persistent isolates may have developed ways of evading the immune responses and this may relay to altered virulence patterns in the host. We sought to investigate the virulence of a range of *A. fumigatus* clinical isolates including a persistent and a non-persistent coloniser by employing the *G. mellonella* insect model. In a comparison between, 14 different clinical *A. fumigatus* isolates there were different patterns of mortality in the *G. mellonella* infection model (Figure 4.3). Some isolates caused a statistically significant increase in mortality after only 1 day compared to the uninfected controls, while others took between 2 and 5 days to produce a significant increase in mortality. These preliminary results suggest that different isolates of *A. fumigatus* have different levels of virulence, which may be important when considering *A. fumigatus* in the clinical setting. Other studies on bacteria have shown the varied pathogenicity of different strains of the same species over time in the CF airways [259] .

In CF bacteria such as *P. aeruginosa* and *Burkholderia cenocepacia* are routinely genotyped as different genotypes cause different levels of infection however this is not routinely performed with respect to *A. fumigatus*. Specific genotypes of *Burkholderia cenocepacia* for example ST-28 and ST-32 are associated with different levels of progression of infection and 5 year survival outcome [323]. *P. aeruginosa* strains such as LES (which is associated with poor prognosis in CF patients) and MES (known to be highly resistant to many antibiotics used to treat *P. aeruginosa*) are also routinely screened for [324]. No overall significant difference between the virulence of the persistent and non-persistent genotypes was noted (Figure 4.5). Although the persistent and non-persistent *A. fumigatus* genotypes caused similar levels of mortality in the *G. mellonella* model, this may not reflect the variable impacts these genotypes may have on inflammation and damage to local tissues and this requires further investigation.

Examination of the LD_{50} for four clinical isolates and the control strain AF26933 showed that mortality was concentration- and time-dependent (Figure 4.4). The concentration required to cause 50% mortality by day 7 was lower than the concentration required to cause 50% mortality

by day 4 for all isolates (Figure 4.4). This illustrated that the effect of *A. fumigatus* on survival in the *G. mellonella* host was concentration- and time-dependent, where higher concentrations caused reduced survival of the larvae over time compared to lower concentrations. This suggests that in the absence of antifungal drug treatment even low concentrations were able to cause mortality in the insect model. This supports the possibility that in CF patients, speed of diagnosis and early treatment may correlate with patient outcome [4, 41, 260] .

Persistently colonising *Pseudomonas aeruginosa* has been shown to genetically adapt to their environment, becoming less virulent in the host over time, presumably to aid in their persistence [259]. Whether this happens with *A. fumigatus* in the CF airways has yet to be established. CF patient 10 in our study had persistent colonisation with 4 isolates collected bi-monthly over 6 months (month 0, 2, 4 and 6) showing an indistinguishable genotype. The virulence of all four isolates was investigated employing the *G. mellonella* infection model. Our results illustrated that the fourth isolate caused a significant increase in mortality on days 1 and 2 post inoculation in the *G. mellonella* compared to the three earlier isolates (Figure 4.6). Although all isolates resulted in similar mortality on day 3, the increased rate of kill earlier in infection may be important in the clinical setting. Our results that *A. fumigatus* became more virulent over time are contradictory to the *P. aeruginosa* study [259] and to our initial hypothesis that they would become less virulent so as to be able to persist. These preliminary results suggest that *A. fumigatus* isolates, of the same genotype, may behave differently in the same host and indicate that persistently colonising isolates of *A. fumigatus* may modify their behaviour over time in the host. The genetic evolution of *A. fumigatus* in order to become resistant to triazole antifungal drugs has been reported [320]. Hagiwara *et al* showed that sequential isolates from a patient with IA and a patient with aspergilloma demonstrated genetic changes (*cyp51A* mutations and other non-synonymous mutations) in isolates which were indistinguishable over time by microsatellite typing [320]. Whether there is genetic evolution of *A. fumigatus* over time in the CF airways or how this may impact on the virulence of the genotype has not yet been reported to our knowledge and this requires further investigation (section 6.1).

Antifungal treatment in the *G. mellonella* model showed a significant reduction in mortality for the reference AF26933, the persistent and non-persistent isolates compared to those infected with *A. fumigatus* conidia who did not receive itraconazole (Figure 4.8). This supports the efficacy of itraconazole as a treatment for *A. fumigatus* colonisation or infection and supports our earlier conclusions that itraconazole treatment correlates with improved patient status [59]. Our findings in the *G. mellonella* model that itraconazole treatment that achieved sub-MIC concentration resulted in a greater mortality due to the clinical isolates compared to treatment with a dose equal

to or higher than the MIC (Figure 4.8) promotes our suggestion that antifungal susceptibility testing of clinical isolates is important and could avoid sub-optimal treatment.

6.4 The interaction of *A. fumigatus* and its CSNs with Respiratory Epithelial cell tight junctions

The lung epithelium integrity relies on the individual epithelial cells to form tight junctions between all neighbouring cells [268]. With the daily exposure of *A. fumigatus* to the lung epithelium, the potential for *A. fumigatus* to affect the tight junctions exists and furthermore could potentially contribute to inflammation in the CF lung. Some microorganisms have the ability to open these tight junctions by direct interaction such as *Burkholderia cepacia* complex [269] or by release of compounds such as proteases from the house dust mite [270, 271] in order to gain access to blood vessels or other organs. *Burkholderia cepacia* complex has been shown to open the tight junctions of epithelial cell tight junctions and then translocate from the apical side to the basolateral side of intact epithelial monolayers *in vitro* [273]. Wan *et al.* demonstrated that the proteases, Der p1 (a cysteine protease) from the house dust mite *Dermatophagoides pteronyssinus* allowed transepithelial migration of allergens by disrupting the epithelial tight junctions [270, 271]. This disruption and transepithelial delivery of allergens led to an enhanced release of proinflammatory cytokines from immortalised and primary bronchial epithelial cells [270, 271]. Several hundred conidia are inhaled on a daily basis [4, 25, 272] and *A. fumigatus* is found in 6-80% of CF patient respiratory samples [59, 124-126]. Considering this, an understanding of the effect of *A. fumigatus* and/or its secretions on the tight junction of the lung epithelium may provide more insight into allergic *Aspergillus* disease in CF and additionally provide insight into the effects of colonisation on the lung epithelium.

Here the ability of different *A. fumigatus* isolates to open epithelial cell tight junctions was investigated. Tight junctions of HBE and CFBE cells infected with live *A. fumigatus* conidia began to open tight junctions at 10 hr post infection and continued to open up to 24 hr post infection. Conidia of AF293 initiated tight junction opening of CFBEs earlier (10 hr) than the conidia of the persistent and non-persistent clinical isolates both of which did not begin opening tight junctions until 24 hr post infection. A small number of other groups have looked at the effect of *A. fumigatus* conidia on the integrity of the epithelium [64, 281-283]. Amitani *et al* found conidia within the tight junctions of cells in an organ culture of human bronchial mucosal tissue and the authors noted that conidia within the tight junctions were associated with dilation of the intercellular space [64]. Their study supports our findings that conidia cause a disruption of the epithelium integrity. However, our findings provide a quantifiable description of the disruption of tight junctions.

Whether the conidia need to viable to cause changes in the lung epithelium is unclear. Antifungal treatment of *A. fumigatus* may inhibit growth and kill conidia however this provides the opportunity for the respiratory epithelium to come into contact with these dead conidia. Heat inactivation of the conidia, killed conidia but conidia remained intact. Heat inactivated conidia of AF293, the persistent and non-persistent isolates caused a significant disruption of tight junction integrity in a similar pattern to that of live conidia. It took the same amount of time for the live and heat inactivated conidia to cause a significant decrease in tight junction integrity compared to the uninfected controls. After 24 hours post infection live conidia caused a statistically significant greater disruption of tight junction integrity compared to heat inactivated conidia. However this later disruption could be attributed to the ability of live conidia to form hyphae that can penetrate through tight junctions [64]. The ability of heat inactivated conidia to open tight junctions suggests that the presence of dormant colonising *A. fumigatus* conidia in the airways of CF patients could cause damage to the epithelial barrier. It is interesting to note that while the *G. mellonella* infection model provides a good guide of the virulence of an isolate, it does not always transfer to the host setting as the heat inactivated conidia caused no mortality in the *G. mellonella* model despite being able to open tight junctions of bronchial epithelial monolayers. This illustrates that heat inactivated conidia may not affect survival but may still negatively affect the host and cause damage. The investigation of heat inactivated microorganisms on tight junctions does not appear to be common practice, particularly with respiratory epithelial tight junctions where live organisms or antigens or culture supernatants appear to be the topic of investigation in most publications [64, 93, 269-271, 273, 281, 285]. Our results suggest that the structure of the *A. fumigatus* conidial wall may be contributing to the ability of conidia to open tight junctions. When the effect of conidia on the tight junction integrity was investigated, we found no significant difference between the ability of the conidia of persistent and non-persistent isolates to open tight junctions corroborating results in the *G. mellonella* infection model. Collectively these results suggest that persistent and non-persistent isolates do not have different levels of virulence and again point towards persistence being a host factor as opposed to a strain factor.

We are interested in the asymptomatic *A. fumigatus* colonisation of the CF airways and the contribution to airway damage this may cause. In light of this we investigated the effects of *A. fumigatus* culture supernatants (CSNs) on the integrity of the tight junctions of HBEs and CFBEs. By removing the conidia from the scenario we eliminate the possibility of effects seen being attributed by hyphal formation, which is already known to disrupt the epithelial barrier [64]. Here differences between the persistent and non-persistent isolates were observed. In response to persistent 4 hr and 8 hr CSNs the tight junctions of CFBEs significantly tightened in the first 6 hr of exposure. Although this trend was also observed with the non-persistent 4 hr and 8 hr CSNs it was not statistically significant. Whether this tightening is beneficial to the CF host or the isolate

is unclear, however it may be possible that the tightening of the tight junctions which aids the prevention of translocation of conidia out of the lungs to blood vessels and other organs, might also have a limiting effect on immune cell transport between the epithelial cells which in theory would be an advantage to a persistent isolate. This statistically significant tightening of the tight junctions was also observed in 4 hr CSNs from AF293 and *ΔgliG*. This tightening was only observed in CFBEs and no significant tightening of HBEs was observed following exposure to any *A. fumigatus* CSNs. CFBE monolayer tight junctions have been reported to be less tight than HBEs however our results reported here are percentage change relative to the uninfected control for each cell line therefore differences reported between CFBEs and HBEs are relative. The difference in response to *A. fumigatus* between CFBEs and HBEs has been reported previously [291]. Coyne *et al* found that CFBEs tight junctions were more vulnerable to proinflammatory cytokines IL-1β, TNF-α and IFN-ϒ compared to HBEs. This supports the hypothesis that inflammation in the CF lung contributes to lung damage. Reihill *et al* found CFBEs produced greater levels of the pro-inflammatory cytokines IL-6 and IL-8 in response to *A. fumigatus* compared to HBEs however this study investigated the response to *A. fumigatus* conidia but not CSNs [291]. The tightening of tight junctions has been reported in tight junction repair [292], however we are not aware of any publications studying the tightening of tight junctions in response to a microorganisms culture supernatant in the respiratory epithelium. As the tightening effect of these early CSNs is transient we can hypothesise that the molecule causing tightening breaks down or is used up within 4 to 6 hours. The molecule causing the tightening of the tight junctions in these earlier CSNs was not identified as part of this thesis however this would be an interesting avenue for future work (section 6.1) as identification of a compound that can tighten the epithelial tight junctions could have therapeutic potential.

All AF293 CSNs caused a disruption of tight junctions in CFBEs within 24 hours post infection. All persistent isolate CSNs from 8 hr CSN upward caused a disruption of tight junctions in CFBEs within 24 hours post infection. All non-persistent isolate CSNs from 12 hr CSN upward caused a disruption of tight junctions in CFBEs within 24 hours post infection. We have demonstrated that while no significant differences were observed in both the *G. mellonella* model and tight junction integrity work when the conidia of persistent and non-persistent isolates were compared, the CSNs of the isolates showed significant differences in their ability to disrupt CFBE and HBE tight junctions. This suggests that differences between persistent and non-persistent isolates might only be seen when the effect of CSNs are analysed.

From our results it was evident that CSNs derived from later cultures produced the greatest and fastest disruption of tight junction integrity. This was best illustrated by the CSNs of AF293 where shorter culturing times such as the 4 hr CSN took longer to elicit a significant reduction in tight

junction integrity (24 hr) compared to longer culturing times such as the 72 hr CSN which caused significant disruptions in tight junction integrity at just 2 hr of exposure. This trend was also seen for CSNs from the persistent and non-persistent isolates but to a lesser extent. Comparison of the 72 hr CSN of AF293, persistent and non-persistent revealed that AF293 72 hr CSN caused the greatest and fastest disruption of tight junction integrity. Importantly, even though CSNs cultured for a longer time, caused a greater and more rapid disruption of tight junction integrity, the earlier CSNs, namely the 8 hr CSNs of all isolates except the NP-Af isolate caused a significant decrease in tight junction integrity after 24 hr exposure. These results suggest that asymptomatically colonising *A. fumigatus* isolates could be disrupting the epithelial barrier as early as 4 hr post-inhalation and from as little as 3 days post-inhalation, lung damage could be accumulating. It is worth noting that these experiments are *ex-vivo* and do not take into consideration factors such as responses of immune cells and the environment of the CF airway and therefore should be interpreted with caution. However, clinical studies have reported that asymptomatically colonising *A. fumigatus* causes lung damage (increased mosaic pattern and bronchiectasis) and increased respiratory exacerbations [59, 166].

As the 72 hr CSNs caused the most obvious affect we hypothesised that gliotoxin, a secondary metabolite of *A. fumigatus* that is produced in highest quantities at 72-96 hr in vitro [63, 72, 294], may be contributing to the disruption in tight junction integrity observed. CSNs of 96hr and beyond were not included in this study as elastase production is known to occur from 96 hr of culture [304, 305] and elastase is completely destructive to epithelial tissue [303]. To test our hypothesis we compared the ability of AF293 and an AF293 mutant *ΔgliG* (gift from Dr Sean Doyle) which is deficient in gliotoxin production [67] to open tight junctions and in particular, alter tight junction protein arrangement. Live conidia of AF293 and *ΔgliG* were both capable of opening CFBE and HBE tight junctions after 24 hr. This is unsurprising as both are capable of producing hyphae. The AF293 opened tight junctions earlier (10 hr) in both CFBEs and HBE than the *ΔgliG* (24 hr). The 4 hr and 8 hr CSNs of AF293 and *ΔgliG* both caused similar reductions in tight junction integrity at 24 hr in HBEs, however caused no opening of tight junctions in CFBEs. The later CSNs gave more interesting results with the 72 hr CSN of AF293 significantly disrupting the tight junction integrity, as early as 2 hr post-treatment. In contrast the 72 hr CSN of *ΔgliG* caused no significant disruption at any time point. This effect was seen for both HBEs and CFBEs. The 24 hr exposure of CFBEs to the 72 hr CSNs of AF293 caused an 80% decrease in tight junction integrity compared to the uninfected control and the *ΔgliG* 72 hr CSN caused a 24% decrease in tight junction integrity. The 24 hr exposure of HBEs to the 72 hr CSNs of AF293 caused a 38% decrease in tight junction integrity compared to the uninfected control and the *ΔgliG* 72 hr CSN caused a 14% decrease in tight junction integrity. Davis *et al.* demonstrated that AF293 produced gliotoxin after 72 hr culture but that *ΔgliG* did not [67]. This suggests that gliotoxin is

likely a contributing factor to the reduction in tight junction integrity of CFBEs and HBEs observed in our study.

To further investigate whether gliotoxin contributed to a reduction in tight junction integrity we exposed CFBEs or HBEs to gliotoxin alone. Results demonstrated that gliotoxin was capable of causing a reduction of tight junction integrity (Fig 5.6). CFBEs appeared to be more susceptible to disruption of tight junction integrity in response to gliotoxin, where all gliotoxin concentrations caused a significant reduction in integrity compared to the control and this was observed in a concentration dependent manner with higher concentrations causing a faster decrease (Figure 5.6). In comparison only the highest concentration of gliotoxin (80μM) caused a significant reduction compared to the control in HBEs (Figure 5.6). Studies investigating other effects of gliotoxin on the respiratory epithelium have been published. Amitani *et al*, reported that gliotoxin slowed ciliary beat frequency (CBF) in an organ culture of human bronchial mucosal tissue and the slowing in CBF was associated with epithelial cell damage and disruption [64]. Coughlan *et al.* found that *in vivo* gliotoxin was increased in the lungs of CF patients positive for *A. fumigatus* and a reduction in bioburden correlated with a decrease in gliotoxin expression [59]. Gliotoxin was also shown to cause a down-regulation of vitamin D receptor in macrophages and respiratory epithelial cells of the CF patients in the study [59]. We have now contributed to the ever increasing amount of evidence demonstrating the ill effect that gliotoxin has on the respiratory epithelium *in vitro* and furthermore these findings highlight the level of damage that colonising *A. fumigatus* conidia may be causing in the CF airway.

Finally in order to determine if the reduction in tight junction integrity by AF293 CSNs was caused by effects on the tight junction proteins we investigated the quantitative presence and distribution of two tight junction proteins. We investigated the quantitative presence and distribution of two tight junction proteins, ZO-1 and JAM-A in the monolayers of CFBEs and HBEs exposed to AF293 and *ΔgliG* CSNs. The tight junctions are formed by a complex of different proteins including ZO-1, JAM-A, occluding and claudin [267, 296-298]. ZO-1 is a peripheral membrane protein found on the cytoplasm surfaces of epithelial cell membranes such as the airways mucosa where it contributes to the intercellular seal that makes a tight junction. ZO-1 interacts with ZO-2 and ZO-3 and has been suggested to be having both structural and signalling roles in the tight junction [267, 296-298]. Additionally ZO-1, ZO-2 and ZO-3 can associate with other tight junction proteins including claudin and occludin at tight junctions, where they provide a linkage between the actin cytoskeleton and the tight junction [267, 296-298]. JAM-A is a member of the immunoglobulin superfamily and is expressed in the tight junctions of epithelial cells [267, 296, 299]. JAM-A localises with F-actin in epithelial cell tight junctions and is responsible for cell to cell adhesion, homophilic interactions and leukocyte

migration [267, 296, 299]. Furthermore JAM-A dimerisation has been reported to be necessary for tight junction barrier regulation [300]. Exposure of *Burkholderia cenocepacia* has been shown to cause a dramatic loss of ZO-1 from the tight junctions of HBEs and CFBEs [269, 273]. In our results, CFBEs exposed to 72 hr CSN of AF293 had markedly reduced levels of ZO-1 and JAM-A. In contrast CFBEs exposed to the *ΔgliG* 72h CSN had no reduction in ZO-1 and JAM-A proteins with levels being comparable to control untreated cells (Figure 5.7). This was observed in CFBEs and HBEs. These results were confirmed by confocal imaging of the tight junction proteins ZO-1 and JAM-A in CFBEs or HBEs following 24 hr exposure to 72 hr CSNs of both AF293 and *ΔgliG* (Figure 5.8 and 5.9). In parallel to western blot analysis, the 72 hr CSN of AF293 caused a complete breakdown of the structure of the tight junction while 72 hr CSN from *ΔgliG* demonstrated distinct contiguous rings of ZO-1 and JAM-A at the tight junctions in par with the untreated control (Figure 5.8 and 5.9). Considering the role that ZO-1 and JAM-A play in tight junction integrity, the disruption that is caused by 72 hr CSNs could potentially have serious implications in the CF airway.

The results from this study suggest that exposure to *A. fumigatus* conidia or CSNs of *A. fumigatus* has a disruptive effect on tight junction integrity. The 72 hr CSNs were particularly destructive to tight junction integrity and we have shown that gliotoxin contributes to this disruption. Earlier assay time points may be interesting to analyse to further investigate the initiation of tight junction protein disruption and perhaps at the 24 hr time point a lot of damage has been caused to the epithelial monolayers. This warrants further investigation (Section 6.1). It has been proposed in a number of publications [293, 297, 301] that disruption of airway epithelial tight junction integrity would permit allergens access to the underlying capillaries. Therefore opening of tight junctions could allow translocation of *A. fumigatus* conidia, hyphae and potentially allergens into the blood stream which may exacerbate or initiate an allergic reaction or ABPA. McMahon *et al,* reported that patients with *A. fumi*gatus colonisation and no ABPA showed greater radiological abnormalities (more severve bronchiectasis) compared to patients not colonised by *A. fumigatus* [166]. It is not currently known why CF patients chronically colonised with asymptomatic *A. fumigatus* for long periods of time suddenly develop ABPA and tight junction integrity could play a role here.

6.5 In summary, this project has demonstrated;

- Detection and quantification of *A. fumigatus* in CF sputum may be more reliably determined by a combination of CFU counts and qPCR. Itraconazole treatment caused a significant reduction in *Aspergillus* bioburden whichcorrelated with improved patient status and itraconazole treatment resulted in no emergence of triazole resistance of any of the *A. fumigatus* isolates. Furthermore no triazole resistance was detected in any of the 187 CF and non-CF isolates collected.
- Genotyping of the isolates from this study revealed CF patients persistently and non-persistently colonised and additionally CF samples could be found to contain a single genotype or multiple genotypes.
- These different genotypes caused different rates of mortality in the *G. mellonella* model however no significant difference in mortality could be found between the persistent and non-persistent isolates. Virulence of a persistent isolate over time did however demonstrate that later isolates were more virulent than earlier ones, suggesting development of virulence or adaptation to host over time in the patient.
- Both *A. fumigatus* conidia and CSNs were capable of opening CFBE and HBE tight junctions within 24 hr of exposure. The CSNs of persistent and non-persistent isolates produced different rates of disruption of tight junction integrity of the respiratory epithelium and only early CSNs from the persistent isolate were capable of significantly tightening the tight junction in the first 6 hours post exposure. Later CSNs from the persistent isolate also opened tight junctions more readily. These results illustrate that differences between the persistent and non-persistent isolates exist.
- Finally, later CSNs revealed the greatest disruption of tight junction integrity which is in part due to gliotoxin. The production of gliotoxin by *A. fumigatus* plays a role in the breakdown of tight junction integrity which could have implication in the human airways, particularly the CF airways where isolates may persist over time while remaining viable.

6.6 Future Work

- While there have been a number of studies investigating the difference in virulence between clinical isolates or genotypes of *A. fumigatus* [249, 290], there appears to be a lack of comparative information on persistent and non-persistent isolates in CF patients. We intend to perform a more in-depth analysis of differences in virulence factors and inflammatory responses to these different types of colonisers.
- The ability of persistent *P. aeruginosa* to genetically evolve within the CF airway over time has been reported [259]. Therefore we propose to carry out whole genome sequencing of sequential *A. fumigatus* persistent isolates identified in this study from at least one CF patient to investigate whether virulence factors have evolved over time in the genotype.
- The ability of heat inactivated conidia to open tight junctions was observed in this study. Therefore we propose to carry out an investigation to determine how heat inactivated conidia open tight junctions.
- Early CSNs from the persistent isolate and AF293 showed a significant tightening of the tight junction within 2 to 6 hr exposure. We aim to carry out an investigation to identify what molecule is being secreted from *A. fumigatus* during early growth phase that is responsible for the tightening of tight junctions in the first 6 hours of exposure to CFBEs.

