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Molecular mechanisms of Aspergillosis:

contribution and impact of inter- and intrastrain Aspergillus fumigatus variations on the influence of cyclic AMP expression in host airway epithelial cells

LISA-MARIE SMALE, BSC
Department of Molecular Pharmacology, University of Groningen, The Netherlands
Department of Respiratory Medicine, Hunter Medical Research Institute, University of Newcastle, Australia

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under supervision of Jane Read, prof dr. Darryl Knight and prof dr. Martina Schmidt

Abstract

Aspergillus (A.) fumigatus relies on host immune status and comorbidities to cause a range of fungal syndromes, with Invasive Aspergillosis (IA) as a worst-case outcome due to mortality rates of 50-90%. Key features in IA include complex pathogen-host interactions, causing dysfunctional conidial internalisation in airway epithelial cells (AECs). Host cyclic adenosine monophosphate (cAMP) regulates similar autophagic pathways, and is often exploited by microbes. This thesis aims to clarify the participation of cAMP in the pathophysiology of IA via determining the effect of *A. fumigatus,* and its inter- and intrastrain variations, on cAMP production of AECs.

Intracellular cAMP expression was measured in cell lysate via a competitive cAMP ELISA assay with 3-isobutyl-1-methylxanthine (IBMX) added to obtain sufficient baseline cAMP recovery. Resting conidia (RC), swollen conidia (SC) and ultraviolet (UV)-inactivated conidia of *A. fumigatus* B5233 and three clinical isolates (P1ms, P1ms and P2cs) were used to infect AECs. RC of all strains, at a multiplicity of infection (MOI) of 10, decreased cAMP production with an average of approximately 60%, but no differences were observed between effects of the clinical isolates. Intra-strain differences were found for B5233 and P2cs only, with RC being more potent than SC.

This thesis suggests AEC cAMP signalling as a new potential target in IA, because it indicates that *A. fumigatus* conidia downregulate AEC cAMP. Furthermore, it indicates that fungal swelling coordinates this process, while interstrain differences only affect the dynamics of germination processes. Continued efforts need to make these pathways more accessible for development of treatment strategies.

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1. Introduction

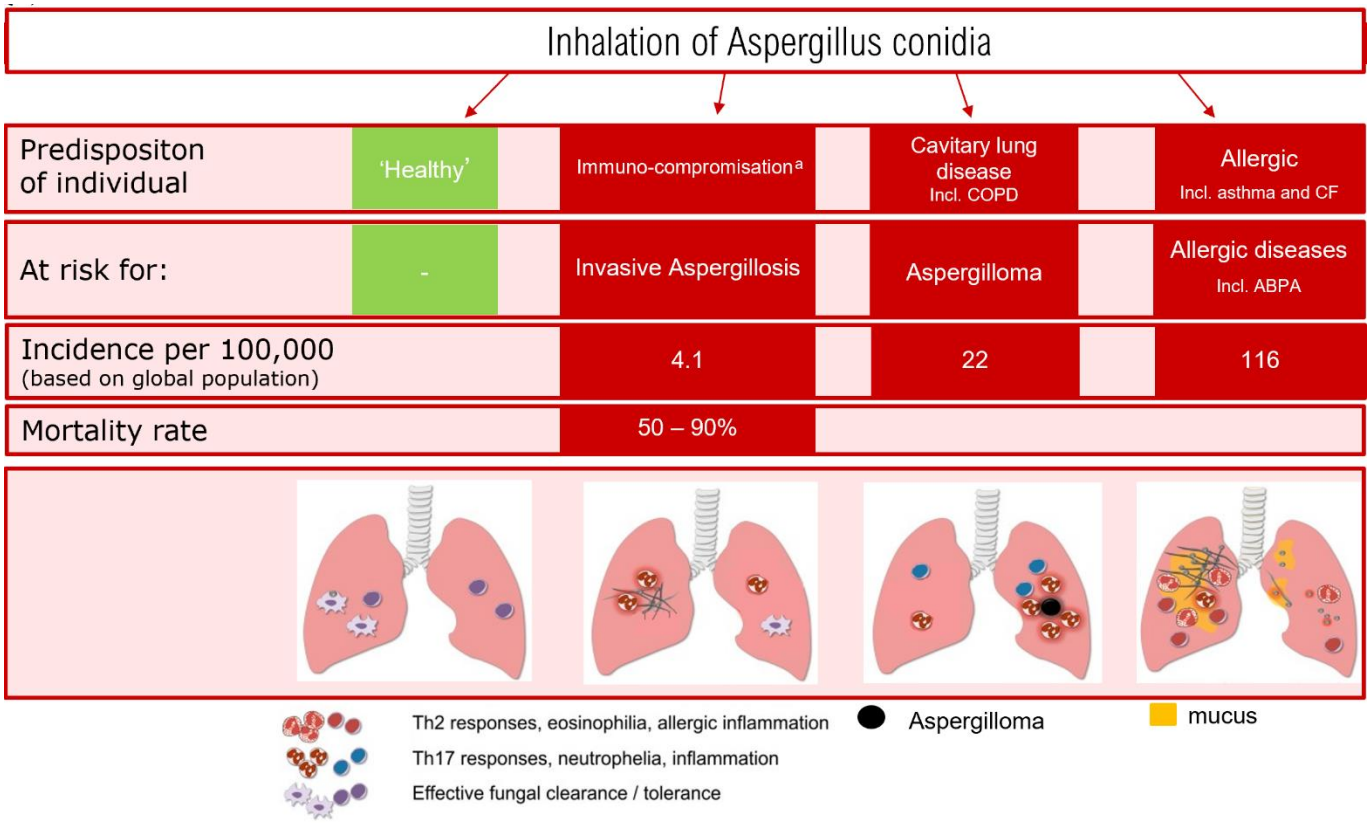
Invasive fungal infections have emerged in the last three decades as an important cause of human disease with global mortality rates of 1.6 million patients each year[1, 2]. Effective prevention and treatment of this ever-increasing range of invasive fungal infections is at risk due to an growing amount of anti-microbial resistance (AMR)[3]. *Aspergillus (A.) fumigatus* is a well-known pathogenic fungus, because it is the main agent responsible for pulmonal fungal diseases and one of the most life-threatening fungi, being responsible for 90% of all cases of Invasive Aspergillosis (IA)[2]. Despite the fact that an average person inhales up to several hundred *A. fumigatus* infectious particles each day, disease only occurs when the host pulmonary immune response is either incompetent or overcompensated[4], revealing the complex balance between host (pulmonary) defence and *A. fumigatus'* potential pathogenic character. In order to enable targeted treatment strategies, this thesis will contribute to a more detailed understanding of the host-pathogen processes underlying fungal clearance, along with divergences driving fungal pathologies.