Chapter 7

References

Chapter 7

References

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Chapter 8

Appendices

Appendix I

Appendix I

Table of nucleotide sequence BLAST results for all clinical isolates collected through the project

Sample Patient no (p), sample no (s), colony no (c)	**Accession Number**	**Description**	**Max Score**	**Identity**
p1 s1	HQ026746.1	*Afum*	1151	100%
p1 s2	HQ285578.1	*Afum*	822	100%
p2 s1	AB354187.1	*Afum*	941	100%
p2 s2	AB354187.1	*Afum*	878	100%
p3 s1	HQ285578.1	*Afum*	884	100%
p4 s1	AB354187.1	*Afum*	813	100%
p5 s1	HQ285578.1	*Afum*	882	100%
p6 s1	AB354187.1	*Afum*	968	100%
p6 s2	AB354187.1	*Afum*	964	99%
p6 s3	JN227083.1	*Afum*	871	100%
p7 s1	AB354187.1	*Afum*	917	100%
p7 s2	HQ285578.1	*Afum*	817	100%
p7 s3	HQ285578.1	*Afum*	822	100%
p8 s1	HQ026746.1	*Afum*	1147	99%
p9 s1	AB354187.1	*Afum*	815	100%
p10 s1	HQ285578.1	*Afum*	968	100%
p10 s2	HQ026746.1	*Afum*	1142	100%
p10 s3	HQ026746.1	*Afum*	1140	100%
p10 s4	HQ026746.1	*Afum*	1109	100%
P14 s1 c1	KJ948647.1	*Afum*	999	100%
p14 s1 c2	NR121481.1	*Afum*	956	100%
p14 s1 c3	KF267254.1	*Afum*	1025	100%
p14 s1 c4	KJ567462.1	*Afum*	1068	100%
p14 s1 c5	KJ433647.1	*Afum*	999	100%
p14 s2 c1	KJ192188.1	*Afum*	862	100%
p14 s2 c2	KJ156821.1	*Afum*	985	100%
p14 s2 c3	FJ867935.1	*Afum*	984	100%
p14 s2 c4	KC237291.1	*Afum*	963	99%
p14 s2 c5	KC119200.1	*Afum*	1095	100%
p15 s1 c1	KC119199.1	*Afum*	888	100%
p15 s1 c2	JX501382.1	*Afum*	852	100%
p15 s1 c3	JX231009.1	*Afum*	962	100%
p15 s1 c4	JX232280.1	*Afum*	969	99%
p15 s1 c5	JX092088.1	*Afum*	999	100%
p15 s2 c1	JX092088.1	*Afum*	1025	100%
p15 s2 c2	HQ285554.1	*Afum*	1043	100%
p15 s2 c3	EU664488.1	*Afum*	1047	100%
p15 s2 c4	NR121481.1	*Afum*	1035	100%
p15 s2 c5	KC119200.1	*Afum*	992	100%
p16 s1 c1	FR733867.1	*Afum*	898	100%
p16 s1 c2	GQ461909.1	*Afum*	1000	100%
p16 s1 c3	KC119199.1	*Afum*	854	100%
p16 s1 c4	KJ192188.1	*Afum*	963	100%

Sample Patient no (p), sample no (s), colony no (c)	**Accession Number**	**Description**	**Max Score**	**Identity**
p16 s1 c5	FJ867935.1	*Afum*	1065	100%
p17 s1 c1	AB354187.1	*Afum*	977	100%
p17 s1 c2	AB354187.1	*Afum*	1080	100%
p17 s1 c3	AB354187.1	*Afum*	1111	99%
p17 s1 c4	AB354187.1	*Afum*	985	100%
p17 s1 c5	AB354187.1	*Afum*	1001	100%
p18 s1 c1	FM999059.1	*Afum*	1015	100%
p18 s1 c2	FM999058.1	*Afum*	1035	100%
p18 s1 c3	FM999057.1	*Afum*	952	99%
p18 s1 c4	KC119200.1	*Afum*	1036	100%
p18 s1 c5	KC119199.1	*Afum*	964	100%
p19 s1 c1	JX501382.1	*Afum*	812	100%
p19 s1 c2	KC237292.1	*Afum*	953	100%
p19 s1 c3	NR121481.1	*Afum*	953	100%
p19 s1 c4	HQ026746.1	*Afum*	955	100%
p19 s1 c5	HQ026746.1	*Afum*	826	100%
p20 s1	KF267254.1	*Afum*	845	100%
p21 s1	KC237292.1	*Afum*	945	100%
p22 s1	KC119200.1	*Afum*	925	100%
p23 s1	KC119199.1	*Afum*	935	100%
p23 s2	JX501382.1	*Afum*	933	100%
p24 s1	NR121481.1	*Afum*	925	100%
p25 s1	KC237291.1	*Afum*	985	99%
p26 s1	HQ026746.1	*Afum*	964	100%
p27 s1	NR121481.1	*Afum*	1035	100%
p28 s1	KJ948647.1	*Afum*	1038	100%
p29 s1	HG518523.1	*Afum*	899	100%
p30 s1	HG518522.1	*Afum*	1077	99%
p30 s2	NR121481.1	*Afum*	900	100%
p31 s1	KC237291.1	*Afum*	1033	100%
p32 s1	NR121481.1	*Afum*	1041	100%
p33 s1	JX501382.1	*Afum*	1055	100%
p34 s1	HQ285569.1	*Afum*	964	100%
p35 s1	HQ285578.1	*Afum*	1001	100%
p36 s1	EU664466.1	*Afum*	896	100%
p37 s1	FM999059.1	*Afum*	965	100%
p38 s1	FM999058.1	*Afum*	947	100%
p39 s1	FM999057.1	*Afum*	913	100%
p40 s1	KM066593.1	*Afum*	908	100%
p41 s1	JX501382.1	*Afum*	1068	100%
p42 s1	JX232280.1	*Afum*	1110	100%
p43 s1	NR121481.1	*Afum*	999	99%
p44 s1	KJ175461.1	*Afum*	985	100%
p45 s1	AB354187.1	*Afum*	962	100%
p46 s1	HQ285578.1	*Afum*	934	100%
p47 s1	KJ175459.1	*Afum*	916	100%
p48 s1	NR121481.1	*Afum*	854	99%
p49 s1	KJ175457.1	*Afum*	888	100%
p50 s1	KJ156821.1	*Afum*	977	100%

Sample Patient no (p), sample no (s), colony no (c)	**Accession Number**	**Description**	**Max Score**	**Identity**
p51 s1	KC237291.1	*Afum*	1038	100%
p52 s1	KC237292.1	*Afum*	1031	100%
p53 s1	JQ776545.1	*Afum*	1050	100%
p54 s1	KC237291.1	*Afum*	1042	100%
p55 s1	KC237292.1	*Afum*	1038	100%
p56 s1	JX501382.1	*Afum*	1055	100%
p56 s2	JQ966581.1	*Afum*	905	100%
p56 s3	AF455431.1	*Afum*	983	100%
p56 s4	KC237292.1	*Afum*	1027	100%
p57 s1	JQ966581.1	*Afum*	929	100%
p57 s2	KC237292.1	*Afum*	1035	100%
p57 s3	AB354187.1	*Afum*	1028	100%
p58 s1	KC689332.1	*Afum*	951	100%
p58 s2	KC119200.1	*Afum*	1022	100%
p59 s1	KC237291.1	*Afum*	1035	99%
p59 s2	JX092088.1	*Afum*	1025	100%
p60 s1	KC237291.1	*Afum*	1038	100%
p61 s1	JX501382.1	*Afum*	1038	100%
p61 s2	HQ285578.1	*Afum*	984	100%
p61 s3	FJ867935.1	*Afum*	1065	99%
p62 s1	JX501382.1	*Afum*	1055	100%
p63 s1 c1	KC237291.1	*Afum*	1017	100%
p63 s1 c2	KC237291.1	*Afum*	1038	100%
p63 s1 c3	KC237291.1	*Afum*	1008	100%
p63 s1 c4	KC237291.1	*Afum*	998	100%
p63 s1 c5	HQ248184.1	*Afum*	1048	100%
p63 s2	AB354187.1	*Afum*	990	100%
p63 s3	JX232280.1	*Afum*	896	100%
p64 s1	KJ156821.1	*Afum*	888	100%
p65 s1	JX501382.1	*Afum*	862	100%
p65 s2	EF669931.1	*Afum*	936	100%
p66 s1	EF669932.1	*Afum*	916	100%
p67 s1 c1	KJ156821.1	*Afum*	1019	100%
p67 s1 c3	FJ867935.1	*Afum*	1020	100%
p67 s1 c4	NR121481.1	*Afum*	1000	99%
p67 s1 c5	HQ026746.1	*Afum*	954	100%
p68 s1	KJ156821.1	*Afum*	1039	100%
p69 s1 c1	DQ837535.1	*Afum*	897	100%
p69 s1 c2	KJ850340.1	*Afum*	1033	100%
p69 s1 c3	KJ820681.1	*Afum*	998	99%
p69 s1 c4	AB354187.1	*Afum*	965	100%
p69 s1 c5	KJ175456.1	*Afum*	1037	100%
p70 s1 c1	KC119200.1	*Afum*	985	100%
p70 s1 c2	KC119199.1	*Afum*	1063	100%
p70 s1 c3	KJ192188.1	*Afum*	934	100%
p70 s1 c4	JX501382.1	*Afum*	1038	100%
p70 s1 c5	FJ867935.1	*Afum*	1088	100%
p71 s1	EF634403.1	*Afum*	1050	100%
p72 s1	EU664488.1	*Afum*	987	100%

Sample Patient no (p), sample no (s), colony no (c)	**Accession Number**	**Description**	**Max Score**	**Identity**
p72 s2	JX041523.1	*Afum*	832	100%
p73 s1	NR121481.1	*Afum*	1031	100%
p74 s1	HE864321.1	*Afum*	1079	100%
p74 s2	JX232280.1	*Afum*	931	100%
p75 s1	KC119199.1	*Afum*	1085	99%
p75 s2	JX006238.1	*Afum*	1030	100%
p76 s1	KC237291.1	*Afum*	971	100%
p77 s1	FM999059.1	*Afum*	1017	100%
p78 s1	EU664466.1	*Afum*	888	100%
p79 s1	AF455465.1	*Afum*	1055	99%
p79 s2	KC119200.1	*Afum*	1027	100%
p80 s1	KC119199.1	*Afum*	1057	100%
p81 s1	JX501382.1	*Afum*	978	100%
p82 s1	FM999061.1	*Afum*	902	100%
p83 s1	NR121481.1	*Afum*	897	100%
p84 s1	HQ392478.1	*Afum*	1091	100%
p84 s2	KF267254.1	*Afum*	932	100%
p85 s1	NR121481.1	*Afum*	1067	100%
p86 s1	KC237291.1	*Afum*	911	100%
p87 s1	DQ837535.1	*Afum*	992	100%
p88 s1	HQ026746.1	*Afum*	997	100%
p89 s1	JF754466.1	*Afum*	1036	100%
p90 s1	KJ156821.1	*Afum*	880	100%
p91 s1	GU982937.1	*Afum*	945	99%
p92 s1	KJ850340.1	*Afum*	1012	100%
p93 s1	FJ867935.1	*Afum*	1038	100%
p94 s1	NR121481.1	*Afum*	973	100%
p95 s1	KF267254.1	*Afum*	1006	100%
p96 s1	KC237291.1	*Afum*	889	99%
p97 s1	KJ192188.1	*Afum*	1000	100%
p98 s1	KJ820681.1	*Afum*	1045	100%
p99 s1	JX092088	*Afum*	922	100%
p100 s1	JX006238.1	*Afum*	1030	100%
p101 s1	KC689313.1	*Afum*	964	100%
p102 s1	FM999057.1	*Afum*	946	100%
p103 s1	FM999061.1	*Afum*	891	100%
p104 s1	FM999059.1	*Afum*	999	100%
p105 s1	FM999058.1	*Afum*	966	99%
p106 s1	FM999057.1	*Afum*	1047	100%
p107 s1	EU664467.1	*Afum*	846	100%
p108 s1	KJ156821.1	*Afum*	968	100%
p109 s1	DQ981399.1	*Afum*	857	100%
p110 s1	FM999058.1	*Afum*	895	100%

Afum = A. fumigatus

Appendix II

Appendix II

Susceptibility of clinical CF and non-CF *A. fumigatus* isolates and three *A. fumigatus* reference strains to nine antifungal drugs.

Sample Patient no (p), sample no (s), colony no (c)	Echinocandins				Azoles				
	Anid	Mica	Caspo	5-fluc	Posa	Vori	Itra	Fluc	Amph B
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
p1 s1	<0.015	<0.008	0.015	16	0.015	0.12	0.03	256	2
P1 s2	<0.015	0.015	0.03	16	0.015	0.12	0.03	>256	2
p2 s1	<0.015	<0.008	0.03	16	0.015	0.12	0.03	>256	1
p2 s2	<0.015	<0.008	0.015	8	0.015	0.12	0.03	256	1
p3 s1	<0.015	<0.008	<0.008	1	0.03	0.25	0.06	>256	2
p4 s1	<0.015	0.015	0.03	32	0.015	0.12	0.03	>256	2
p5 s1	<0.015	0.015	0.03	32	0.015	0.12	0.03	256	2
p6 s1	<0.015	<0.008	0.03	32	0.015	0.12	0.03	256	1
p6 s2	<0.015	<0.008	0.015	64	0.015	0.25	0.06	>256	1
p6 s3	<0.015	<0.008	0.015	32	0.015	0.25	0.03	>256	1
p7 s1	<0.015	<0.008	0.03	16	0.015	0.12	0.03	256	1
p7 s2	<0.015	<0.008	0.03	32	0.015	0.12	0.03	>256	1
p7 s3	<0.015	0.015	0.03	2	<0.008	0.12	0.03	256	1
p8 s1	<0.015	0.015	0.03	8	0.015	0.12	0.03	>256	1
p9 s1	<0.015	<0.008	0.03	8	0.015	0.12	0.03	>256	1
p10 s1	<0.015	<0.008	0.03	32	0.015	0.12	0.03	>256	2
p10 s2	<0.015	<0.008	0.03	32	0.015	0.12	0.03	256	2
p10 s3	<0.015	0.015	0.03	32	0.015	0.015	0.03	256	2
p10 s4	<0.015	<0.008	0.03	16	<0.008	0.12	0.03	256	2
p14 s1 c1	0.015	0.03	0.12	16	0.25	0.25	0.25	>256	1
p14 s1 c2	0.015	0.03	0.12	16	0.25	0.25	0.25	>256	1
p14 s1 c3	0.015	0.03	0.12	16	0.25	0.25	0.25	>256	1
p14 s1 c4	0.015	0.03	0.12	16	0.25	0.25	0.25	>256	1
p14 s1 c5	0.015	0.03	0.12	16	0.25	0.25	0.25	>256	1
p14 s2 c1	0.5	0.25	1	32	0.25	0.25	0.5	>256	1
p14 s2 c2	0.5	0.25	1	32	0.25	0.25	0.5	>256	1
p14 s2 c3	0.5	0.25	1	32	0.25	0.25	0.5	>256	1
p14 s2 c4	0.5	0.25	1	32	0.25	0.25	10.5	>256	1
p14 s2 c5	0.5	0.25	1	32	0.25	0.25	0.5	>256	1
p15 s1 c1	0.015	0.5	0.015	8	<0.008	0.12	0.03	256	1
p15 s1 c2	0.03	0.03	0.06	16	0.03	0.12	0.03	>256	1
p15 s1 c3	0.015	0.5	0.015	8	<0.008	0.12	0.03	256	1
p15 s1 c4	0.03	0.03	0.12	16	0.12	0.12	0.25	>256	1
p15 s1 c5	0.03	0.06	0.12	16	0.12	0.12	0.25	>256	1
p15 s2 c1	0.05	0.05	1	16	0.12	0.25	0.03	256	1
p15 s2 c2	0.06	0.03	0.12	8	0.12	0.25	0.25	>256	1
p15 s2 c3	0.05	0.05	1	16	0.12	0.25	0.03	256	1
p15 s2 c4	0.05	0.05	1	16	0.12	0.25	0.03	256	1
p15 s2 c5	0.06	0.03	0.12	8	0.12	0.25	0.25	>256	1
p16 s1 c1	1	1	1	8	<0.008	0.12	<0.015	256	1
p16 s1 c2	<0.015	<0.008	0.03	16	0.06	0.12	0.12	256	1
p16 s1 c3	0.03	0.008	1	8	0.12	0.12	0.12	>256	1
p16 s1 c4	<0.015	<0.008	0.03	16	0.06	0.12	0.12	256	1

Sample Patient no (p), sample no (s), colony no (c)	Echinocandins				Azoles				
	Anid	**Mica**	**Caspo**	**5-fluc**	**Posa**	**Vori**	**Itra**	**Fluc**	**Amph B**
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
p16 s1 c5	0.06	0.03	0.03	8	0.03	0.06	0.12	256	1
p17 s1 c1	0.5	0.5	0.5	16	0.12	0.25	0.25	>256	1
p17 s1 c2	0.5	0.5	0.5	16	0.12	0.25	0.25	>256	1
p17 s1 c3	0.5	0.5	0.5	16	0.12	0.25	0.25	>256	1
p17 s1 c4	0.5	0.5	0.5	16	0.12	0.25	0.25	>256	1
p17 s1 c5	1	0.5	0.5	16	0.12	0.25	0.25	>256	1
p18 s1 c1	<0.015	<0.008	0.03	64	0.015	0.12	0.03	256	2
p18 s1 c2	0.015	0.008	0.03	32	0.015	0.12	0.03	256	2
p18 s1 c3	0.015	0.008	0.03	32	0.015	0.12	0.03	256	2
p18 s1 c4	0.015	0.008	0.03	32	0.015	0.12	0.03	256	2
p18 s1 c5	0.015	0.008	0.03	32	0.015	0.12	0.03	256	2
p19 s1 c1	0.5	0.03	0.03	16	0.12	0.25	0.25	>256	1
p19 s1 c2	0.5	0.03	0.03	16	0.12	0.25	0.25	>256	1
p19 s1 c3	0.5	0.03	0.03	16	0.12	0.25	0.25	>256	1
p19 s1 c4	0.5	0.03	0.03	16	0.12	0.25	0.25	>256	1
p19 s1 c5	0.5	0.03	0.03	16	0.12	0.25	0.25	>256	1
p20 s1	<0.015	<0.008	0.03	8	<0.008	0.12	0.03	256	1
p21 s1	<0.015	<0.008	0.015	16	0.03	0.12	0.06	256	1
p22 s1	<0.015	<0.008	0.03	4	0.03	0.06	0.06	256	0.5
p23 s1	<0.015	<0.008	0.03	32	0.015	0.12	0.06	256	0.5
p23 s2	1	1	1	64	0.015	0.25	0.03	>256	2
p24 s1	<0.015	<0.008	0.015	16	0.015	0.06	0.03	>256	1
p25 s1	<0.015	<0.008	0.03	8	0.015	0.06	0.12	>256	1
p26 s1	<0.015	<0.008	0.015	16	0.015	0.12	0.12	>256	0.5
p28 s1	<0.015	<0.008	<0.008	0.5	0.03	0.06	0.12	256	0.5
p27 s1	<0.015	<0.008	<0.008	1	0.03	0.06	0.06	256	0.5
p29 s1	<0.015	<0.008	0.03	2	0.03	0.06	0.06	>256	1
p30 s1	<0.015	<0.008	0.03	2	0.03	0.06	0.12	256	1
p30 s2	0.06	0.06	0.5	64	0.015	0.25	0.03	>256	1
p31 s1	<0.015	<0.008	0.03	1	0.03	0.06	0.06	128	0.5
p32 s1	<0.015	<0.008	0.03	2	0.015	0.06	0.06	128	0.5
p33 s1	<0.015	<0.008	0.03	2	0.03	0.06	0.06	128	0.5
p34 s1	0.5	1	1	64	0.015	0.12	0.03	>256	1
p35 s1	2	1	1	32	0.015	0.25	0.03	>256	1
p36 s1	0.03	0.03	0.06	32	0.03	0.5	0.06	>256	0.5
p37 s1	0.12	0.03	0.03	64	0.015	0.25	0.03	>256	1
p38 s1	1	1	1	64	0.015	0.25	0.03	>256	1
p39 s1	0.5	4	1	64	0.015	0.12	0.03	>256	1
p40 s1	0.06	0.06	1	64	0.03	0.25	0.06	>256	1
p41 s1	1	1	1	16	0.015	0.25	0.03	>256	1
p42 s1	0.015	0.015	0.06	64	0.015	0.25	0.03	>256	1
p43 s1	0.015	0.015	0.12	32	0.03	0.5	0.06	>256	1
p44 s1	0.12	0.03	0.12	32	0.015	0.25	0.06	>256	2
p45 s1	1	0.5	1	64	0.015	0.12	0.03	>256	1
p46 s1	0.03	0.03	0.12	32	0.015	0.12	0.03	>256	1
p47 s1	0.03	0.03	1	64	0.015	0.25	0.03	>256	2
p48 s1	1	0.5	1	32	<0.008	0.25	0.03	>256	1
p49 s1	0.5	1	1	64	0.015	0.25	0.03	>256	1
p50 s1	1	1	1	64	0.015	0.25	0.03	>256	1
p51 s1	<0.015	<0.008	<0.008	32	0.015	0.12	0.06	>256	1
p52 s1	<0.015	<0.008	<0.008	64	0.03	0.12	0.12	>256	1

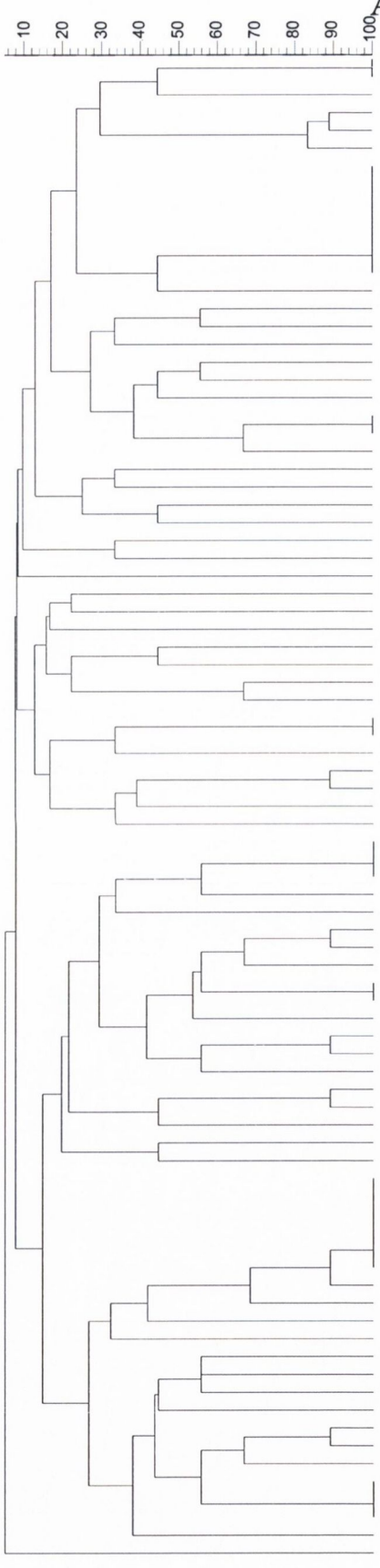
Sample Patient no (p), sample no (s), colony no (c)	Echinocandins				Azoles				
	Anid	Mica	Caspo	5-fluc	Posa	Vori	Itra	Fluc	Amph B
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
p53 s1	<0.015	<0.008	<0.008	16	0.015	0.12	0.06	256	1
p54 s1	<0.015	<0.008	<0.008	64	0.06	0.25	0.12	>256	1
p55 s1	<0.015	<0.008	0.03	64	0.03	0.12	0.12	>256	1
p56 s1	1	1	1	8	0.06	0.12	0.25	256	1
p56 s2	0.015	0.015	0.03	64	0.06	0.25	0.12	>256	2
p56 s3	0.03	0.03	0.008	64	0.06	0.12	0.12	>256	1
p56 s4	1	<0.008	1	32	0.03	0.25	0.06	>256	1
p57 s1	1	2	1	64	0.015	0.12	0.03	>256	0.5
p57 s2	0.03	0.06	0.25	64	0.06	0.12	0.25	>256	1
p57 s3	0.25	0.25	0.25	64	0.015	0.25	0.03	>256	1
p58 s1	1	0.06	0.12	64	0.06	0.12	0.25	>256	1
p58 s2	1	1	1	32	0.015	0.12	0.03	>256	1
p59 s1	1	0.25	0.5	8	0.015	0.25	0.03	>256	1
p59 s2	0.03	0.12	0.03	16	0.12	0.25	0.25	>256	1
p60 s1	1	1	0.5	64	0.03	0.25	0.06	>256	1
p61 s1	0.5	1	0.5	64	0.03	0.12	0.12	>256	1
p61 s2	0.06	0.25	0.5	64	0.015	0.25	0.03	>256	1
p61 s3	0.5	0.06	1	32	0.03	0.25	0.06	>256	2
p62 s1	<0.015	<0.008	0.015	16	0.06	0.25	0.25	>256	1
p63 s1 c1	<0.015	<0.008	<0.008	16	0.03	0.12	<0.015	>256	1
p63 s1 c2	0.015	0.015	0.03	8	0.03	0.12	0.06	>256	1
p63 s1 c3	0.015	0.015	0.03	8	0.03	0.12	0.06	256	1
p63 s1 c4	0.015	0.015	0.03	8	0.03	0.12	0.06	>256	1
p63 s1 c5	0.03	<0.008	0.015	16	0.03	0.25	0.03	>256	1
p63 s2	<0.015	<0.008	0.015	8	0.015	0.25	0.03	256	1
p63 s3	1	1	1	32	0.03	0.25	0.06	>256	2
p64 s1	1	1	1	64	0.03	0.25	0.06	>256	2
p65 s1	1	0.5	0.25	8	0.015	0.25	0.03	>256	1
p65 s2	0.5	0.06	0.25	8	0.015	0.25	<0.015	256	2
p66 s1	0.03	<0.008	0.12	16	0.25	0.5	0.5	>256	1
p67 s1 c1	0.015	0.03	0.03	8	0.25	0.5	1	>256	1
p67 s1 c3	0.015	0.06	0.06	16	0.12	0.12	0.03	>256	1
p67 s1 c4	0.015	0.03	0.03	8	0.25	0.5	1	>256	1
p67 s1 c5	0.015	0.06	0.06	16	0.12	0.12	0.1	256	1
p68 s1	0.06	0.03	0.06	16	0.12	0.25	0.25	>256	1
p69 s1 c1	<0.015	0.03	0.03	16	0.12	0.25	0.25	>256	2
p69 s1 c2	0.015	0.06	0.06	16	0.12	0.12	0.1	256	1
p69 s1 c3	<0.015	0.03	0.03	16	0.12	0.25	0.25	>256	2
p69 s1 c4	0.015	0.03	0.03	8	0.25	0.06	0.03	>256	1
p69 s1 c5	0.015	0.06	0.06	16	0.12	0.12	0.1	256	1
p70 s1 c1	0.03	0.03	0.06	16	0.25	1	1	>256	1
p70 s1 c2	0.12	0.06	0.03	16	0.12	0.12	0.25	256	1
p70 s1 c3	0.03	0.03	0.06	16	0.25	1	1	>256	1
p70 s1 c4	0.03	0.03	0.06	16	0.25	1	1	>256	1
p70 s1 c5	0.06	0.25	0.5	64	0.015	0.25	0.03	>256	1
p71 s1	<0.015	<0.008	0.25	64	0.03	0.12	0.12	>256	1
p72 s1	<0.015	<0.008	<0.008	64	0.015	0.12	0.03	>256	0.5
p72 s2	<0.015	<0.008	0.03	2	0.015	0.12	0.03	>256	1
p73 s1	<0.015	<0.008	0.03	64	0.015	0.12	0.03	>256	1
p74 s1	<0.015	<0.008	0.06	16	0.015	0.12	0.03	256	1
p74 s2	<0.015	<0.008	0.015	4	0.03	0.06	0.06	256	0.25

Sample Patient no (p), sample no (s), colony no (c)	Echinocandins				Azoles				
	Anid	**Mica**	**Caspo**	**5-fluc**	**Posa**	**Vori**	**Itra**	**Fluc**	**Amph B**
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
p75 s1	<0.015	<0.008	0.12	16	0.06	0.25	0.25	>256	1
p75 s2	<0.015	<0.008	0.06	8	0.03	0.12	0.12	>256	1
p76 s1	<0.015	<0.008	0.03	8	0.03	0.06	0.12	256	1
p77 s1	<0.015	<0.008	<0.008	4	0.015	0.12	0.03	>256	1
p78 s1	<0.015	<0.008	0.06	4	0.03	0.06	0.12	256	0.5
p79 s1	<0.015	<0.008	0.03	16	0.03	0.25	0.06	>256	1
p79 s2	<0.015	<0.008	0.015	2	<0.008	0.12	<0.015	256	1
p80 s1	<0.015	<0.008	0.03	32	0.015	0.12	0.03	256	1
p81 s1	<0.015	<0.008	0.03	4	0.03	0.12	0.12	256	1
p82 s1	<0.015	<0.008	0.03	16	0.015	0.12	0.03	>256	1
p83 s1	<0.015	<0.008	0.03	32	0.03	0.12	0.12	256	0.5
p84 s1	<0.015	<0.008	<0.008	1	0.015	0.06	0.06	256	0.5
p84 s2	<0.015	<0.008	0.03	32	0.015	0.12	0.03	>256	1
p85 s1	<0.015	0.015	0.03	16	0.015	0.25	0.03	>256	1
p86 s1	<0.015	<0.008	0.03	16	0.015	0.12	0.03	>256	1
p87 s1	<0.015	<0.008	0.25	16	0.06	0.25	0.25	>256	1
p88 s1	<0.015	<0.008	0.03	32	0.015	0.12	0.03	256	1
p89 s1	<0.015	<0.008	0.12	32	0.12	0.25	0.25	>256	1
p90 s1	<0.015	<0.008	0.03	4	0.015	0.12	0.03	>256	2
p91 s1	<0.015	<0.008	0.03	16	0.015	0.25	0.06	>256	1
p92 s1	<0.015	<0.008	0.03	64	0.015	0.12	0.03	>256	1
p93 s1	<0.015	0.015	0.03	64	0.03	0.25	0.06	>256	1
p94 s1	<0.015	<0.008	<0.008	8	0.015	0.12	0.03	256	1
p95 s1	<0.015	<0.008	0.06	16	0.03	0.25	0.06	>256	1
p96 s1	<0.015	<0.008	0.015	16	0.015	0.12	0.03	>256	1
p97 s1	<0.015	<0.008	<0.008	4	<0.008	0.12	<0.015	256	1
p98 s1	<0.015	<0.008	0.06	32	0.015	0.12	0.03	256	2
p99 s1	<0.015	<0.008	0.03	16	0.015	0.12	0.03	256	1
p100 s1	<0.015	<0.008	0.03	32	0.015	0.25	0.06	>256	1
p101 s1	<0.015	0.015	0.03	16	0.015	0.12	0.03	>256	1
p102 s1	<0.015	<0.008	0.015	4	0.015	0.12	0.06	>256	2
p103 s1	0.06	0.015	0.06	32	0.06	0.12	0.25	256	1
p104 s1	0.06	0.03	0.03	32	0.12	0.25	0.25	>256	2
p105 s1	0.015	0.06	0.06	16	0.12	0.12	0.25	>256	2
p106 s1	0.015	0.015	0.03	8	0.12	0.25	0.25	>256	2
p107 s1	0.03	0.03	0.06	16	0.25	0.25	0.25	>256	1
p108 s1	<0.015	<0.008	0.06	32	0.25	0.5	0.5	256	1
p109 s1	0.03	0.06	0.12	64	0.12	0.12	0.25	256	2
p110 s1	0.5	0.5	1	16	0.03	0.12	0.03	256	1
AF293	<0.015	<0.008	0.015	1	0.03	0.12	0.03	256	0.5
AF26933	<0.015	<0.008	0.015	4	0.015	0.12	0.03	256	1
Susceptible ref AF NCPF2109	<0.015	<0.008	0.03	16	0.06	0.12	0.12	256	1

Anid = anidulafungin, Mica= micafungin, Caspo= caspofungin, 5-fluc= 5-flucytosine, Posa= posaconazole, Itra=itraconazole, Vori= voriconazole, Flu= fluconazole, Ampho B=amphotericin B.

Appendix III

Appendix III

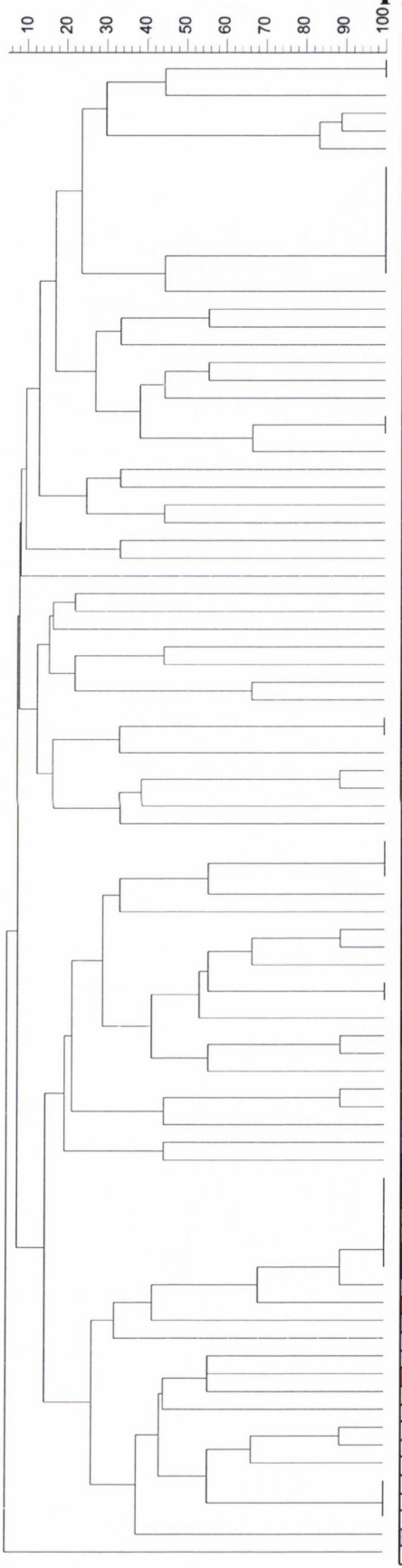


Patient nº	Sample nº	Patient Type	Hospital nº	Azole Sensitive
64	1	CF	4	✓
14	1	CF	1	✓
50	1	CF	2	✓
34	1	CF	2	✓
51	1	CF	3	✓
45	1	CF	2	✓
17	1	CF	1	✓
5	1	CF	1	✓
6	1	CF	1	✓
10	1	CF	1	✓
10	2	CF	1	✓
10	3	CF	1	✓
10	4	CF	1	✓
48	1	CF	2	✓
20	1	CF	2	✓
7	3	CF	1	✓
27	1	CF	2	✓
61	3	CF	4	✓
39	1	CF	2	✓
3	1	CF	1	✓
56	2	CF	4	✓
58	1	CF	4	✓
6	3	CF	1	✓
62	1	CF	4	✓
29	1	CF	2	✓
56	3	CF	4	✓
37	1	CF	2	✓
25	1	CF	2	✓
30	2	CF	2	✓
18	1	CF	1	✓
21	1	CF	2	✓
49	1	CF	2	✓
15	1	CF	1	✓
28	1	CF	2	✓
54	1	CF	3	✓
47	1	CF	2	✓
15	2	CF	1	✓
63	3	CF	4	✓
63	1	CF	4	✓
46	1	CF	2	✓
1	1	CF	1	✓
1	2	CF	1	✓
44	1	CF	2	✓
43	1	CF	2	✓
56	1	CF	4	✓
56	4	CF	4	✓
65	1	CF	4	✓
7	1	CF	1	✓
38	1	CF	2	✓
2	1	CF	1	✓
2	2	CF	1	✓
23	2	CF	2	✓
26	1	CF	2	✓
33	1	CF	2	✓
63	2	CF	4	✓
23	1	CF	2	✓
55	1	CF	3	✓
32	1	CF	2	✓
59	1	CF	4	✓
59	2	CF	4	✓
16	1	CF	1	✓
31	1	CF	2	✓
14	1	CF	1	✓
57	1	CF	4	✓
57	2	CF	4	✓
61	1	CF	4	✓
61	2	CF	4	✓
57	3	CF	4	✓
58	2	CF	4	✓
4	1	CF	1	✓
7	2	CF	1	✓
6	2	CF	1	✓
41	1	CF	2	✓
60	1	CF	4	✓
65	2	CF	4	✓
22	1	CF	2	✓
35	1	CF	2	✓
24	1	CF	2	✓
42	1	CF	2	✓
40	1	CF	2	✓
19	1	CF	1	✓
8	1	CF	1	✓
9	1	CF	1	✓
30	1	CF	2	✓
36	1	CF	2	✓

Figure 3.7: Dendrogram of microsatellite typing of CF isolates colour coded based on hospital. Metadata associated with the dendrogram is colour coded based on hospital number; hospital 1(■), hospital 2 (■), hospital 3(■) and hospital 4 (□). Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate.

Appendix IV

Appendix IV

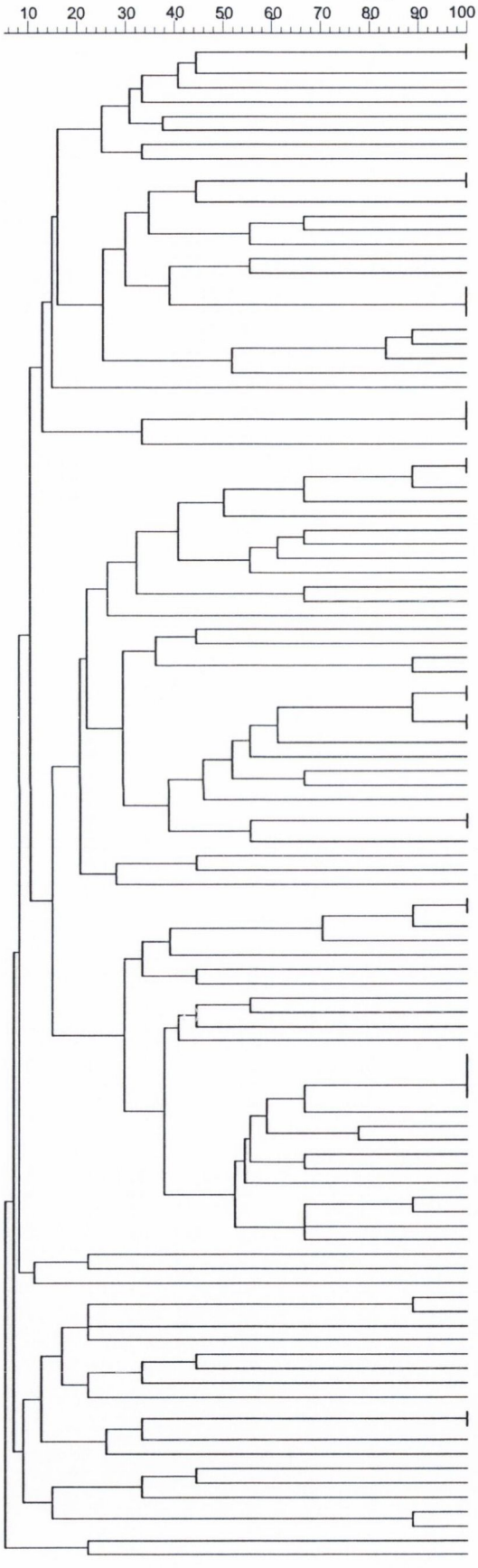


Patient nº	Sample nº	Patient Type	Hospital nº	Azole Sensitive
64	1	CF	4	✓
14	1	CF	1	✓
50	1	CF	2	✓
34	1	CF	2	✓
51	1	CF	3	✓
45	1	CF	2	✓
17	1	CF	1	✓
5	1	CF	1	✓
6	1	CF	1	✓
10	1	CF	1	✓
10	2	CF	1	✓
10	3	CF	1	✓
10	4	CF	1	✓
48	1	CF	2	✓
20	1	CF	2	✓
7	3	CF	1	✓
27	1	CF	2	✓
61	3	CF	4	✓
39	1	CF	2	✓
3	1	CF	1	✓
56	2	CF	4	✓
58	1	CF	4	✓
6	3	CF	1	✓
62	1	CF	4	✓
29	1	CF	2	✓
56	3	CF	4	✓
37	1	CF	2	✓
25	1	CF	2	✓
30	2	CF	2	✓
18	1	CF	1	✓
21	1	CF	2	✓
49	1	CF	2	✓
15	1	CF	1	✓
28	1	CF	2	✓
54	1	CF	3	✓
47	1	CF	2	✓
15	2	CF	1	✓
63	3	CF	4	✓
63	1	CF	4	✓
46	1	CF	2	✓
1	1	CF	1	✓
1	2	CF	1	✓
44	1	CF	2	✓
43	1	CF	2	✓
56	1	CF	4	✓
56	4	CF	4	✓
65	1	CF	4	✓
7	1	CF	1	✓
38	1	CF	2	✓
2	1	CF	1	✓
2	2	CF	1	✓
23	2	CF	2	✓
26	1	CF	2	✓
33	1	CF	2	✓
63	2	CF	4	✓
23	1	CF	2	✓
55	1	CF	3	✓
32	1	CF	2	✓
59	1	CF	4	✓
59	2	CF	4	✓
16	1	CF	1	✓
31	1	CF	2	✓
14	1	CF	1	✓
57	1	CF	4	✓
57	2	CF	4	✓
61	1	CF	4	✓
61	2	CF	4	✓
57	3	CF	4	✓
58	2	CF	4	✓
4	1	CF	1	✓
7	2	CF	1	✓
6	2	CF	1	✓
41	1	CF	2	✓
60	1	CF	4	✓
65	2	CF	4	✓
22	1	CF	2	✓
35	1	CF	2	✓
24	1	CF	2	✓
42	1	CF	2	✓
40	1	CF	2	✓
19	1	CF	1	✓
8	1	CF	1	✓
9	1	CF	1	✓
30	1	CF	2	✓
36	1	CF	2	✓

Figure 3.8: Dendrogram of microsatellite typing of CF isolates colour coded based on consecutive CF isolates over time. Metadata associated with the dendrogram is colour coded based on patients with a number of samples over time; patients; p1-(■), p2-(■), p6-(■), p7-(■), p10-(■), p14-(■), p15-(■), p23-(■), p30-(■), p56-(■), p57-(■), p58- (■), p59-(■), p61-(■), p63-(■) and p65-(■). All unshaded boxes (□) are CF samples where only one samples was taken. . Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate.

Appendix V

Appendix V

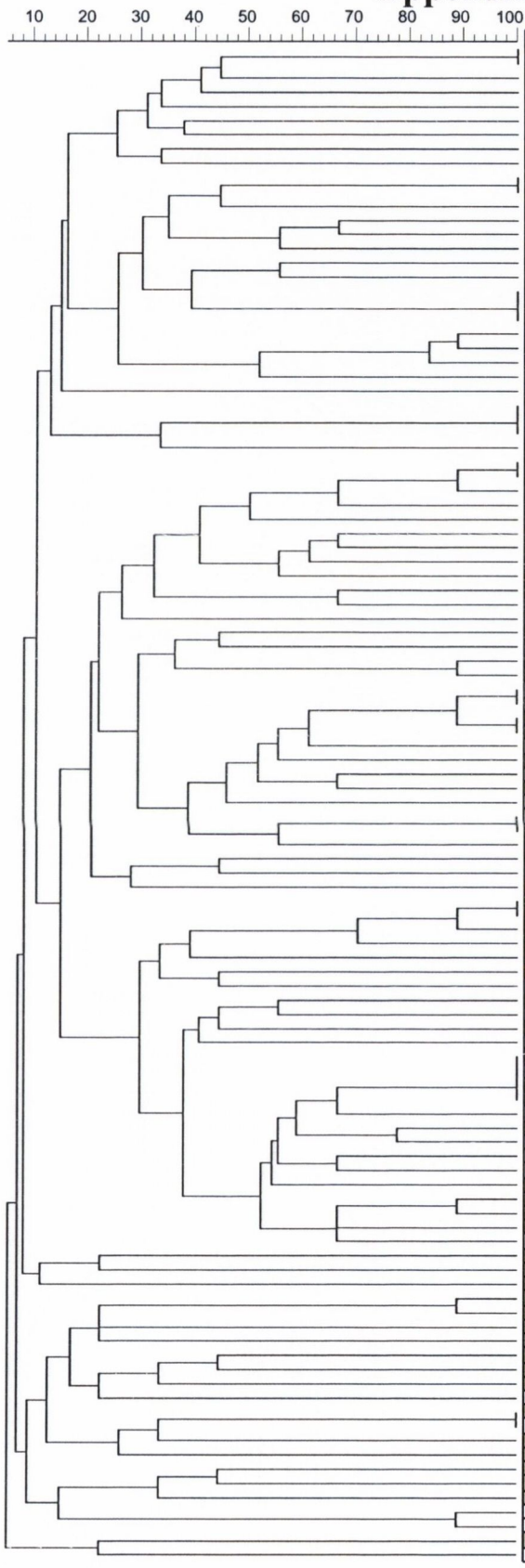


Patient type	Patient no	Hospital no	Sample no	Azole Sensitive?
CF	58	1	1	✓
Non-CF	85	5	1	✓
CF	3	4	1	✓
Non-CF	86	5	1	✓
Non-CF	84	5	1	✓
CF	39	2	1	✓
Non-CF	104	5	1	✓
CF	20	2	1	✓
CF	27	2	1	✓
CF	64	1	1	✓
CF	14	4	1	✓
CF	21	2	1	✓
Non-CF	87	5	1	✓
Non-CF	88	5	1	✓
Non-CF	89	5	1	✓
CF	48	2	1	✓
Non-CF	90	5	1	✓
CF	17	4	1	✓
CF	5	4	1	✓
CF	10	4	1	✓
CF	34	2	1	✓
CF	51	3	1	✓
CF	45	2	1	✓
Non-CF	79	5	1	✓
Non-CF	100	5	1	✓
CF	62	1	1	✓
Non-CF	67	4	1	✓
Non-CF	70	4	1	✓
CF	29	2	1	✓
CF	56	1	1	✓
CF	65	1	1	✓
Non-CF	110	5	1	✓
Non-CF	73	5		✓
Non-CF	109	5	1	✓
Non-CF	105	5	1	✓
Non-CF	108	5	1	✓
Non-CF	74	5	1	✓
CF	16	4	1	✓
CF	41	2	1	✓
Non-CF	80	5	1	✓
Non-CF	71	5	1	✓
CF	31	2	1	✓
Non-CF	91	5	1	✓
CF	23	2	1	✓
CF	55	3	1	✓
Non-CF	76	5	1	✓
Non-CF	77	5	1	✓
CF	26	2	1	✓
CF	33	2	1	✓
Non-CF	75	5	1	✓
CF	32	2	1	✓
Non-CF	103	5	1	✓
CF	2	4	1	✓
Non-CF	78	5	1	✓
Non-CF	82	5	1	✓
Non-CF	83	5	1	✓
Non-CF	81	5	1	✓
CF	59	1	1	✓
Non-CF	106	5	1	✓
Environmental		Soil	-	x
CF	57	1	1	✓
CF	61	1	1	✓
CF	56	4	1	✓
CF	59	4	1	✓
CF	58	4	1	✓
Environmental		Soil	-	x
Non-CF	99	5	1	✓
CF	30	2	1	✓
Non-CF	94	5	1	✓
CF	35	2	1	✓
Non-CF	93	5	1	✓
CF	19	4	1	✓
Non-CF	68	4	1	✓
CF	8	4	1	✓
CF	9	4	1	✓
Non-CF	95	5	1	✓
CF	22	2	1	✓
Non-CF	96	5	1	✓
Non-CF	97	5	1	✓
Non-CF	98	5	1	✓
CF	60	1	1	✓
CF	24	2	1	✓
CF	42	2	1	✓
CF	40	2	1	✓
Non-CF	94	5	1	✓
CF	25	2	1	✓
CF	38	2	1	✓
CF	18	1	1	✓
CF	47	2	1	✓
CF	102	5	1	✓
CF	28	4	1	✓
CF	49	2	1	✓
CF	28	2	1	✓
CF	54	3	1	✓
CF	37	2	1	✓
Non-CF	107	5	1	✓
CF	46	2	1	✓
Non-CF	101	5	1	✓
CF	63	1	1	✓
CF	21	2	1	✓
CF	44	2	1	✓
CF	1	4	1	✓
CF	43	2	1	✓
Non-CF	66	4	1	✓
Non-CF	69	4	1	✓
CF	36	2	1	✓
Non-CF	92	5	1	✓

Figure 3.10: Microsatellite typing of CF and Non-CF *A. fumigatus* isolates based on hospital Metadata associated with the dendrogram is colour coded based on hospital number; hospital 1(■), hospital 2 (■), hospital 3(■) and hospital 4 (□).Two environmental isolates colour coded (■). Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate.

Appendix VI

Appendix VI



Patient type	Patient no	Hospital no	Sample no	Azole Sensitive?
CF	58	1	1	P
Non-CF	85	5	1	P
CF	3	4	1	P
Non-CF	86	5	1	P
Non-CF	84	5	1	P
CF	39	2	1	P
Non-CF	104	5	1	P
CF	20	2	1	P
CF	27	2	1	P
CF	64	1	1	P
CF	14	4	1	P
CF	21	2	1	P
Non-CF	87	5	1	P
Non-CF	88	5	1	P
Non-CF	89	5	1	P
CF	48	2	1	P
Non-CF	90	5	1	P
CF	17	4	1	P
CF	5	4	1	P
CF	10	4	1	P
CF	34	2	1	P
CF	51	3	1	P
CF	45	2	1	P
Non-CF	79	5	1	P
Non-CF	100	5	1	P
CF	62	1	1	P
Non-CF	67	4	1	P
Non-CF	70	4	1	P
CF	29	2	1	P
CF	56	1	1	P
CF	65	1	1	P
Non-CF	110	5	1	P
Non-CF	73	5		P
Non-CF	109	5	1	P
Non-CF	105	5	1	P
Non-CF	108	5	1	P
Non-CF	74	5	1	P
CF	16	4	1	P
CF	41	2	1	P
Non-CF	80	5	1	P
Non-CF	71	5	1	P
CF	31	2	1	P
Non-CF	91	5	1	P
CF	23	2	1	P
CF	55	3	1	P
Non-CF	76	5	1	P
Non-CF	77	5	1	P
CF	26	2	1	P
CF	33	2	1	P
Non-CF	75	5	1	P
CF	32	2	1	P
Non-CF	103	5	1	P
CF	2	4	1	P
Non-CF	78	5	1	P
Non-CF	82	5	1	P
Non-CF	83	5	1	P
Non-CF	81	5	1	P
CF	59	1	1	P
Non-CF	106	5	1	P
Environmental		Soil	-	O
CF	57	1	1	P
CF	61	1	1	P
CF	56	4	1	P
CF	59	4	1	P
CF	58	4	1	P
Environmental		Soil	-	O
Non-CF	99	5	1	P
CF	30	2	1	P
Non-CF	94	5	1	P
CF	35	2	1	P
Non-CF	93	5	1	P
CF	19	4	1	P
Non-CF	68	4	1	P
CF	8	4	1	P
CF	9	4	1	P
Non-CF	95	5	1	P
CF	22	2	1	P
Non-CF	96	5	1	P
Non-CF	97	5	1	P
Non-CF	98	5	1	P
CF	60	1	1	P
CF	24	2	1	P
CF	42	2	1	P
CF	40	2	1	P
Non-CF	94	5	1	P
CF	25	2	1	P
CF	38	2	1	P
CF	18	1	1	P
CF	47	2	1	P
CF	102	5	1	P
CF	28	4	1	P
CF	49	2	1	P
CF	28	2	1	P
CF	54	3	1	P
CF	37	2	1	P
Non-CF	107	5	1	P
CF	46	2	1	P
Non-CF	101	5	1	P
CF	63	1	1	P
CF	21	2	1	P
CF	44	2	1	P
CF	1	4	1	P
CF	43	2	1	P
Non-CF	66	4	1	P
Non-CF	69	4	1	P
CF	36	2	1	P
Non-CF	92	5	1	P

Figure 3.11: Microsatellite typing of CF and Non-CF *A. fumigatus* isolates

Metadata associated with the dendrogram is colour coded based on CF (■) or Non-CF (□) or environmental (■) isolates. Dendrograms were constructed by BioNumerics version 3.5 software. All markers were given an equal weight. In the dendrogram, the indicated percentages reflect the number of corresponding markers. A 100% similarity was considered an indistinguishable isolate.

Appendix VII

Publications and Presentations

Publications

Coughlan CA, Chotirmall SH, Renwick J, Hassan T, Boon Low T, Bergsson G, Eshwika A, Bennett K, **Dunne K**, Greene CM, Gunaratnam C, Kavanagh K, Logan PM, Murphy P, Reeves EP, McElvaney NG. The Effect of *Aspergillus fumigatus* Infection on Vitamin D Receptor Expression in Cystic Fibrosis. Am J Respir Crit Care Med, 2012. 186(10): p999-1007.

Christina O. Maher, **Katie Dunne**, Ross Comerford, Siobhán O'Dea, Aisling Loy, Jim Woo, Thomas Rogers, Fiona Mulcahy, Pádraic J. Dunne, Derek G. Doherty. *Candida albicans* stimulates IL-23 release by human dendritic cells and downstream IL-17 secretion by Vδ1 T cells. J Immunol, 2015. 194(12):5953-60.

Bojana Mirković, Gillian M. Lavelle, Ahmed Abdul Azim , Kristine Helma , Fatma S. Gargoum1, Kevin Molloy , Yael Gernez , **Katie Dunne**, Julie Renwick, Philip Murphy, Richard B. Moss, Catherine M. Greene, Cedric Gunaratnam, Sanjay H. Chotirmall, Noel G. McElvaney. The Basophil Surface Marker CD203c Identifies *Aspergillus* Sensitization in Cystic Fibrosis. Submitted to the Journal of Allergy and Clinical Immunology June 2015.

Dunne K., McClean S., Doyle S., Rogers T., Murphy P. and Renwick J. Disruption of Cystic Fibrosis Bronchial Epithelial Monolayers by Culture Supernatants of *Aspergillus fumigatus.* Submitted to the Journal of Cystic Fibrosis June 2015.

Katie Dunne, Anna-Rose Prior, Kate Murphy, Niall Wall, Geraldine Leen, Thomas Rogers, Basil Elnazir, Peter Greally, Julie Renwick and Philip Murphy. Emergence of Persistent *Aspergillus terreus* Colonisation in a Child with Cystic Fibrosis. Submitted to Medical Mycology Case Reports June 2015.

Oral Presentations

Dunne K, Renwick J, McElvaney G , Chotirmall S , Meis J F , Klaassen C, Murphy P, Rogers T.R. Epidemiology and Antifungal Susceptibility of *Aspergillus fumigatus* in an Irish Cystic Fibrosis Patient Cohort. Irish Fungal Society Meeting 2012. June 2012. The abstract for this presentation was published in The Ulster Medical Journal. Abstracts Presented at the 2nd Annual Meeting of the Irish Fungal Society 21 & 22 June 2012. Ulster Med J 2012; 81(3): 1-5.

Dunne K. Epidemiology and Antifungal Susceptibility of *A. fumigatus* in Irish Cystic Fibrosis patients. Trinity College Department of Clinical Microbiology Research Day 2012. December 2012. 1st prize PhD oral presentation

Dunne K. *Aspergillus* in Cystic Fibrosis. Trinity College Dublin, Department of Clinical Microbiology Research Day 2014. April 2014. 1st prize PhD oral presentation

Katie Dunne, Julie Renwick, Gerard McElvaney, Sanjay Chotirmall, Ferry Hagen, Jacques F, Meis, Philip Murphy and Thomas Rogers. Patterns of *Aspergillus fumigatus* colonisation and Antifungal drug susceptibility in Irish Cystic Fibrosis Patients. Third Meeting of the ECMM/ISHAM Working group in Fungal respiratory infections in Cystic Fibrosis June 2014.

Dunne K, Renwick J, McElvaney G , Chotirmall S , Meis J F , Murphy P, Rogers T.R. Epidemiology of *Aspergillus fumigatus* and Antifungal drug Susceptibility in Irish Cystic Fibrosis Patients; an update. Irish Fungal Society Meeting 2014. June 2014.

Posters

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The Effect of *Aspergillus fumigatus* Infection on Vitamin D Receptor Expression in Cystic Fibrosis

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Rationale: *Aspergillus fumigatus* (*A. fumigatus*) in cystic fibrosis (CF) is increasingly recognized. Although allergic bronchopulmonary aspergillosis (ABPA) leads to deterioration of pulmonary function, the effect of *A. fumigatus* colonization in the absence of ABPA remains unclear.

Objectives: To address this, we examined individuals with CF with *A. fumigatus* who were ABPA negative to identify the effects of itraconazole therapy on *Aspergillus*-induced lung inflammation.

Methods: The effect of *A. fumigatus* on nuclear vitamin D receptor (VDR) expression was investigated using qRT-PCR and Western blotting. IL-5 and IL-13 levels were quantified by ELISA. The effect of itraconazole was assessed by a combination of high-resolution computed tomography, lung function test, and microbiological analysis.

Measurements and Main Results: We demonstrate that *A. fumigatus* down-regulates VDR in macrophages and airway epithelial cells and that the fungal metabolite gliotoxin (Gt) is the main causative agent. Gt overcame the positive effect of 1,25-OH vitamin D_3 on VDR expression *in vitro*, resulting in increased IL-5 and IL-13 production. *In vivo*, *A. fumigatus* positivity was associated with increased Gt in CF bronchoalveolar lavage fluid and increased bronchoalveolar lavage fluid levels of IL-5 and IL-13. After airway eradication of *A. fumigatus* with itraconazole, we observed decreased Gt, IL-5 and IL-13, improved respiratory symptoms, and diminished high-resolution computed tomography mosaic pattern consistent with sustained pulmonary function.

Conclusions: This study provides a rationale for the therapeutic effect of itraconazole and implied that the therapeutic potential of vitamin D supplementation in preventing ABPA is only feasible with concurrent elimination of *A. fumigatus* to permit VDR expression and its positive functional consequences.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

In individuals with cystic fibrosis, the effect of azole treatment on patients with *Aspergillus fumigatus* colonization in the absence of allergic bronchopulmonary aspergillosis is unknown.

What This Study Adds to the Field

Aspergillus colonization increases T-helper 2 cytokine production by structural and innate immune cells of the airways. The mechanism of action involves down-regulation of vitamin D receptor expression. The overall effect of itraconazole treatment was elimination of *A. fumigatus* bioburden, thereby permitting vitamin D receptor expression, resulting in decreased T-helper 2 cytokine production and significant clinical improvement.

Keywords: cystic fibrosis; vitamin D receptor; *Aspergillus fumigatus*; itraconazole; inflammation

Cystic fibrosis (CF) is a systemic heritable disorder. Pathogenesis of lung disease induced by CF is a direct result of decreased chloride secretion and hyperabsorption of sodium, resulting in the retention of dehydrated mucus within the airways. Pathogenic colonization and chronic infection occurs, giving rise to recurrent exacerbations, respiratory failure, and death.

Our understanding of the airway microbiome within the CF milieu has evolved with fungi emerging as recognized colonizers and potential pathogens (1–4). Although yeasts and filamentous fungi are identified in CF, the most commonly isolated member of the latter group is *Aspergillus fumigatus*. In the immune-competent host, exposure to *A. fumigatus* spores leads to an immune response stimulating prompt fungal clearance. Occasionally, however, in immunocompromised individuals, this ubiquitous fungus can cause life-threatening invasive disease. In most cases, although it is acknowledged as a potential problem, the impact of *A. fumigatus* on airway disease and inflammation in CF remains unclear (4).

Colonization rates vary between centers. In one decade-long study, *A. fumigatus* was isolated in over one third of lung cultures from people with CF (5), a proportion similar to that detected by our group (6). Additionally, we and others have demonstrated that the frequency of *A. fumigatus* isolation from CF sputum does not correlate with rates of allergic bronchopulmonary aspergillosis (ABPA), gender, pancreatic status, or cirrhosis but is associated with higher recovery rates of *Pseudomonas aeruginosa* infection

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(6, 7). Moreover, exposure of cystic fibrosis transmembrane conductance regulator (CFTR)-deficient mice to *A. fumigatus* antigens leads to a severe pulmonary Th2-biased inflammatory phenotype, suggesting that in CF, even in the absence of ABPA, *A. fumigatus* acts unfavorably (8). In addition, vitamin D deficiency has been proposed as a risk factor for CF-ABPA as reduced levels of IL-5 and IL-13 expression were recorded after *in vitro* treatment with vitamin D (9). Further support for this theory is provided by research illustrating that deletion of vitamin D receptor (VDR) results in elevated IL-5 and IL-13 production (10) and pulmonary inflammation (11).

The role of *A. fumigatus* colonization in the absence of ABPA remains a subject of uncertainty. It has been shown that pulmonary radiological abnormalities on high-resolution computed tomography (HRCT) are more pronounced in an *A. fumigatus*–colonized cohort compared with a matched non–*Aspergillus*-colonized CF group, a finding not reflected by pulmonary function testing alone (12). *A. fumigatus* colonization has additionally been shown to be an independent risk factor for hospital admission in CF (1, 13), yet studies assessing the effect of eradication of *A. fumigatus* from the CF airway unaffected by ABPA are limited. Although work on azole-based agents (e.g., itraconazole or voriconazole) in CF-ABPA is ongoing, the anti-inflammatory effects of vitamin D and other VDR agonists in the setting of *A. fumigatus* colonization without ABPA remain unaddressed. This study clarifies the effect of *A. fumigatus* on VDR expression in the CF airway and for the first time evaluates treatment effectiveness of itraconazole in *A. fumigatus* colonization without ABPA. Some of the results of these studies have been previously reported in the form of an abstract (14–16).

METHODS

Patient Selection and Recruitment

All patients with CF attending Beaumont Hospital, Dublin, Ireland, between July 2008 and June 2010 (2-yr period) were screened for study eligibility (n = 117) (*see* Figure E1 in the online supplement). Inclusion criteria required a confirmed diagnosis of CF by sweat chloride concentrations greater than 60 mmol/l on at least two separate occasions and subsequent confirmatory genotyping. Patients included had to be colonized with *A. fumigatus*. Colonization was defined as detection of the fungus in sputum and/or bronchoalveolar lavage fluid (BALF) on at least two separate and consecutive occasions at least 12 weeks apart in the year before study commencement. Before recruitment, patients had to be exacerbation free over the preceding 6-week period with no prior diagnosis of ABPA (as per classical consensus conference criteria) (17). Exclusion criteria included allergies to any azole related substances, prior lung transplantation, and either a confirmed or suspected diagnosis of ABPA or prior administration of systemic corticosteroid therapy in any form. All participants had serum IgE levels less than 450 ng/ml and negative cutaneous reactivity to *Aspergillus* antigens before the start of the study. Ethical approval from Beaumont Hospital Institutional Review Board was acquired, and written informed consent was obtained from all study participants during the screening/baseline (pretreatment) visit (Figure E1). Demographics of recruited patients (n = 13) are outlined in Table E1 in the online supplement. CT imaging, pulmonary function testing, Cystic Fibrosis Questionnaire Revised (CFQ-R) respiratory scoring, and observational study protocols are outlined in the online supplement.

Sputum, Bronchoalveolar Lavage, and Bronchial Brushing Samples

Spontaneously expectorated sputum from a deep cough was collected at each study visit. Sputum was divided into two sterile containers and transported immediately for evaluation. Suitability of sputum samples for study inclusion was confirmed by the criteria used to determine representative sputum samples (3). A single patient was a nonproducer; hence, BALF was used instead and obtained by standard methods (18). A total of four samples per patient were obtained and processed over the 1-year study course (52 samples). Bronchial brushings were also obtained before and after itraconazole treatment (n = 3) from *A. fumigatus*–positive or *A. fumigatus* –negative patients (n = 3) not on treatment (age in years ± SD, 24.33 ± 4.45; gender [% male/female], 50:50; FEV_1% predicted ± SD, 38.83 ± 13.37; BMI ± SD, 20.95 ± 1.84; plasma vitamin D levels ± SD, 47.66 ± 26.31 ng/ml) and processed as previously described (19). A full description of the procedures pertaining to the bronchial brushings can be found in the online supplement.

For processing of CF sputum or BALF for *A. fumigatus* colonization, an equal volume of Sputasol (Oxoid Ltd, Basingstoke, UK) was added to each sputum sample (no Sputasol was added to BALF), which was then shaken for 15 minutes at 37°C. The *A. fumigatus* status of homogenized sputum or BALF from each patient sample was determined in triplicate as colony forming units (CFU), and the concurrent equivalent number of conidia per gram of sputum as evaluated (a full protocol can be found in the online supplement). The occurrence of airway colonizers (e.g., *P. aeruginosa* and *Staphylococcus aureus*) were identified by standard microbiological methods as outlined by the Cystic Fibrosis Trust (20). *In vitro* cell culture, preparation of *Aspergillus* culture supernatants, and treatment of human bronchial epithelial cells or macrophages is described in the online supplement.

Statistical Analysis

Descriptive analyses are presented as means (± SD or SE), medians, or proportions depending on whether the data were normal, nonnormal, or categorical. Continuous data were tested for normality (Kolmogorov-Smirnoff or Shapiro Wilk tests as appropriate) and where normal were compared using a Student *t* test (paired or unpaired where appropriate). For nonnormal data, the Mann-Whitney U test was performed for comparisons. Tests for trends over time were conducted using repeated measures ANOVA for normally distributed data, and *post hoc* multiple comparison tests (Tukey's test) were applied. Friedmans chi-square test was used to compare trends over time for nonnormal (nonparametric) data. For count data (e.g., number of infective exacerbations), log-linear (poisson) regression analysis was performed using Generalized Estimating Equations for repeated measures within each patient. All statistical analyses were performed using the PRISM 4.0 software package (GraphPad, San Diego, CA) and SAS (v9.1; SAS Institute Inc., Cary, NC). Differences were considered significant at $P \leq 0.05$.

RESULTS

The Effect of *A. fumigatus* Culture Filtrates on VDR Gene and Protein Expression in CF Tracheal Epithelial and CF Bronchial Epithelial Cells

We and others have previously shown that *A. fumigatus* colonization affects approximately one third of people with CF (5, 6), an observation further confirmed in this study (Figure E1). The percentages of dominant immune cells in *A. fumigatus*–colonized CF BALF has been previously reported to include neutrophils (61%) and macrophages (31%) and, to a much lower extent, lymphocytes (4%) (21). By Western blotting, however, VDR protein expression was found abundant in macrophages but not in neutrophils and at a very low level in T cells (Figure E2). CF airway epithelial cells were also found to express VDR protein, and consequently this study examined the effect of *A. fumigatus* on VDR expression by structural (bronchial and tracheal epithelial cells) and immune cells (macrophages) of the airways.

VDR gene expression was significantly down-regulated by approximately 80% in CF tracheal epithelial (CFTE) cells ($P = 0.001$) (Figure 1A) and was completely inhibited in CF bronchial epithelial (CFBE) cells ($P = 0.04$) (Figure 1B) in response to treatment with 3-day culture filtrates for 16 hours. VDR gene expression was decreased by approximately 33% 2 hours after addition of *Aspergillus* culture filtrate in CFTE

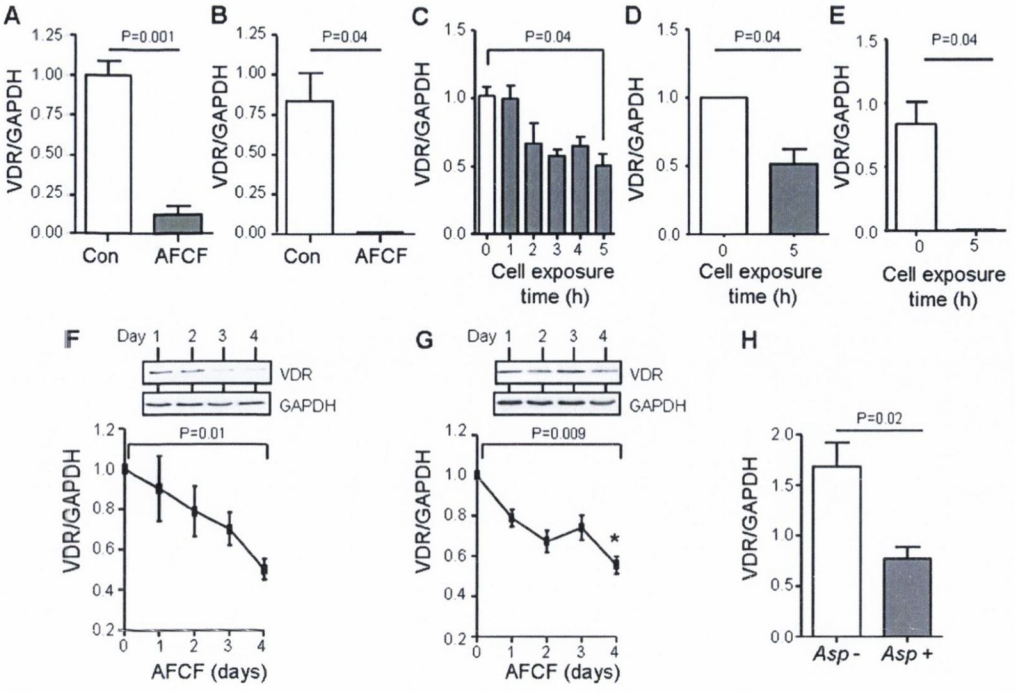

Figure 1. Culture filtrates of *Aspergillus fumigatus* down-regulate vitamin D receptor (VDR) gene and protein expression. Cystic fibrosis tracheal epithelial (CFTE) (*A* and *C*) and cystic fibrosis bronchial epithelial (CFBE) (*B* and *D*) cells (1×10^5) were untreated (Con) or treated with 3 days of *A. fumigatus* (ATCC-26933) culture filtrates (AFCF) for 16 hours (*A* and *B*) or 0 to 5 hours (*C* and *D*). RNA was isolated and analyzed for VDR gene expression by qRT-PCR and standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). VDR gene expression was significantly down-regulated at 16 hours and at the earlier time point of 5 hours after treatment (*C* and *D*). CFBE cells were treated for 5 hours with 4-day fungal culture filtrates, with total down-regulation of VDR gene expression observed (*E*). (*F* and *G*) Western blot analysis of VDR protein expression of CFTE (*F*) and CFBE (*G*) cells (1×10^6) treated with AFCF (0- to 4-d cultures). Western blots were probed with mouse monoclonal VDR or rabbit polyclonal GAPDH (loading control) antibodies. The Western blot illustrated is a representative result of one out of three separate experiments and converted to densitometry units in respective graphs. VDR protein expression was significantly down-regulated by 4-day cultures. (*H*) Bronchial brushings were obtained from subjects with CF who were positive (*Asp*+) (n = 3) and negative (*Asp*−) (n = 3) for *Aspergillus* colonization and analyzed for VDR gene expression by qRT-PCR. VDR gene expression was significantly down-regulated in subjects with CF colonized with *Aspergillus* compared with those noncolonized by *Aspergillus*. All experiments were performed in triplicate on three consecutive days. Data shown are mean ± SE.

cells (Figure 1C) and was significantly down-regulated in CFTE and CFBE cells by approximately 50% after 5 hours of exposure to 3-day cultures ($P = 0.04$) (Figures 1C and 1D, respectively). Maximal effect was observed by treating CFBE cells with 4-day fungal culture filtrates for 5 hours, with total elimination of VDR gene expression observed ($P = 0.04$) (Figure 1E).

The effect of *A. fumigatus* culture filtrates (1–4 d) on VDR protein expression was evaluated by Western blotting. Significant down-regulation of VDR protein expression was observed in CFTE and CFBE cells after treatment with 4-day culture filtrates for 5 hours ($P = 0.01$ and $P = 0.009$, respectively) (Figures 1F and 1G). To confirm the effect of *Aspergillus* colonization on VDR gene expression *in vivo*, bronchial brushings were performed on *Aspergillus*-positive (Asp^+; n = 3) and *Aspergillus*-negative (Asp^-; n = 3) patients. qRT-PCR confirmed significant down-regulation of VDR gene expression *in vivo* in *Aspergillus*-colonized patients, in line with *in vitro* results ($P = 0.02$) (Figure 1H). Collectively these results indicate *in vitro* and *in vivo* down-regulation of VDR gene and protein expression by *A. fumigatus*.

Down-regulation of VDR Expression by Gliotoxin, an Immunomodulatory Mycotoxin from *A. fumigatus*

The VDR modulator present within culture filtrates of *A. fumigatus* (ATCC 26933) was characterized. The inhibitory activity was stable within culture filtrates stored at −70°C and subsequently used against CFBE cells at 37°C but was significantly abolished by heat inactivation (99°C) for 20 minutes ($P = 0.003$) (Figure 2A). Passage of culture filtrates through a 3 kD exclusion filter removed approximately 65% of the VDR inhibitory activity ($P = 0.007$), which suggested the presence of predominantly low-molecular-weight (< 3 kD) VDR inhibitory factors.

Secreted by the hyphal form of *A. fumigatus*, gliotoxin (Gt) has previously been characterized as a small, heat-inactivated toxin of 326.4 daltons (22). Gt has been shown to provoke airway inflammation and tissue damage with consequential long-term airway remodeling (23). To confirm Gt as a fungal agent capable of VDR down-regulation, the effect of a low–Gt-producing *A. fumigatus* strain (CaF1) on VDR expression was explored. Strain CaF1 produced minimal levels of Gt compared with the high–Gt-producing strain ATCC-26933 after 96 hours of fungal growth (2 ± 0.05 and 252 ± 2.5 ng/mg hyphae, respectively) (Figure E3). Moreover, VDR expression in CFTE and CFBE cells remained relatively unchanged when treated with 1- to 4-day culture filtrates of CaF1, in comparison to the ATCC-26933 strain, which resulted in significant down-regulation of VDR ($P = 0.01$ and $P = 0.009$ for CFTE and CFBE cells compared with untreated cells, respectively) (Figures 2C and 2D). The clinical relevance of these findings was confirmed when BALF obtained from *Aspergillus*-positive patients illustrated high levels of Gt, as quantified by HPLC analysis of chloroform extracts (Figure 2E).

The effect of purified Gt on VDR expression by CF and non-CF cells was next explored. By HPLC analysis of cultures of *A. fumigatus* strain ATCC-26933, no significant difference was detected in Gt levels produced on Day 3 (280 ± 29 ng/mg hyphae) compared with Day 4 (252 ± 2.5 ng/mg hyphae) ($P > 0.05$) (Figure E3A). This concentration produced by the fungus equates to approximately 0.8 μM commercial Gt, which was therefore used in this study. Gt used at this concentration significantly down-regulated VDR protein expression in CFBE

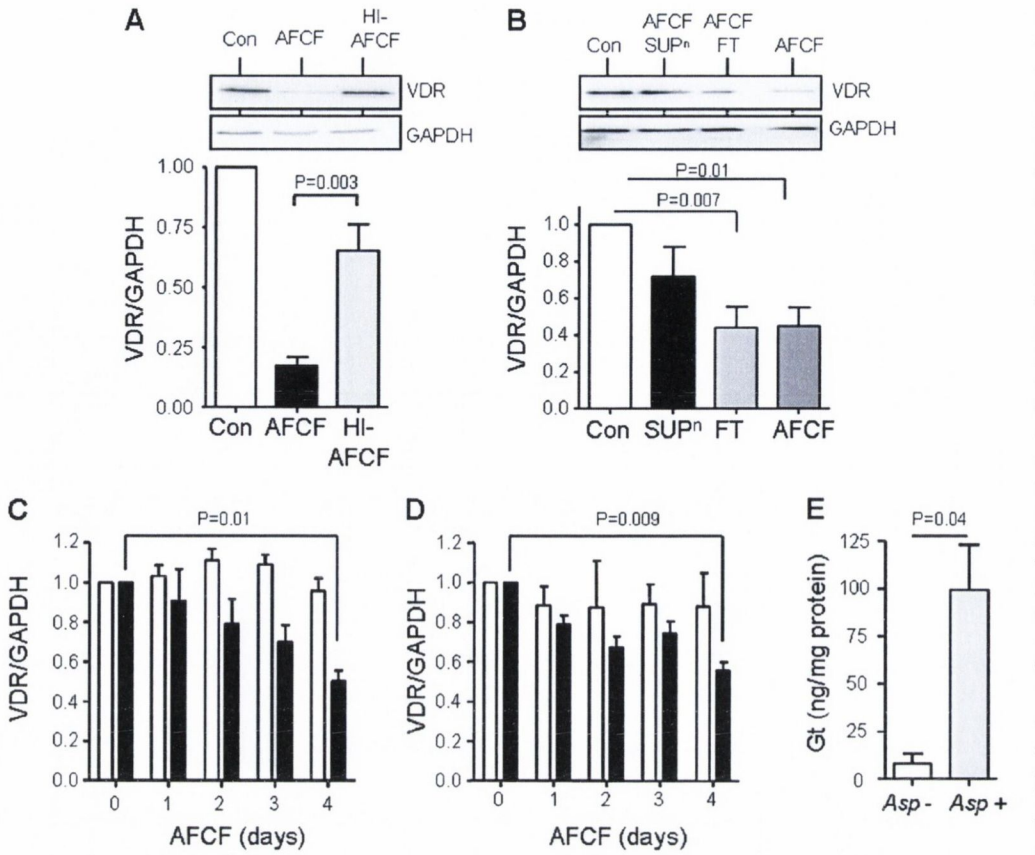

Figure 2. Characterization of the *Aspergillus* virulence factor responsible for vitamin D receptor (VDR) down-regulation. (*A*) Down-regulation of VDR protein expression was reversed by heat inactivation (HI) of *A. fumigatus* (ATCC-26933) culture filtrates (AFCF). Cystic fibrosis bronchial epithelial (CFBE) cells (1×10^6) were untreated (Con) or exposed to 4 days of AFCF or HI-AFCF for 5 hours. The Western blots illustrated are from one representative experiment out of three and converted to densitometry units in respective graphs. (*B*) VDR is down-regulated by a small (< 3 kD) secreted molecule. AFCF (4 d) was passed through a 3 kD membrane, and the resulting flow through (FT < 3 kD) or top supernatant (AFCF SUP^n >3 kD) was added to CFBE cells (1×10^6) for 5 hours. (*C* and *D*) The effect of CaF1 (*white bars*) and ATCC-26933 (*black bars*) AFCF grown for 1 to 4 days on VDR protein expression in cystic fibrosis tracheal epithelial (CFTE) (*C*) and CFBE cells (*D*). (*E*) Levels of Gt in *A. fumigatus* colonized cystic fibrosis BALF (n = 3, *Asp+*) compared with noncolonized samples (n = 3; *Asp−*). Gt was quantified by HPLC and standardized to total BALF protein (mg). All assays were performed in triplicate on three consecutive days. Data shown are mean ± SE.

cells ($P = 0.03$) to a similar level obtained after exposure to 4-day culture filtrates of *A. fumigatus* (ATCC-26933), indicating that Gt is most likely the main VDR inhibitory factor secreted by the fungus (Figure E3B).

In vitro studies further revealed that 0.8 μM Gt significantly down-regulated VDR gene expression in CFTE ($P = 0.001$), CFBE ($P = 0.02$), and human bronchial epithelial (HBE) ($P = 0.003$) cell lines (Figures 3A–3C, respectively). HBE cells were used in this latter experiment to provide evidence that down-regulation of VDR by Gt was independent of dysfunctional CFTR. In addition, CFBE cells cultured under submerged monolayer (Figure 3D) or air–liquid interface (Figure

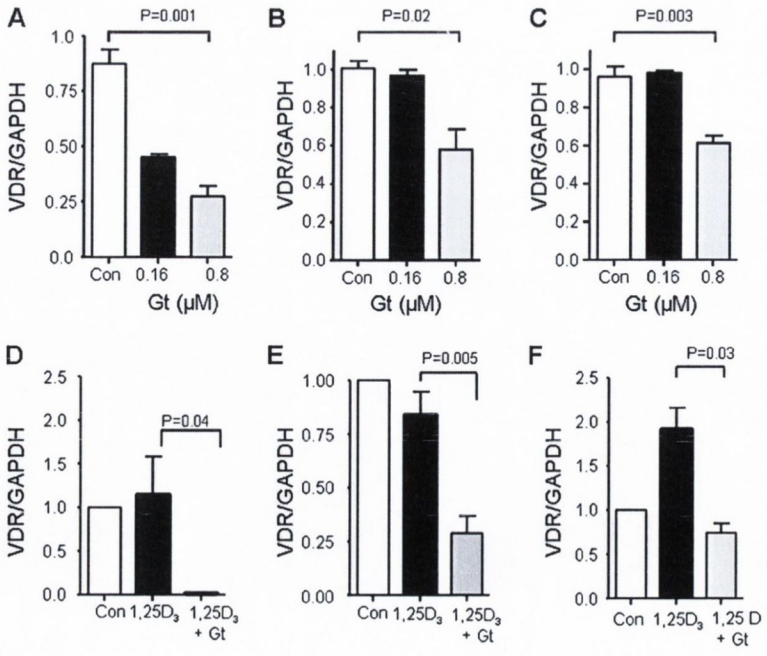

Figure 3. Effect of purified gliotoxin on vitamin D receptor (VDR) expression. (*A–C*) Purified gliotoxin (Gt) down-regulates VDR in a dose-dependent manner. VDR gene expression of cystic fibrosis tracheal epithelial (CFTE) (*A*), cystic fibrosis tracheal bronchial (CFBE) (*B*), and human bronchial epithelial (HBE) (*C*) cells (1×10^5) treated for 5 hours with 0.16 or 0.8 μM Gt. RNA was analyzed by qRT-PCR, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and compared with untreated cells (Con). (*D–F*) Gt down-regulates vitamin D–induced VDR protein expression *in vitro*. CFBE cells cultured under submerged monolayer (1×10^6) (*D*) or air–liquid interface (5×10^5) (*E*) or U937 differentiated macrophages (1×10^6) (*F*) were untreated (Con) or exposed to $1,25D_3$ (10^{-9} M) in the presence or absence of Gt (0.8 μM) for 24 hours, and VDR protein expression was analyzed by Western blotting using mouse monoclonal VDR and rabbit polyclonal GAPDH antibodies. All assays were standardized to GAPDH and were performed in triplicate on three separate days. Data shown are mean ± SE.

3E) treated with $1,25D_3$ in the presence of 0.8 μM Gt exhibited significant down-regulation of VDR protein expression compared with cells treated with $1,25D_3$ only ($P = 0.04$ and $P = 0.005$, respectively). This effect was also observed by treating macrophages with Gt ($P = 0.03$) (Figure 3F). Control experiments indicated that Gt did not affect cell viability or induce cell apoptosis as determined by trypan blue exclusion assays or a lack of cleaved (17/19 kD) caspase-3, respectively (result not shown). Collectively these experiments suggest an overwhelming inhibitory effect of Gt on VDR expression.

Gliotoxin Enhances the Production of Th2 Cytokines, Which Are Reduced after Itraconazole Treatment

It has been shown that VDR knockout mice have elevated levels of IL-5 and IL-13 production (10), and vitamin D has also been observed to modulate *Aspergillus*-induced Th2 cytokines IL-5 and IL-13 expression in $CD4^+$ T cells (9). In line with this, we investigated the effect of Gt on IL-5 and IL-13 expression *in vitro*. CFBE cells or macrophages were treated with $1,25D_3$ (10^{-9} M) in the presence or absence of 0.8 μM Gt. Gt significantly increased IL-5 and IL-13 gene (Figures 4A and 4B) and protein (Figures 4C–4F) expression in CFBE submerged monolayers ($P = 0.05$ and $P = 0.05$, respectively) (Figures 4A and 4B), air–liquid interface CFBE cultures ($P = 0.05$ and $P = 0.01$, respectively) (Figures 4C and 4D), and macrophages ($P = 0.05$ and $P = 0.05$, respectively) (Figures 4E and 4F). In support of this result, high levels of IL-5 and IL-13 were observed in CF BALF samples before itraconazole treatment (Figures 4G and 4H). After itraconazole treatment, a significant decrease in IL-5 ($P = 0.01$) and IL-13 ($P = 0.002$) protein expression was detected *in vivo*, suggesting a role for itraconazole in reducing the Th2 inflammatory response in these patients.

Itraconazole Treatment Effectively Reduces *A. fumigatus* Bioburden in CF Airways

Itaconazole therapy administered orally at 400 mg once daily for a 6-week period significantly reduced *A. fumigatus* bioburden within the CF airway over time as determined by CFU and conidia counts per gram of sputum and/or BALF (Figures 5A and 5B). Itraconazole therapy was well tolerated in the study cohort, with no reports of adverse effects or liver dysfunction during treatment and follow-up. Although CFU counts remained low at the 12-month follow-up (Figure 5A), *A. fumigatus* conidia burden was increased when compared with the 6-month follow-up sample (Figure 5B), suggesting that qPCR is a more reliable method of *Aspergillus* detection in CF sputum. In addition, corresponding to a reduction in *Aspergillus* bioburden at the 12-month follow-up, levels of Gt were significantly reduced *in vivo* from approximately 250 ± 80 to 75 ± 50 ng/mg BALF protein ($P = 0.04$) (Figure 5C). Moreover, after 6 weeks of itraconazole (400 mg daily) treatment for *Aspergillus* infection, VDR gene expression was significantly increased by greater than 2-fold ($P = 0.04$) (Figure 5D). This set of experiments demonstrates that itraconazole therapy significantly reduced *A. fumigatus* colonization within the CF airways and by corollary diminished Gt levels initiating up-regulation of VDR expression.

A. fumigatus Is Associated with a Mosaic Pattern on HRCT That Significantly Attenuates after Itraconazole Therapy

It has been shown that *A. fumigatus* colonization in CF has adverse effects upon the lung parenchyma (on HRCT) that do not correlate with pulmonary function testing (12). To establish the presence of radiological changes after eradication of *A. fumigatus* from the CF airway, HRCT scans were performed

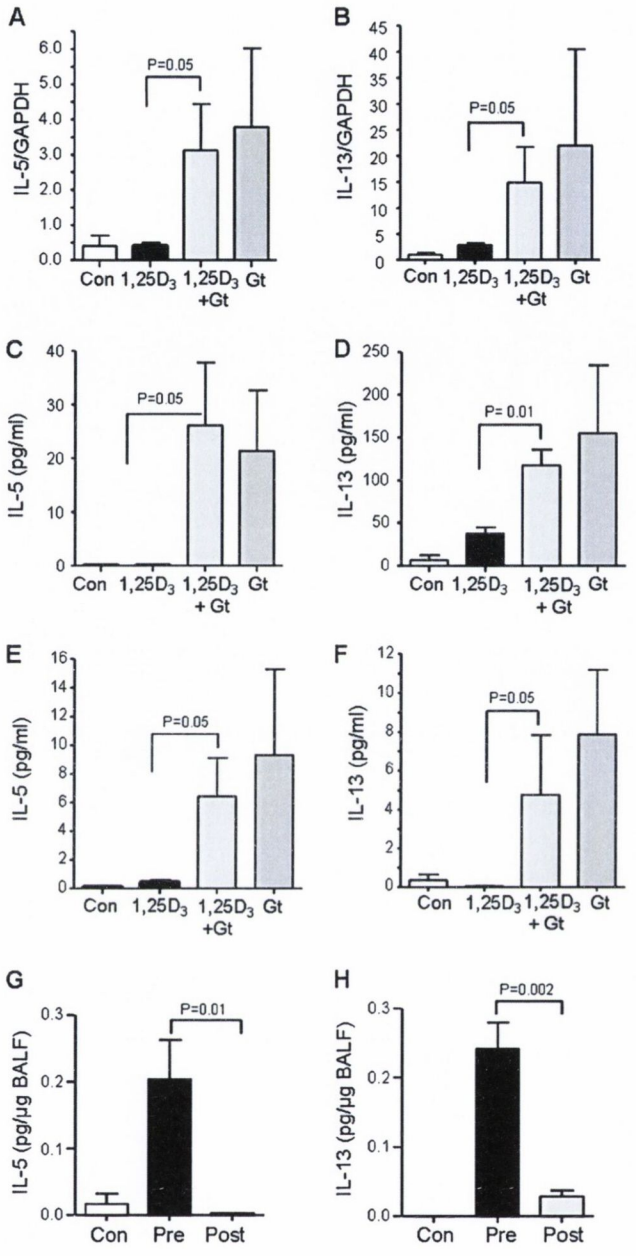

Figure 4. Gliotoxin (Gt)-enhanced production of Th2 cytokines is reduced after itraconazole treatment. (*A–D*) Gt exposure results in increased production of Th2 cytokines IL-5 and IL-13 *in vitro*. Cystic fibrosis bronchial epithelial (CFBE) monolayer cells (1×10^5) (*A* and *B*), CFBE cells grown under air liquid conditions (5×10^5) (*C* and *D*), and U937 differentiated macrophages (1×10^6) (*E* and *F*) were untreated (Con) or exposed to Gt (0.8 μM) alone or to $1,25D_3$ (10^{-9} M) in the presence or absence of Gt for 24 hours. IL-5 (*A*) or IL-13 (*B*) gene expression was analyzed by qRT-PCR and standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). IL-5 (*C* and *E*) or IL-13 (*D* and *F*) protein expression was quantified by ELISA. Assays were performed in triplicate on three separate days. (*G* and *H*) IL-5 and IL-13 *in vivo* cytokine expression is reduced after itraconazole treatment. IL-5 (*G*) and IL-13 (*H*) levels in healthy control subjects (n = 3, Con) and in *Aspergillus*-positive cystic fibrosis bronchoalveolar lavage (BALF) before (n = 3) or after itraconazole treatment (n = 3) were quantified by ELISA. Assays illustrated in *C* and *D* were performed a minimum of three times in duplicate and normalized to total BALF protein. Data shown are mean ± SE.

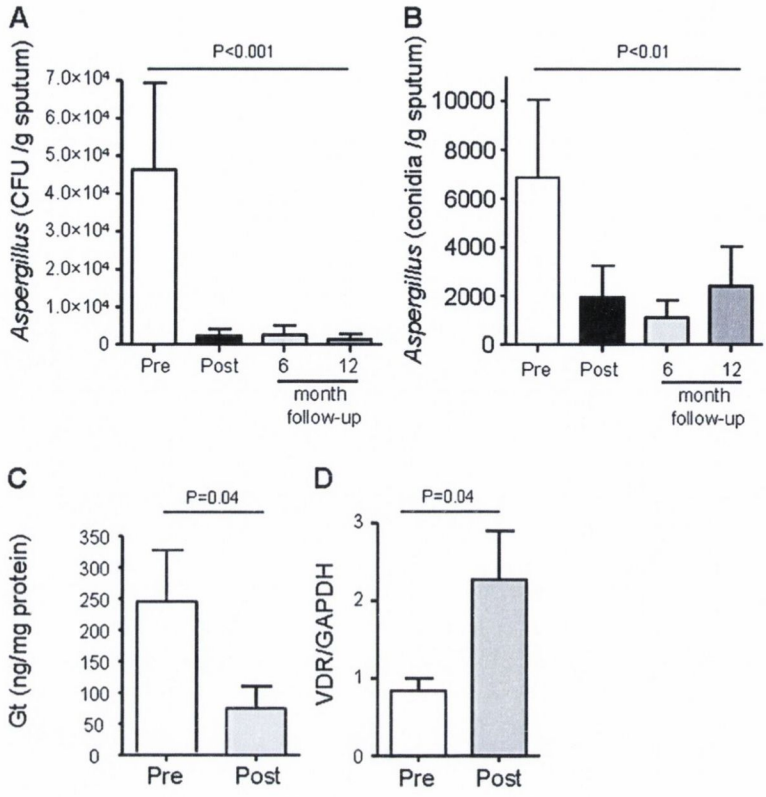

Figure 5. *Aspergillus* bioburden is significantly reduced in the cystic fibrosis (CF) lung after itraconazole therapy, resulting in increased vitamin D receptor (VDR) expression. (*A*) Colony-forming units (CFU) of *A. fumigatus* per gram of spontaneously expectorated sputum were determined in subjects with CF (n = 13) before (Pre) and after (Post) treatment with itraconazole (400 mg orally once daily for 6 wk) and at 6 and 12 months. All CFU counts were performed in triplicate for each patient sample. $^{**}P < 0.001$ (Friedman's test; Q = 20.59). (*B*) The equivalent *A. fumigatus* conidia number was determined in the same patient group (n = 13) using qPCR and *A. fumigatus* primer probe sets targeting the 28S rRNA region. DNA extraction for each patient sample was performed in duplicate, and qPCR was performed at least in triplicate. $^{**}P < 0.01$ (Friedman's test; Q = 15.12). (*C*) Gliotoxin (Gt) levels *in vivo* are reduced as a consequence of itraconazole treatment. Gt levels were quantified in cystic fibrosis bronchoalveolar lavage (BALF) before (n = 3) or after itraconazole treatment (n = 3) by HPLC and normalized to total BALF protein (mg) and analyzed by paired *t* test. (*D*) After itraconazole treatment, VDR gene expression is upregulated in *Aspergillus*-colonized patients *in vivo*. Bronchial brushings were obtained from people with cystic fibrosis before (n = 3) or after itraconazole treatment (n = 3), and VDR gene expression was analyzed by qRT-PCR standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The assay was performed in triplicate. Data shown are the mean ± SD (*A* and *B*) or ± SE (*C* and *D*).

in people with CF colonized by *A. fumigatus* with no prior history of ABPA or corticosteroid treatment for suspected ABPA (n = 13). Three HRCT scans were performed at baseline (pretreatment), at 10 weeks after completion of itraconazole (posttreatment), and at 12-month follow-up.

No significant differences in the overall or component parts of the modified Bhalla score was identified after treatment or during follow-up. However, 92.3% (n = 12) of baseline (pretreatment) scans illustrated a mosaic pattern, which was significantly less evident after itraconazole eradication of *A. fumigatus*. Fewer scans after treatment (76.9%; n = 10) or during follow-up (53.8%; n = 7) illustrated evidence of the mosaic pattern observed at baseline (Figures 6A and 6B). In patients in whom attenuation rather than elimination of the pattern occurred after itraconazole treatment, we further quantified the mosaic pattern by its conspicuity (obvious, subtle, or present on expiratory images only) and by percentage of lung parenchyma involved. A significantly diminished conspicuity and a lower percentage of lower lobe lung parenchymal involvement after treatment and during follow-up was observed (Figure 6C).

Pulmonary function (FEV_1 and forced expiratory flow, midexpiratory phase [FEF_{25-75}]) remained stable without significant deterioration over the study course (Figure 6D). However, eradication of *A. fumigatus* from the CF airway resulted in a significant reduction in the number of infective exacerbations (Figure 6E) and requirement for intravenous antibiotic therapy (Figure E4), findings that were sustained over the study follow-up period. Respiratory symptoms (assessed by CFQ-R) illustrated significant improvement during follow-up that was maintained to study conclusion (Figure 6F). Additionally, mean serum levels of IgE decreased after treatment (178.2 ± 88.4 to 136.9 ± 60.7 IU/ml) and during follow-up (90.5 ± 32.0 IU/ml), albeit not significantly ($P > 0.05$). No patient developed ABPA over the course of the study. Radioallergosorbent test *Aspergillus* sampling validated this. In summary, these results indicate that eradication of *A. fumigatus* from the CF airway resulted in improved HRCT appearances, less infective exacerbations, fewer requirements for intravenous antibiotics, and improved CFQ-R respiratory symptom scores.

DISCUSSION

In the current study, we illustrate that *A. fumigatus* colonization down-regulates VDR and identify Gt as one fungal agent responsible for mediating this effect. We also found that *in vivo* *A. fumigatus* colonization is associated with increased Gt in CF BALF, mosaic pattern on HRCT, and an enhanced production of the Th2 cytokines IL-5 and IL-13. Treatment with the antifungal agent itraconazole *in vivo* rescued VDR expression, significantly attenuated Gt levels, and decreased the production of the Th2 cytokines IL-5 and IL-13, which drive the allergic process leading to ABPA (24, 25). Such eradication of *A. fumigatus* from the CF airway resulted in improved respiratory symptoms and diminished HRCT mosaic pattern, consistent with sustained pulmonary function.

Vitamin D influences the inflammatory and immune state in CF and acts via cognate nuclear VDR (26, 27). Although insufficient vitamin D (< 30 ng/ml) is problematic for bone health in CF, it is also acknowledged as a risk factor for ABPA in those colonized with *A. fumigatus* (9, 28). Consequently, supplemental administration of vitamin D is advocated for its potential to prevent ABPA (9). As a result, the protein level of VDR through which vitamin D functions is clinically relevant but has not been investigated thoroughly in the context of CF complicated by *A. fumigatus*. In this study, we illustrate that *A. fumigatus* colonization in the absence of ABPA down-regulates VDR and enhances Th2 cytokine production through Gt, a toxin released by the hyphal form of the fungi. Compromised VDR in the face of *A. fumigatus* disrupts the inflammatory state in CF, with vitamin D unable to exert its positive impact. One method by which

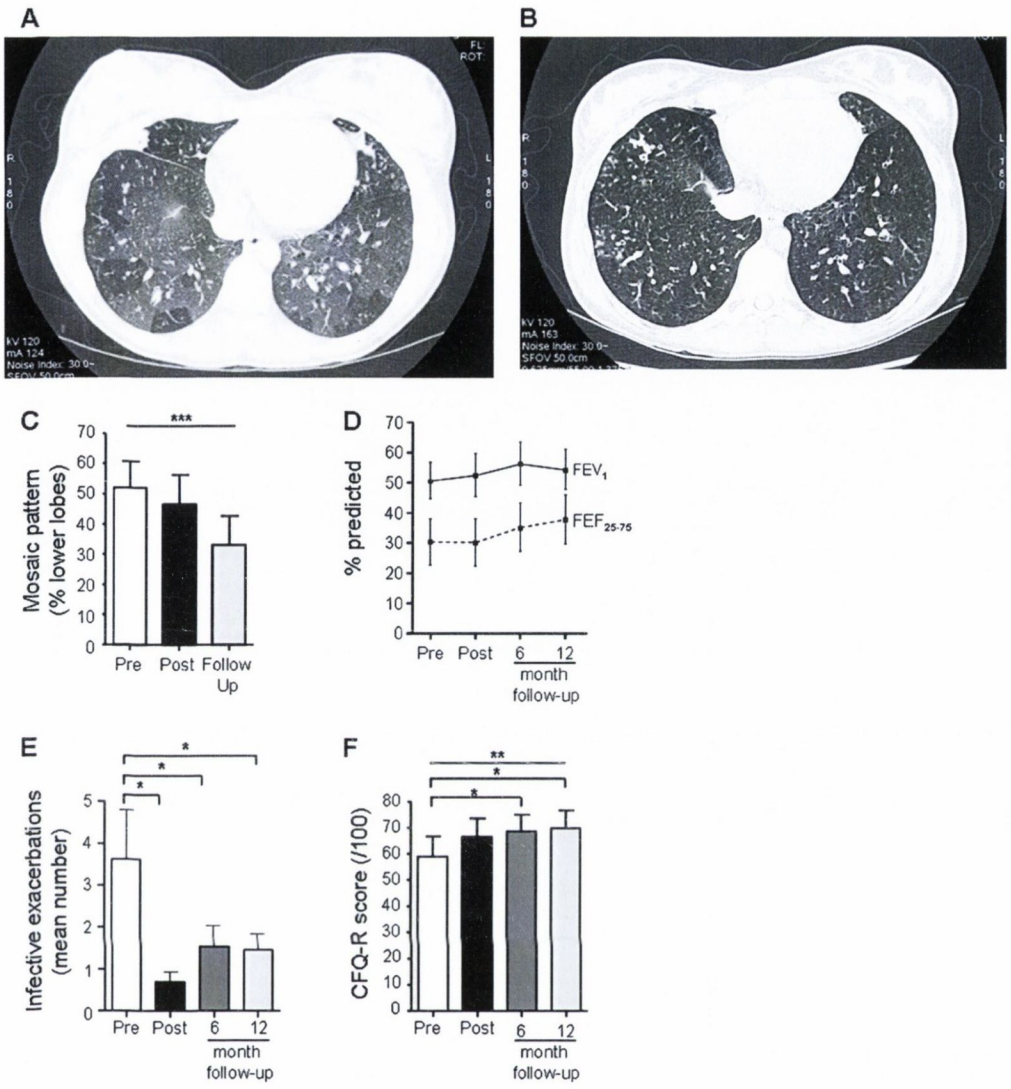

Figure 6. Itraconazole therapy in *Aspergillus fumigatus*–colonized patients demonstrates radiological and clinical improvement. Sixty-four–slice high-resolution computed tomography (HRCT) imaging of the thorax was performed in 13 subjects with cystic fibrosis before treatment, 10 weeks after treatment, and at 12-month follow-up. (*A*) A representative pretreatment HRCT image through the lower lobes obtained at end expiration. There is an obvious and conspicuous mosaic pattern involving 80% of the lung parenchyma. (*B*) Follow-up HRCT image at the same level in the same patient at 12 months obtained at end-expiration. There is a dramatic reduction in the degree of mosaic pattern in conspicuousness and extent. (*C*) The percentage of lung parenchyma (within the lower lobes) illustrating a mosaic pattern was determined. A significant attenuation of mosaic pattern was detected over time, greatest at late follow-up. $***P = 0.0019$ (Friedman's test; $Q = 12.56$). (*D*) Pulmonary function as percent predicted FEV_1 and forced expiratory flow, midexpiratory phase ($FEF_{25–75}$) was evaluated before and after treatment and during follow-up at 6 and 12 months. No significant deterioration of FEV_1 or $FEF_{25–75}$ occurred over the course of the study. (*E*) Mean number of infective exacerbations defined by the criteria outlined by Fuchs and colleagues (38) was recorded for the 3-month period before- and after treatment and reassessed at 6 and 12 months. Infective exacerbations significantly decreased after treatment and during follow-up, $*P < 0.001$ (log-linear Poisson regression analysis). (*F*) Changes in respiratory symptoms determined by the cystic fibrosis questionnaire-revised (CFQ-R) respiratory symptom scale. Respiratory symptoms significantly improved at 6- and 12-month follow-up after treatment. $**P = 0.015$ (repeat measures ANOVA; $F = 3.99$); $*P < 0.05$ between pretreatment and 6- or 12-month follow-up. Data shown are the mean scaled score for the respiratory domain of the CFQ-R (out of maximum 100) ± SD.

vitamin D confers benefit is by attenuating Th2 responses to *A. fumigatus* by CD4+ T cells in CF-ABPA (9). Th2 cytokines IL-5 and IL-13 are not restricted to CD4+ cells alone, and, although this source remains of primary concern in the ABPA context, release of these cytokines by eosinophil and mast cells (29, 30), and by airway epithelial cells and macrophages as shown in the present study, is of importance in people with CF colonized by *A. fumigatus* but unaffected by ABPA.

The unique structural motif of Gt is essential for its damaging effects, including the inhibition of immune cell activation, phagocytosis, and ciliary beat frequency, together preventing fungal clearance (22, 31, 32). Gt concentrations have been reported in the setting of a murine model of invasive aspergillosis (33); however, to our knowledge this is the first study to report Gt levels *in vivo* in the context of CF and its relationship to VDR expression. However, Gt-mediated VDR down-regulation is independent of CFTR dysfunction; this phenomenon was observed during *in vitro* experiments using non-CF HBE cells. Gt is also able to overcome the positive effects conferred by vitamin D on VDR expression, a novel mode of action within the pulmonary milieu. Placing our findings in the context of those reported by Kriendler and colleagues (9), vitamin D may attenuate Th2 cytokines and potentially reduce or prevent CF-ABPA, although it requires the presence of VDR to carry out such functions. Hence, the elimination of Gt remains as crucial as the administration of vitamin D.

This observational study provided unique insights into the treatment effectiveness of itraconazole on microbiological, radiological, and clinical outcomes, the former two not addressed by prior work (34). Sputum culture alone has been previously reported as insufficient at detecting the presence of *A. fumigatus* in CF (6, 35). Despite this, CFU counts for *A. fumigatus* remain the most accessible and affordable method of assessment and therefore remain in routine clinical practice. Failure to detect *A. fumigatus* because of a negative sputum culture can have deleterious clinical consequences. In view of this, we chose

to assess for the presence of *A. fumigatus* from sputum using two separate approaches, CFU counts and qPCR for *A. fumigatus* conidia burden. Although itraconazole was effective at eliminating *A. fumigatus* after treatment, these effects were sustained over the 1-year follow-up by both methodologies. However, despite the reassuring CFU counts obtained at the 1-year time point, escalations of *A. fumigatus* conidia burden were detectable by qPCR at this time as compared with earlier time points, suggesting a slow return of *A. fumigatus* colonization and the potential need for retreatment. These data provide evidence to support use of qPCR as the optimal detection method for *A. fumigatus* from respiratory CF samples. However, the clinical parameters assessed in this study continue to show positive effects at 12 months after treatment, a time when qPCR counts for *A. fumigatus* conidia begin to slowly rise (35). Consequently, cost and clinical importance of such methods for *A. fumigatus* detection must be considered before recommending their routine use. Most benefit from such methodology is probably gained from its use in clinical scenarios of strongly suspected *A. fumigatus*–associated disease in the presence of negative sputum culture where a treatment decision is necessary and further microbiological evidence for such treatment is sought. This may be applicable to *A. fumigatus* beyond CF.

Further work performed by our group described the radiological evaluation of *A. fumigatus* colonization in CF (12) and reported a novel mosaic pattern associated with such colonization in the CF setting unrelated to ABPA. Quantification of this mosaic pattern over the study course and after itraconazole treatment illustrated elimination or significant attenuation, suggestive of an inflammatory benefit in the individuals with CF recruited to this study. Significant time-dependent effects, greatest at 12-month follow-up, were observed. Whether such findings confer prolonged benefit or represent a radiological lag in appearance compared with clinical benefit remains to be elucidated. Although images from an untreated placebo controlled group of *A. fumigatus* colonized patients with CF without ABPA would have provided stronger evidence for this radiological relationship, we feel it would have been unethical to recruit an untreated group in view of our previously published work illustrating worse radiological appearances in patients who are *A. fumigatus* colonized (12). Although our study findings are of interest, the small study population and the uncontrolled and open-label nature of the trial are notable weaknesses. However, itraconazole treatment produced positive outcomes across all the clinical parameters assessed, including decreasing exacerbations and stabilizing pulmonary function while improving CFQ-R respiratory scores, findings contrasting prior work (34). Moreover, itraconazole is a potent inhibitor of CYP3A4 activity, and corticosteroids are partly metabolized through such enzymes; hence, it should be noted that a proportion of our patient cohort received inhaled corticosteroids at a low dose during the course of the study (Table E1). Systemic absorption of such steroid use is accepted to be diminutive; hence, the effects on the CYP3A4 system conferred by itraconazole treatment during the study are probably minimal. However, this is a potential confounding factor to be considered when interpreting our results. No patient during the study developed ABPA by consensus criteria (17), and a reduced likelihood of ABPA occurring was demonstrated by decreasing levels of IgE, radioallergosorbent test to *Aspergillus*, and an attenuated Th2 cytokine response after treatment.

Despite the fact that IL-5 and IL-13 are linked to ABPA, none of the patients recruited to this study had ever had ABPA, although high levels of these cytokines were present in their lungs. These Th2 cytokines were significantly reduced after itraconazole therapy, which is suggestive of an important antiinflammatory role for the drug in addition to its antifungal properties. Minimal clinical data exist within the CF literature to assist the clinician in identifying individuals at risk of ABPA or those that can be predicted to develop ABPA in the setting of *A. fumigatus* colonization alone. Although our group has previously shown that sputum isolation of *A. fumigatus* does not correlate with occurrence of ABPA (6), the current study has uncovered high levels of Th2 cytokines IL-5 and IL-13 existing *in vivo* in the setting of *A. fumigatus* colonization without ABPA that may be attenuated by empirical itraconazole treatment. Whether such therapy can prevent ABPA in the long term in those asymptomatically colonized by *A. fumigatus* remains to be determined.

In summary, although published work has suggested the therapeutic potential of vitamin D in preventing ABPA (9), our data imply that this approach is only feasible with concurrent elimination of *A. fumigatus* with itraconazole to permit VDR expression. This study raises awareness of the clinical importance of antifungal resistance (36, 37) and acknowledges the need for a more long-term study on the antifungal activity of triazole derivatives in the treatment of *A. fumigatus* in individuals with CF.

Author disclosures are available with the text of this article at www.atsjounals.org.

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Candida albicans Stimulates IL-23 Release by Human Dendritic Cells and Downstream IL-17 Secretion by Vδ1 T Cells

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γδ T cells expressing the Vδ1 TCR are expanded in patients with HIV infection. We show in this article that circulating Vδ1 T cell numbers are particularly high in patients with HIV and candidiasis, and that these cells expand and produce IL-17 in response to *Candida albicans* in vitro. Although *C. albicans* could directly stimulate IL-17 production by a subset of Vδ1 T cells, fungus-treated dendritic cells (DCs) were required to expand *C. albicans*–responsive Vδ1 T cells to generate sufficient numbers of cells to release IL-17 at levels detectable by ELISA. *C. albicans* induced the release of IL-1β, IL-6, and IL-23 by DCs, but addition of these cytokines or supernatants of *C. albicans*–treated DCs to Vδ1 T cells was not sufficient to induce proliferation. We found that direct contact with DCs was required for Vδ1 T cell proliferation, whereas IL-23R–blocking studies showed that IL-23 was required for optimal *C. albicans*–induced IL-17 production. Because IL-17 affords protection against both HIV and *C. albicans*, and because Vδ1 T cells are not depleted by HIV, these cells are likely to be an important source of IL-17 in HIV-infected patients with candidiasis, in whom $CD4^+$ Th17 responses are impaired. These data show that *C. albicans* stimulates proliferation and IL-17 production by Vδ1 T cells by a mechanism that involves IL-23 release by DCs. *The Journal of Immunology*, 2015, 194: 5953–5960.

Candida albicans is a commensal yeast fungus that colonizes the oropharynx, intestine, and vagina in healthy individuals (1, 2). In HIV-infected individuals and other immunocompromised hosts, *C. albicans* can be a major pathogen, causing oropharyngeal, esophageal, or vulvovaginal candidiasis (3), and it is the fourth most common cause of nosocomial bloodstream infections in the United States with a 38% mortality rate (4). Identification of the mechanisms by which the immune system protects against *C. albicans* infection is critical for the development of treatments and vaccines against this pathogen.

Host defense against *C. albicans* requires the IL-17 pathway of cells and cytokines (5–8). IL-17 is an inflammatory cytokine released by a subset of $CD4^+$ T cells (Th17 cells), some $CD8^+$ T cells, NK cells, NK T cells, and innate lymphoid cells (9–11). IL-17 induces the production of antimicrobial peptides and chemokines, which recruit neutrophils to sites of infection where they kill extracellular bacteria and fungi by phagocytosis or the release of antimicrobial agents (6, 12, 13). Pathological *C. albicans* infections are common in humans with inherited deficiencies of the IL-17 pathway (such as IL-17 deficiency or mutations in the IL-17R or STAT1) (14, 15) and in knockout mice that lack IL-17 or IL-23 (6, 16). *C. albicans*–specific T cells in healthy donors are preferentially Th17 cells that coproduce IFN-γ (7).

T cells expressing γ and δ TCR chains are an important source of innate IL-17. Fenoglio and coworkers (17) showed that the Vδ1 subset of human γδ T cells proliferates and produces IFN-γ and IL-17 in response to *C. albicans*. However, the mechanisms by which *C. albicans* stimulates Vδ1 T cells are poorly understood. Murine γδ T cells expressing TLR2 and dectin-1, which bind to β-glucans present in cells walls of fungi, are implicated in responses to *C. albicans* (8, 18–20). Murine γδ T cells can also release IL-17A and the Th17-associated cytokines, IL-17F, IL-21, and IL-22, in response to treatment with IL-1 and IL-23, or IL-18 and IL-23 in the absence of TCR stimulation (21, 22). Cellular sources of IL-1, IL-18, and IL-23 include monocytes, macrophages, dendritic cells (DCs), or neutrophils, which can express receptors that mediate immune recognition of *C. albicans*, such as TLR2, dectin-1, dectin-2, and mannose receptors (23, 24). IL-1β, IL-6, and IL-23 contribute to $CD4^+$ Th17 cell induction by *C. albicans* in humans (7). However, the factors that induce and regulate IL-17 production by human Vδ1 T cells are not known.

Vδ1 T cells are expanded in the circulation of patients with HIV infection (25–27). In this study, we show that *C. albicans* infection is a major determinant of Vδ1 T cell expansions in patients with HIV, and we confirm that *C. albicans* drives expansion of and IL-17 production by human Vδ1 T cells in vitro. Although some Vδ1 T cells could produce IL-17 upon direct contact with the fungus, a robust IL-17 response to *C. albicans*, resulting in substantial IL-17 release, required DC-driven proliferation of Vδ1 T cells. *C. albicans* potently induced IL-1β, IL-6, and IL-23 secretion by DCs, but without the presence of DCs, these cytokines were not sufficient to induce Vδ1 T cell proliferation, resulting in elevated

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Abbreviations used in this article: DC, dendritic cell; RORγt, retinoic acid-related orphan receptor gamma t.

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IL-17 secretion, which required direct contact with DCs as well as IL-23 signaling. These results indicate that Vδ1 T cells are likely to be important mediators of immunity against candidiasis, particularly in the setting of HIV infection where $CD4^+$ Th17 cells are depleted.

Materials and Methods

Subjects

Blood samples were obtained from 23 HIV-1–infected subjects with no evidence of *Candida* coinfection (16 white, 6 African, and 1 Asian subjects; 13 male subjects) and 14 HIV-1–infected subjects with candidiasis (9 white and 5 African subjects; 8 male subjects), who were attending the Genito-Urinary and Infectious Diseases Clinic at St. James's Hospital, Dublin, Ireland. Ten HIV^- healthy donors were also studied. All HIV-infected patients were receiving antiretroviral treatment. The CD4 T cell count at the time of blood collection ranged from 55 to 1115 (median 529) per microliter of blood in the patients without candidiasis and 261–1857 (median 575) per microliter in the patients with candidiasis. Eleven patients without candidiasis and eight with candidiasis had HIV viral loads of <50 copies/ml, and the remainder had viral loads ranging up to 72,976 copies/ml in the patients without candidiasis and 32,265 copies/ml in the patients with candidiasis. Three patients without candidiasis were positive for hepatitis B virus surface Ag and two were positive for hepatitis C virus RNA, whereas none of the patients with candidiasis had hepatitis B and one had hepatitis C. Ethical approval for this study was obtained from the Joint Research Ethics Committee of St. James's Hospital and Tallaght Hospitals, Dublin, and all participants gave written, informed consent. Buffy coat packs from healthy blood donors were kindly provided by the Irish Blood Transfusion Service. PBMCs were prepared by density gradient centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway) and used immediately in all procedures.

Abs and flow cytometry

Fluorochrome-conjugated mAbs specific for human Vδ1 TCR (clone TS-1), CD3ε, CD11c, CD14, IFN-γ, IL-17A, and dectin-1 were obtained from Thermo Fisher Scientific (Dublin, Ireland) and Biolegend (San Diego, CA). A total of 10^5 cells were labeled with mAbs and analyzed using a CyAN ADP (Beckman Coulter, High Wycombe, U.K.) or FACSCanto (Becton Dickinson, Oxford, U.K.) flow cytometer. Data were analyzed with FlowJo v7.6 (Tree Star, Ashland, OR) software. Single-stained OneComp Beads (Becton Dickinson) were used to set compensation parameters, and fluorescence minus one and isotype-matched Ab controls were used to set analysis gates.

Preparation of fungi

C. albicans strain 10231 was obtained from the American Type Culture Collection and cultured on malt extract agar according to American Type Culture Collection protocols. Fungi were cultured for 24 h, isolated, counted, and then inactivated by heating at 96°C for 60 min or treating with 70% ethanol for 30 min. Samples were then centrifuged at 5000 × *g* for 10 min, the supernatants discarded, and the pellets washed with PBS. Inactivation was confirmed by plating an aliquot onto malt extract agar and incubating for 1–7 d to check for growth.

Generation of DCs

Monocytes were enriched from PBMCs isolated from buffy coat packs and allowed to differentiate into immature DCs by culturing them for 6 d in the presence of GM-CSF and IL-4 as described previously (28). Maturation of DCs was achieved by plating immature DCs at densities of 100,000/ml and stimulating them overnight with medium only, with the dectin-1 ligand curdlan (100 μg/ml; InvivoGen, Toulouse, France), or with heat- or ethanol-killed *C. albicans* (5×10^6 cells/ml).

In vitro expansion of Vδ1 T cells

Total γδ T cells were enriched from healthy donor PBMCs using human anti-TCR γ/δ Microbeads (Miltenyi Biotec, Gladbach Bergische, Germany). γδ-enriched cells (200,000/ml) were cultured in the absence or presence of *C. albicans*– or curdlan-treated DCs at 2:1 ratios in complete serum-free AIM-V medium (AIM-V containing 0.05 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.02 M HEPES, 55 μM 2-ME, 1X essential amino acids, 1X nonessential amino acids, and 1 mM sodium pyruvate). Cocultures were challenged with PHA-L (1 μg/ml; Sigma-Aldrich, Dublin, Ireland) and cultured with rIL-2 (40 U/ml; Miltenyi Biotec), which was added in fresh medium every 2–3 d. Cultures were restimulated every 2 wk with activated DCs and PHA-L, which resulted in yields of >10 million Vδ1 T cells by day 28.

Vδ1 T cell stimulation and analysis of cytokine production

IFN-γ and IL-17 expression by fresh, unexpanded Vδ1 T cells within γδ T cell–enriched PBMCs was examined by flow cytometry (29) after stimulation of the cells for 6 h with medium alone, 1 ng/ml PMA with 1 μg/ml ionomycin, or *C. albicans* in the absence or presence of DCs. Cytokine expression by Vδ1 T cells that were expanded for 14 d with *C. albicans*–treated DCs was similarly examined. Levels of soluble IFN-γ, IL-1β, IL-6, IL-17A, IL-22, and IL-23 released into supernatants of DCs treated for 24 h with medium, *C. albicans*, or curdlan were measured by ELISAs using Ab pairs purchased from BioLegend (San Diego, CA) and R&D Systems (Abingdon, U.K.). ELISA was also used to measure cytokines released by Vδ1 T cell–enriched PBMCs after treatment for up to 14 d with curdlan- or *C. albicans*–treated DCs, supernatants of immature or activated DCs, or rIL-1β (10 ng/ml; Immunotools, Friesoythe, Germany) or rIL-23 (10 ng/ml; eBioscience, Hatfield, U.K.). In some experiments, a blocking Ab specific for human IL-23R or isotype control Ab (20 μg/ml; eBioscience) was added at times 0, 3 h, 17 h, and 3 d.

Statistical analysis

Prism GraphPad software (San Diego, CA) was used for data analysis. Vδ1 T cell frequencies in subject groups and cytokine levels in treatment groups were compared using the Mann–Whitney *U* test. Matched donor treatments were compared using the Wilcoxon matched pair test. The *p* values <0.05 were considered significant.

Results

Vδ1 T cells are expanded in the circulation of HIV-infected subjects with candidiasis

Previous studies have demonstrated that Vδ1 T cells are expanded in the circulation of patients with HIV infection (25–27). We used flow cytometry to determine the percentages of T cells that express Vδ1 TCRs in fresh PBMCs from 10 healthy donors, 23 treated HIV-infected subjects without *Candida* coinfection, and 14 HIV-infected subjects with candidiasis. Fig. 1 confirms that Vδ1 T cells are expanded in HIV-infected subjects compared with healthy donors and further shows that these cells are significantly more frequent in HIV-infected subjects with candidiasis compared with patients with no evidence of fungal infection (means 4.0

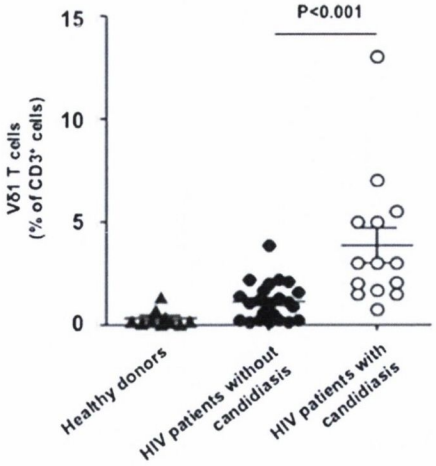

FIGURE 1. Vδ1 T cells are expanded in the circulation of HIV-infected subjects with candidiasis. Frequencies of Vδ1 T cells, as percentages of all T cells, in blood samples taken from 10 healthy donors, 23 HIV-infected subjects with no evidence of *Candida* coinfection, and 14 HIV-infected subjects with candidiasis. Freshly isolated PBMCs were stained with mAbs specific for CD3 and the Vδ1 TCR chain, and the percentages of $CD3^+$ cells that expressed Vδ1 were determined by flow cytometry. Horizontal lines show the mean and error bars show SEM.

versus 1.0%; $p < 0.001$). Vδ1 T cell frequencies were only moderately increased in patients with HIV without candidiasis compared with healthy control subjects (0.32%; Fig. 1; NS). Therefore, *C. albicans* infection is likely to be a major driver of Vδ1 T cell expansion in patients with HIV.

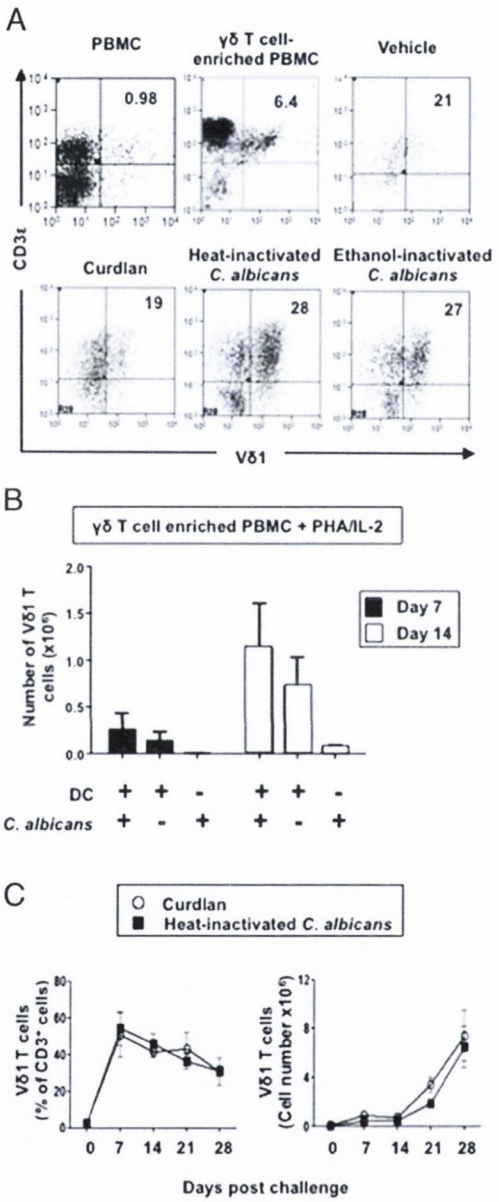

FIGURE 2. *C. albicans* drives the expansion of Vδ1 T cells in vitro. (**A**) Representative flow-cytometry dot plots showing CD3ε and Vδ1 TCR expression by freshly isolated PBMCs (*top left panel*), PBMCs enriched for total γδ T cells by magnetic bead separation (*top center panel*), γδ T cell–enriched PBMCs after stimulation for 7 d with monocyte-derived DCs pulsed overnight with vehicle (*top right panel*), 100 μg/ml curdlan (*bottom left panel*), or 5×10^6 bodies/ml heat-inactivated (*bottom center panel*) or ethanol-inactivated (*bottom right panel*) *C. albicans* in the presence of 1 μg/ml PHA and 40 U/ml IL-2. Numbers indicate the percentages of total cells that express $CD3^+V\delta1^+$ phenotypes. (**B**) Expansion of Vδ1 T cells from γδ T cell–enriched PBMCs in the presence of PHA and IL-2 and in the presence and absence of DCs and *C. albicans*. Results show mean ± SEM of four independent experiments. (**C**) Kinetics of Vδ1 T cell expansion showing purities (*left panel*) and absolute numbers (*right panel*) of Vδ1 T cells obtained after stimulation as described earlier on days 0 and 14, and analysis by flow cytometry on days 0, 7, 14, 21, and 28. Results show mean ± SEM of four independent experiments.

C. albicans *drives the expansion of Vδ1 T cells in vitro*

A number of protocols for the ex vivo expansion of human γδ T cells from healthy donors were tested (30, 31), and the following method was optimized for generating lines of Vδ1 T cells. PBMCs prepared from buffy coat packs were enriched for total γδ T cells using Ab-coated magnetic beads and cultured for 7–14 d in medium containing PHA-L and IL-2, with monocyte-derived DCs that had been matured overnight with curdlan or heat- or ethanol-killed *C. albicans* (Fig. 2A). This resulted in the selective expansion of Vδ1 T cells, with yields of up to 2.1×10^6 Vδ1 T cells for *C. albicans*–stimulated γδ T cells and up to 4.9×10^6 Vδ1 T cells for curdlan-stimulated γδ T cells (starting with 200000 total γδ T cells) by day 7 as demonstrated by flow cytometry (Fig. 2A). Curdlan and heat- or ethanol-inactivated *C. albicans*, when pulsed onto DCs, showed comparable capacity for inducing Vδ1 T cell proliferation. *C. albicans* in the absence of DCs failed to induce Vδ1 T cell proliferation, even when PHA and IL-2 were present, whereas DCs in the absence of *C. albicans* induced moderate proliferation of Vδ1 T cells (Fig. 2B). Numbers of Vδ1 T cells could be greatly increased by restimulating them as de-

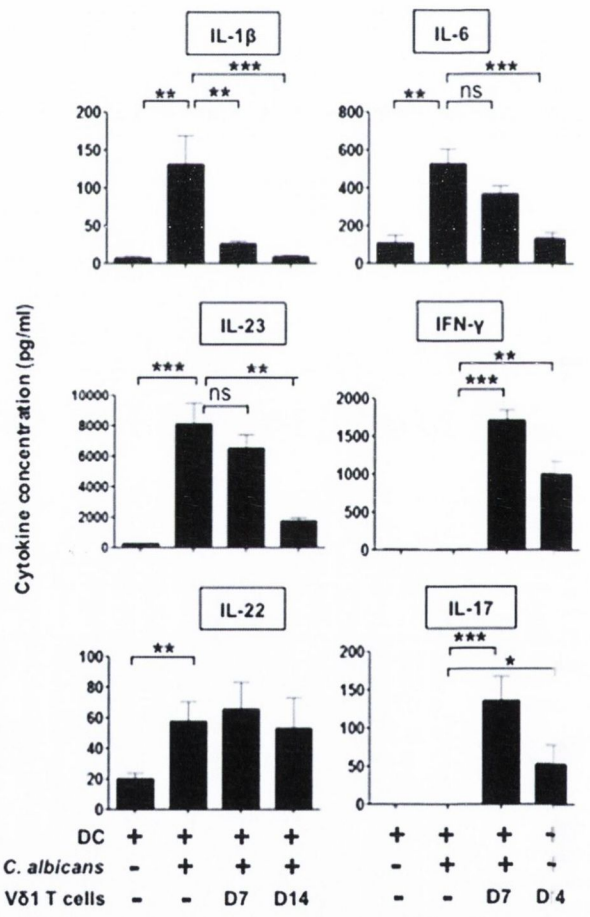

FIGURE 3. *C. albicans* induces IL-1β, IL-6, and IL-23 secretion by DCs and downstream IFN-γ and IL-17 secretion by γδ T cell–enriched PBMCs. Monocyte-derived DCs were pulsed for 24 h in medium alone or with 5×10^6 bodies/ml heat-inactivated *C. albicans*. Cell supernatants were collected for analysis of cytokine production by ELISA (first two bars in each graph) and replaced with fresh medium containing Vδ1 T cells, PHA, and IL-2. Medium was replaced with fresh medium containing IL-2 every 3 d. Supernatants were collected for cytokine analysis after 7 and 14 d (D7 and D14) by ELISA (third and fourth bars in each graph) Data are mean ± SEM of 6–11 experiments. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ using unpaired Mann–Whitney *U* tests.

scribed earlier on day 14, which resulted in yields of up to 10^7 Vδ1 T cells by day 28 starting with 200,000 γδ T cell–enriched PBMCs (Fig. 2C). Purified populations of Vδ1 T cells could be obtained by sorting $CD3^+V\delta1^+$ cells on a MoFlo XDP Cell Sorter (Beckman Coulter). This method proved to be a robust method for generating human Vδ1 T cells lines.

C. albicans *induces IL-1β, IL-6, and IL-23 secretion by DCs and downstream IFN-γ and IL-17 secretion by expanded Vδ1-enriched T cells*

To identify the cytokines produced in response to *C. albicans* stimulation of DCs and the downstream activation of enriched Vδ1 T cells, we pulsed monocyte-derived DCs for 24 h in medium alone or with heat-inactivated *C. albicans*. Cell supernatants were collected for analysis of cytokine production by ELISA and replaced with fresh medium containing γδ T cell–enriched PBMCs, PHA, and IL-2. Medium was replaced with fresh medium containing IL-2 every 3 d. Supernatants were collected after 7 and 14 d and analyzed for cytokine levels by ELISA. Fig. 3 shows that addition of *C. albicans* to immature DCs resulted in potent secretion of IL-1β, IL-6, IL-22, and IL-23, but not IFN-γ or IL-17A within 24 h. Addition of γδ T cell–enriched PBMCs resulted in secretion of IFN-γ and IL-17A, the levels of which peaked by day 7. The levels of IL-1β, IL-6, and IL-23 decreased over the 14-d period of culture with the Vδ1-enriched T cells, presumably because they were mainly produced by *C. albicans*–treated DCs and not by T cells, and then were partially removed when medium was collected for analysis by ELISA and replaced with fresh medium. IL-1β, IL-6, and IL-23 levels may also have decreased because of consumption by the T cells. In contrast, IL-22 levels remained relatively constant over this 14-d period, suggesting that IL-22 is being produced both by DCs and T cells (Fig. 3). These data indicate that *C. albicans* directly induces proinflammatory cytokine production by DCs and that *C. albicans*–treated DCs induce IFN-γ and IL-17 secretion by γδ T cell–enriched PBMCs.

C. albicans *induces IL-17 secretion by Vδ1 T cells*

The expanded γδ-enriched T cells used earlier typically contained <60% Vδ1 T cells; therefore, it is possible that cells other than Vδ1 T cells were releasing the IFN-γ and IL-17. To ascertain whether Vδ1 T cells can produce IL-17 in response to *C. albicans*, we stimulated freshly isolated PBMCs enriched for γδ T cells for 6–12 h with *C. albicans* in the absence or presence of DCs, in the presence of monensin. Intracellular expression of IL-17A by Vδ1 T cells was then analyzed by flow cytometry (Fig. 4A, 4B). Up to 10% of unexpanded Vδ1 T cells expressed intracellular IL-17A when stimulated with *C. albicans*, alone or loaded onto DCs, but no IL-17A was detected in Vδ1 T cells cultured with PMA and ionomycin or with DCs in the absence of *C. albicans*. When expanded Vδ1 T cells, generated by treatment of γδ T cell–enriched

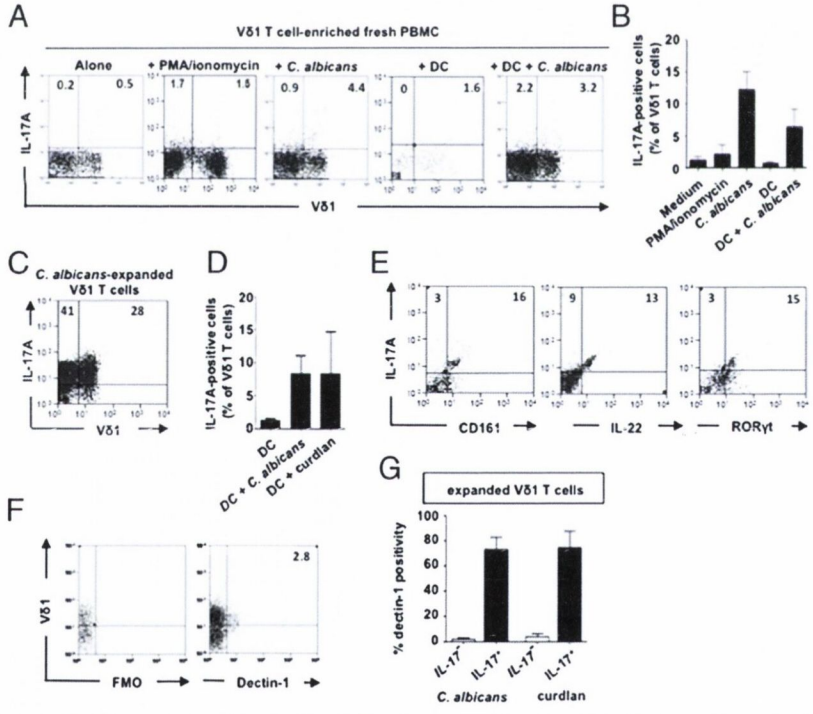

FIGURE 4. *C. albicans* induces IL-17 secretion by Vδ1 T cells. γδ T cells were enriched from PBMCs by positive selection using magnetic beads. (**A** and **B**) Fresh γδ T cell–enriched PBMCs were treated for 6 h with medium alone, PMA and ionomycin, *C. albicans*, DC, or *C. albicans*–treated DCs, and intracellular expression of IL-17A by Vδ1 T cells was then analyzed by flow cytometry. (A) Flow-cytometry dot plots showing IL-17 expression by Vδ1 T cells treated with (from *left* to *right*) medium, PMA and ionomycin, *C. albicans*, DC, or *C. albicans*–treated DCs after gating on $CD3^+$ cells. Numbers in the plots show percentages of Vδ1 T cells and non-Vδ1 T cells that produced IL-17. (B) Mean (± SEM) frequencies of fresh Vδ1 T cells from four γδ T cell–enriched PBMC samples that expressed IL-17 after the different treatments. (**C**–**G**) Vδ1 T cells were expanded from γδ T cell–enriched PBMCs by treatment for 14 d with *C. albicans*–treated DCs in the presence of PHA and IL-2. Cells were then restimulated with PMA and ionomycin, and IL-17, IL-22, CD161, RORγt, and dectin-1 expression by Vδ1 T cells were examined by flow cytometry. (C) Flow-cytometry dot plot showing IL-17 expression by Vδ1 T cells expanded with *C. albicans*–treated DCs. (D) Mean (± SEM) frequencies of expanded Vδ1 T cells in four expanded Vδ1 T cell lines that expressed IL-17 after treatment with DCs alone, *C. albicans*–treated DCs, and curdlan-treated DCs. (E) Flow-cytometric analysis of CD161, IL-22, and RORγt expression by *C. albicans*–expanded Vδ1 T cells. (F) Flow-cytometric detection of dectin-1 expression by *C. albicans*–expanded Vδ1 T cells (*right*) after gating on $CD3^+$ cells. The *left panel* shows a fluorescence minus one (FMO) control omitting the dectin-1 mAb. (G) Mean ± SEM frequencies of $IL\text{-}17^-$ and $IL\text{-}17^+$ Vδ1 T cells, expanded with *C. albicans*– or curdlan-pulsed DCs that expressed dectin-1. Results show mean ± SEM of results from experiments with three Vδ1 T cell lines.

PBMCs for 14 d with *C. albicans*–treated DCs in the presence of PHA and IL-2, were restimulated as described earlier for 6 h, up to 90% of Vδ1 T cells expressed IL-17A (Fig. 4C, 4D). The data in Fig. 4A and 4B indicate that a subset of Vδ1 T cells can produce IL-17 in response to *C. albicans* by a mechanism that does not require DCs. Although it cannot be ruled out that residual DCs or monocytes remaining in the γδ T cell–enriched PBMCs provided the necessary signals, this is unlikely because *C. albicans* stimulated IL-17 expression by Vδ1 T cells within 6 h and this was not augmented by the addition of DCs. However, Fig. 4C–E clearly shows that a 14-d stimulation with *C. albicans*–treated DCs is required to generate large numbers of IL-17A–producing Vδ1 T cells that coexpress markers of Th17 cells, such as CD161, IL-22, and retinoic acid-related orphan receptor gamma t (RORγt). Vδ1$^-$ T cells that produce IL-17A were also expanded (Fig. 4C), and these were found to express CD161 but did not include other γδ T cells (data not shown). Analysis of dectin-1 expression by these Vδ1 T cells indicated that most IL-17–producing Vδ1 T cells expressed this receptor, whereas the IL-17$^-$ Vδ1 T cells did not (Fig. 4F, 4G), indirectly suggesting that *C. albicans* may stimulate subsets of Vδ1 T cells through dectin-1. Therefore, although *C. albicans* can directly stimulate IL-17 production by Vδ1 T cells (Fig. 4), both DCs and *C. albicans* are required for the induction of potent and sustained IL-17 release by Vδ1 T cells (Fig. 3).

IL-17 response to C. albicans *requires DCs*

Because DCs promoted sustained IL-17 secretion by *C. albicans*–treated Vδ1 T cells (Fig. 3) but were not required for intracellular expression of IL-17 by *C. albicans*–stimulated fresh Vδ1 (Fig. 4), we further investigated the role of DCs in this IL-17 response. γδ T cell–enriched PBMCs were cultured, in the presence of PHA and IL-2, with *C. albicans* alone, DCs that were pulsed overnight with heat-inactivated *C. albicans*, or with supernatants of untreated or *C. albicans*–treated DCs. Culture supernatants were collected after 7 d for measurement of IFN-γ and IL-17 release by ELISA. Although *C. albicans*–treated DCs induced IL-17 release by Vδ1-enriched T cells, Fig. 5A shows that neither *C. albicans* alone nor supernatants of untreated or *C. albicans*–treated DCs induced IL-17 production. In contrast, detectable IFN-γ was released by γδ T cell–enriched T cells stimulated with supernatants of *C. albicans*–treated DCs (Fig. 5B). Thus, *C. albicans* alone or soluble factors released by *C. albicans*–treated DCs, such as IL-1β, IL-6, and IL-23, are not sufficient to induce sustained IL-17 release by Vδ1 T cells in the absence of Vδ1 T cells.

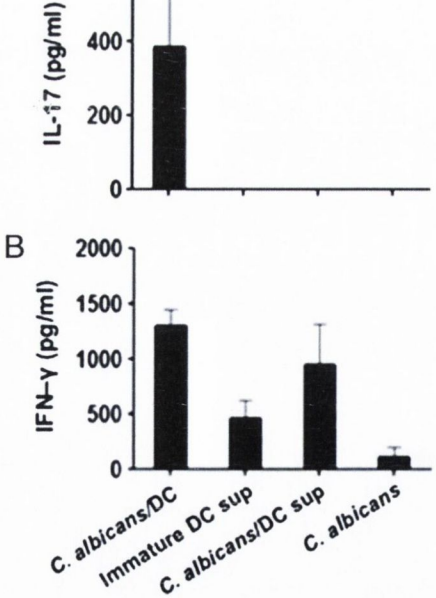

FIGURE 5. IL-17 response to *C. albicans* requires DCs. Vδ1 T cells were cultured with PHA, IL-2, and either DCs that were pulsed overnight with 5×10^6 bodies/ml heat-inactivated *C. albicans*, supernatants of untreated or *C. albicans*–treated DCs, or *C. albicans* alone. Cell supernatants were collected after 7 d for measurement of IL-17 (**A**) and IFN-γ (**B**) release by ELISA. Results are mean ± SEM of three experiments.

Proliferation of Vδ1 T cells in response to C. albicans *requires direct contact with DCs*

We next tested the hypothesis that the IL-17 response of Vδ1 T cells to *C. albicans* requires DCs to induce Vδ1 T cell prolif-

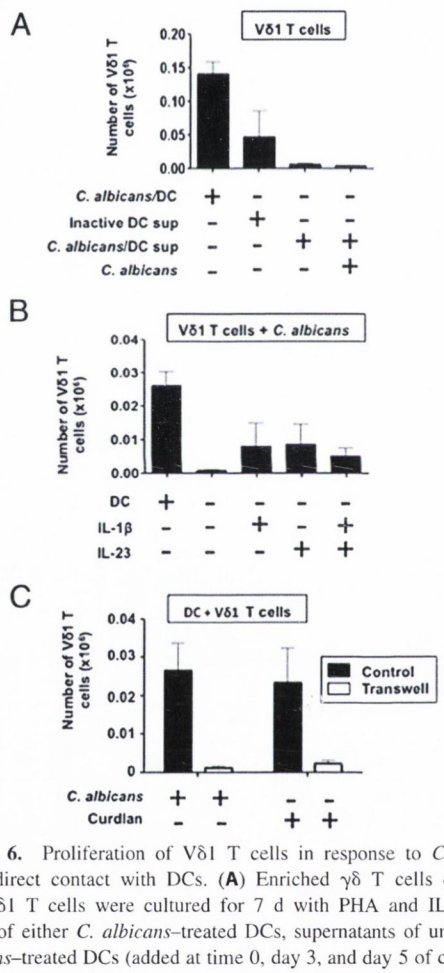

FIGURE 6. Proliferation of Vδ1 T cells in response to *C. albicans* requires direct contact with DCs. (**A**) Enriched γδ T cells containing ~5000 Vδ1 T cells were cultured for 7 d with PHA and IL-2, in the presence of either *C. albicans*–treated DCs, supernatants of untreated or *C. albicans*–treated DCs (added at time 0, day 3, and day 5 of culture), or supernatants of *C. albicans*–treated DCs in the presence of *C. albicans*. The numbers of viable Vδ1 T cells in the cultures were determined by a combination of microscopic cell counting and analysis by flow cytometry. (**B**) Enriched Vδ1 T cells were cultured for 7 d with PHA, IL-2 and *C. albicans* alone or in the presence of either DCs, 10 ng/ml IL-1β, 10 ng/ml IL-23, or IL-1β + IL-23 added at time 0 and day 4 of culture. The numbers of viable Vδ1 T cells in the cultures were determined by a combination of microscopic cell counting and analysis by flow cytometry. (**C**) Enriched Vδ1 T cells were cultured for 7 d with PHA and IL-2 in the presence of *C. albicans*– or curdlan-treated DCs in either a standard flat-bottom, 96-well plate or a transwell plate, which prevented contact between the treated DCs (bottom chamber) and the Vδ1 T cells (transwell insert). The numbers and percentages of Vδ1 cells present in the cultures after 7 d were determined by a combination of microscopic cell counting and flow cytometry. Results show mean ± SEM of three experiments.

eration to generate sufficient numbers of cells releasing IL-17 to allow detection by ELISA. Enriched γδ T cells that contain ~5000 Vδ1 T cells were cultured for 7 d with PHA and IL-2, in the presence of either *C. albicans*–treated DCs, supernatants of untreated or *C. albicans*–treated DCs (added at days 0, 3, and 5 of culture), or supernatants of *C. albicans*–treated DCs in the presence of *C. albicans*. The numbers of viable Vδ1 T cells in the cultures were determined by a combination of microscopic cell counting and analysis by flow cytometry. Fig. 6A shows that, like for IL-17 production, proliferation of Vδ1 T cells required *C. albicans*–treated DC and could not be restored by substituting them with supernatants of untreated or *C. albicans*–treated DCs, even in the presence of *C. albicans*. We also found that recombinant IL-1β, IL-23, or IL-1β+IL-23, added at time 0 and day 4 of culture at concentrations similar to those released by *C. albicans*–treated DCs, failed to restore full proliferation of Vδ1 T cells (Fig. 6B). Furthermore, enriched γδ T cells cultured for 7 d with PHA and IL-2 in the presence of *C. albicans*– or curdlan-treated DCs, but separated using a transwell insert, also failed to proliferate (Fig. 6C). These experiments confirm that direct contact with DCs is required for *C. albicans*–induced proliferation of Vδ1 T cells.

IL-17 production by C. albicans*–treated Vδ1 T cells requires DC-derived IL-23*

Murine γδ T cells can release IL-17 in response to treatment with IL-1 or IL-18 with IL-23 in the absence of TCR stimulation (21, 22). Although IL-23 was not sufficient for inducing proliferation (Fig. 6) or IL-17 production (Fig. 5) by Vδ1 T cells, we investigated whether IL-23 is required for *C. albicans*–induced IL-17 production by Vδ1 T cells. Vδ1 T cells were stimulated with *C. albicans*–pulsed DCs in the presence of PHA and IL-2, and in the presence of a blocking anti–IL-23R mAb or isotype control Ab, which was added at 0 h, 3 h, 17 h, and 3 d post challenge. Cell supernatants were collected after 3, 7, and 14 d and analyzed for cytokine production by ELISA. Fig. 7A shows that blocking IL-23R led to a significant reduction in IL-17, but not IFN-γ, IL-22, or IL-23, release by the Vδ1 T cells. In contrast, blocking IL-23R resulted in an increase in soluble IL-23 levels in the cell supernatants, presumably by preventing IL-23 binding to the Vδ1 T cells. IL-23R blocking did not have any effect on Vδ1 T cell proliferation in response to *C. albicans*–treated DCs (Fig. 7B). This experiment confirms that, although cell contact with DCs is required for *C. albicans*–induced Vδ1 T cell proliferation, IL-23 released by *C. albicans*–treated DCs is a key player in the induction of IL-17 release by Vδ1 T cells.

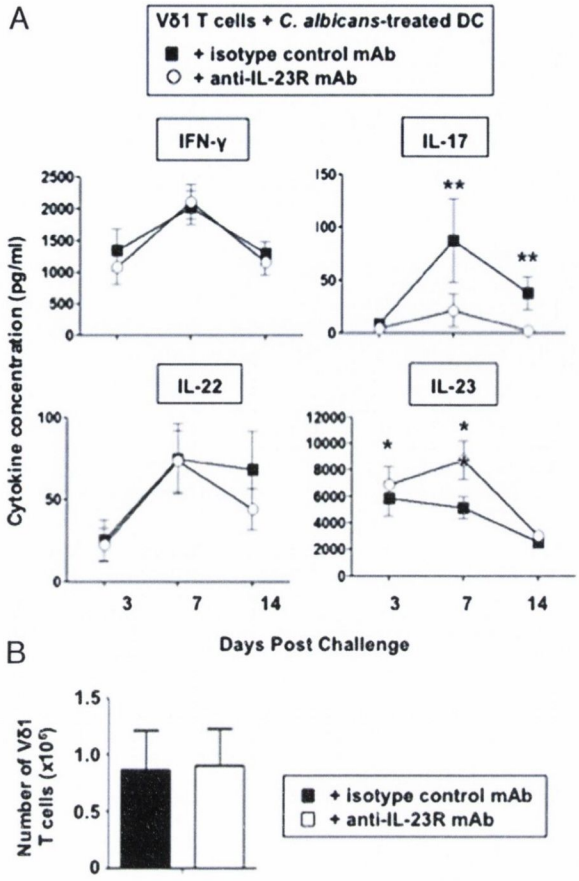

FIGURE 7. IL-17 production by *C. albicans*–treated Vδ1 T cells requires DC-derived IL-23. γδ T cell–enriched PBMCs were cultured for 14 d with DCs that were pulsed overnight with 5×10^6 bodies/ml heat-inactivated *C. albicans* in the presence of PHA and IL-2. A blocking anti–IL-23R mAb (20 μg/ml) or isotype control Ab was added at 0 h, 3 h, 17 h, and 3 d post challenge. Cell supernatants were collected after 3, 7, and 14 d for analysis of cytokine production by ELISA (**A**). Vδ1 T cells numbers were determined after 14 d by a combination of microscopic cell counting flow cytometry (**B**). Data are mean ± SEM of six to eight experiments. $*p < 0.05$, $**p < 0.01$ using paired Wilcoxon tests, comparing Vδ1 T cells stimulated with *C. albicans*–pulsed DCs with anti–IL-23 mAb and isotype control mAb.

Discussion

Vδ1 T cells are thought to play a role in immunity against HIV. They are expanded in the circulation of patients with HIV infection (25–27). They can kill HIV-infected and -uninfected $CD4^+$ T cells (32, 33) and can secrete chemokines that suppress replication of HIV-1 in T cell lines in vitro (34). In this study, we show that *C. albicans* infection is a major determinant of Vδ1 T cell expansion in patients with HIV, with Vδ1 T cells being present in significantly higher numbers in patients with HIV and candidiasis compared with patients with HIV with no evidence of *Candida* infection. Indeed, Vδ1 T cells were only marginally expanded in HIV-infected patients without candidiasis compared with healthy donors. We also confirm a previous report (17) that *C. albicans* drives expansion and IL-17 production by human Vδ1 T cells in vitro. We initially tested several protocols (30, 31) for expanding Vδ1 T cells and found that coculturing PBMCs enriched for γδ T cells with DCs activated with the dectin-1 ligand curdlan in the presence of PHA and IL-2 resulted in the selective expansion of Vδ1 T cells. Subsequently, it was found that substituting curdlan with heat- or ethanol-inactivated *C. albicans*, also known to bind dectin-1 (18–20), could similarly promote Vδ1 T cell expansion in vitro. DCs were required for Vδ1 T cell expansion, and cells could be restimulated every 14 d to induce further expansion and to maintain Vδ1 T cell–rich lines for >6 wk.

Host defense against *C. albicans* requires IL-17 (5, 6) and IL-17–producing cells, including $CD4^+$ Th17 cells (7) and IL-17–secreting innate lymphoid cells (35). γδ T cells have been shown to be an important source of IL-17 in mice (8, 20–22) and humans (17), and IL-17–producing γδ T cells from both species expand in response to *C. albicans* (17, 20). Purified murine γδ T cells can release IL-17 in response to curdlan stimulation in vitro and this response is augmented by addition of IL-23 (20). They can also release IL-17 in response to stimulation with IL-1β and IL-23 or IL-18 and IL-23 in the absence of TCR or dectin-1 stimulation (21, 22). This study using human γδ T cell–enriched PBMCs shows that *C. albicans* induces IL-1β, IL-6, and IL-23 secretion by DCs and downstream IFN-γ and IL-17 secretion by Vδ1 T cells. However, neither IL-1β nor IL-23 alone or together was able to induce expansion of Vδ1 T cells or significant IL-17 secretion as detected by ELISA. Furthermore, supernatants of

C. albicans–stimulated DCs, which contain all cytokines produced by *C. albicans*–treated DCs, failed to induce IL-17 production by Vδ1 T cells. We found that a strong IL-17 response to *C. albicans* was seen only in the presence of DCs and after Vδ1 T cells had proliferated.

Significant and sustained IL-17 production by Vδ1 T cells in response to *C. albicans* required DC-driven proliferation of Vδ1 T cells. However, we found that a proportion of fresh, unexpanded Vδ1 T cells within PBMCs expressed intracellular IL-17 in response to stimulation with *C. albicans*. This occurred within 6 h of stimulation in the absence of DCs and it was not augmented by the presence of DCs, suggesting that *C. albicans* can directly induce IL-17 production by Vδ1 T cells. The β-glucan receptor dectin-1 was expressed by most IL-17–producing Vδ1 T cells expanded with *C. albicans*, adding further support to this notion; however, blocking studies are required to confirm a role for dectin-1 in Vδ1 T cell activation by *C. albicans*. DCs were required to induce proliferation of Vδ1 T cells to generate sufficient numbers of IL-17–secreting cells to detect IL-17 in the supernatants of 7-d cultures of γδ T cell–enriched PBMCs by ELISA. We found that *C. albicans*–treated DCs induced potent proliferation of Vδ1 T cells that expressed markers of Th17 cells, such as CD161 and RORγt, and produced the Th17 cytokines IL-17A and IL-22. However, supernatants of *C. albicans*–treated DCs, recombinant IL-1β, IL-23, or IL-1β together with IL-23 failed to induce Vδ1 T cell proliferation when DCs were not present. Furthermore, separation of DCs and Vδ1 T cells using transwell plates that allow passage of soluble factors, but not cell contact, abrogated *C. albicans*–induced Vδ1 T cell proliferation. Thus, *C. albicans* can directly induce IL-17 production by Vδ1 T cells, but DCs are required for Vδ1 T cell proliferation to generate high numbers of IL-17–producing cells.

IL-23 is required for the induction of IL-17 production by murine γδ T cells (21, 22), by *C. albicans*–stimulated innate lymphoid cells (35), and by human $CD4^+$ T cells (7). We found that DC-derived IL-23 is also a key factor in IL-17 production by Vδ1 T cells in response to *C. albicans*. Blocking the IL-23R potently inhibited IL-17 release, but not proliferation, of Vδ1 T cells. Taken together, our data show that IL-23 is an important factor for IL-17 production by *C. albicans*–stimulated human Vδ1 T cells, and that contact with DCs plays a key role in expanding *C. albicans*–responsive Vδ1 T cells to numbers sufficient to release significant amounts of IL-17.

Therapeutic inhibition of the IL-23/IL-17 axis is currently under consideration for inflammatory and autoimmune diseases (36, 37). In contrast, enhancement of these pathways may be beneficial for the treatment of *C. albicans* infection. The IL-23/IL-17 axis is also thought to contribute to immunity against HIV; therefore, stimulation of these pathways may also be of particular benefit to patients coinfected with HIV and *C. albicans* (38–40). HIV infects and kills IL-17–secreting $CD4^+$ T cells, but Vδ1 T cells are expanded in patients with HIV infection (25–27). Therefore, Vδ1 T cells, which are currently under investigation as effectors in tumor immunotherapy (41), have potential for restoring IL-17 responses in patients with HIV/AIDS.

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Disclosures

The authors have no financial conflicts of interest.

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Investigations of *Aspergillus* Infection in Cystic Fibrosis

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Introduction: *Aspergillus fumigatus* is a ubiquitous saprophytic fungus that is a pathogen in principally immunocompromised hosts. It is the most common fungal pathogen in Cystic Fibrosis (CF), most often associated with Allergic Bronchopulmonary Aspergillosis (ABPA). However, CF patients are also commonly colonised with *A. fumigatus* without displaying any symptoms of ABPA. There is a wide array of antifungal drugs available to treat *A. fumigatus* infections in CF including the triazoles but drug resistance has been reported in a number of countries. No triazole resistance in clinical *A. fumigatus* isolates has been reported in Ireland to date, however antifungal susceptibility testing is not routinely performed in Irish diagnostic microbiology laboratories. The epidemiology of *A. fumigatus* and patterns of colonisation in Irish CF patients is unclear. Furthermore the virulence of isolates that persist in the CF airways over time is unknown. What impact asymptomatic colonisation has on the CF airways over time is also not fully understood.

Methods: The bioburden of *A. fumigatus* in an Irish CF cohort (n=13), who were asymptomatically colonised with the fungus was examined by standard culture and qPCR at pre- and post-itraconazole treatment time points. *A. fumigatus* isolates collected from these patients and additional isolates collected from other CF patients from a total of four CF centres in Ireland were identified to the species level by PCR and sequencing of the *ITS* region. The epidemiology of these *A. fumigatus* isolates was investigated using the *A. fumigatus* specific STR*Af* genotyping assay [1]. Antifungal drug susceptibility of all isolates collected was established using the commercial Trek Sensititre susceptibility system. The virulence of different genotypes of *A. fumigatus* was determined using the *Galleria mellonella* insect model. The ability of the *A. fumigatus* CF isolates to open epithelial cell tight junctions in both human bronchial epithelial cells (HBEs) and cystic fibrosis bronchial epithelial cells (CFBEs) was determined using transepithelial resistance (TER). Changes in concentration and distribution of tight junction proteins zonula occludens-1 (ZO-1) and Junctional adhesion molecule-A (JAM-A) in response to *A. fumigatus* was determined by western blot analysis and confocal microscopy.

Results and Conclusions: *Aspergillus* bioburden, measured by CFU counts (CFU/g) and qPCR, was significantly reduced following itraconazole treatment. These results demonstrate that itraconazole treatment effectively reduces *Aspergillus* burden in the CF airways. All isolates collected were confirmed as *A. fumigatus* with a 99-100% identity. Within our CF patient isolates no antifungal drug resistance was observed for a panel of nine antifungal drugs including three echinocandins and four triazoles (even those isolates collected following itraconazole treatment) with the exception of fluconazole which is known to be inactive against *A. fumigatus*. Two patterns of airway colonisation were observed within our CF patients; persistent colonisation (with an indistinguishable genotype in $\geq$2 consecutive samples) and non-persistent colonisation (with distinguishable genotypes in consecutive samples), furthermore patients sharing an indistinguishable isolate was also observed. Analysis of multiple colonies per sample showed examples of some CF patients being colonised with a unique genotype, while others were colonised with several genotypes. In the *G. mellonella* model different *A. fumigatus* genotypes caused varied rates of mortality however no significant difference could be found between representative persistent and non-persistent colonisers. However, virulence of a persistent isolate over time demonstrated that later isolates were more virulent than earlier ones in the model, suggesting an increase of virulence or adaptation over time in the patient. *A. fumigatus* conidia and culture supernatants (CSNs) were able to cause significant disruption to tight junctions of HBEs and CFBEs. CSNs of persistent and non-persistent isolates produced different rates of disruption of tight junction integrity of the respiratory epithelium and later CSNs from the persistent isolate also opened tight junctions more readily. This suggested that differences between the persistent and non-persistent colonising isolates exist. CSNs from longer culturing times caused the greatest disruption of tight junction integrity and breakdown of tight junction proteins ZO-1 and JAM-A, which is in part due to gliotoxin. These results could have implications for the human lung, particularly the CF lung where isolates may persist over time while remaining viable.