Patient burden

Although interaction of inhaled fungal particles with the lung is a unifying feature in all aspergilloses, patient outcome varies dramatically as a consequence of immune status of the host. In this way *Aspergillus* is able to cause a spectrum of fungal syndromes in humans ranging from local, mild allergic reactions to fatal invasive mycoses (Figure 1)[2,5,6].

In allergic bronchopulmonary aspergillosis (ABPA) an eosinophilic response is caused by *Aspergillus* conidia, usually affecting asthmatic or cystic fibrosis (CF) patients. Consequently, bronchospasm and mucus build-up results in coughing, breathing difficulties, airway obstruction and potentially bronchiectasis. However, despite a global incidence of 4.1 cases/per 100,000, IA is the worst-case outcome of Aspergillosis, because it typically results in long-term hospitalisation, intensive antifungal therapies and high mortality rates[2]. Furthermore, the incidence of IA is likely to increase due to the rise of people at risk. This encompasses almost three million Human Immunodeficiency Virus infection and Acquired Immune Deficiency Syndrome (HIV/AIDS) patients with limited access to antiretroviral therapies[7]. Other susceptible patients include the increasing amount of individuals with immunosuppressive therapies for autoimmune diseases, organ transplantation and neoplastic diseases[1,8]. Recently, a cumulative number of case studies have demonstrated that non-classically immunocompromised patients can be at risk for IA too, due to an *Aspergillus* superinfection provoked by severe Influenza[9–13]. Intriguingly, in 28% of all 128 published cases of Influenza-associated Aspergillosis (IAA) no underlying medical condition was found, and in 87% of the cases the H1N1 virus was responsible[9]. This strongly increases the reach and burden of Aspergillosis, making an adequate diagnosis and treatment of these fungal syndromes more crucial than ever[14].

IA presents itself with complaints such as fever, cough, dyspnea, chest discomfort and hemoptysis[15]. Given that these symptoms are non-specific, positive histopathologic or cytologic evidence is required to diagnose IA[15]. However, culture methods have some major drawbacks; for example the low sensitivity and time required to acquire results[16]. If diagnosed and treated, IA still carries a 50% overall mortality rate, while IA with a missed or delayed diagnosis proves to be nearly 100% fatal[1]. Since timing and accuracy of diagnosis are considered vital to improve patient outcome, non-culture-based diagnostic methods with improved turnaround time and a higher sensitivity should be developed[17]. This further emphasizes the importance of identifying the molecular processes underlying IA, with the aim of determining adequate targets for diagnosis and treatment.



[a] greatest risk factors: neutropenia, allogenic haematopoetic, stem cell or organ transplant, haematological malignancy, cytotoxic cancer chemotherapy, CGD, HIV/AIDS, high-dose treatment with corticosteroids.

Figure 1: Primary manifestations of Aspergillosis, including risk factors, incidence and mortality rate.

COPD = chronic obstructive pulmonary disease, CF = cystic fibrosis, ABPA = allergic broncho-pulmonary aspergillosis. CGD = Chronic granulomatous disease, HIV/AIDS = Human Immunodeficiency Virus infection and Acquired Immune Deficiency Syndrome.
Incidence rates are obtained from *Bongomin et al.* (2017)[2], and illustrations are obtained from *Dewi et al.* (2017)[18].

Besides quality of diagnosis, early and adequate antifungal treatment is of primary importance for surviving IA[17,19]. Triazole antifungal agents, such as itraconazole, voriconazole, isavuconazole and posaconazole, are the first-choice treatment for all fungal syndromes[14,17]. However, due to large-scale environmental use of these azoles, there is a globally increasing appearance of resistant *A. fumigatus* strains with a tripling of the mortality rate in IA patients as a consequence[14,17,20]. Currently, already 6.7% - 16.3% of the diagnosed *A. fumigatus* infections in Dutch hospitals concern azole-resistant strains[21]. Furthermore, azole resistant *A. fumigatus* strains are reported in the majority of Europe, together with Turkey, Iran, Kuwait, Japan, China, Taiwan, Pakistan, India, Tanzania, Australia, United States and Colombia[14]. However, besides mutations of Cyp51A, a gene that is targeted by azoles, little is known about resistance mechanisms, which makes diagnostics and treatment for these infections even more difficult[20].

Internalisation of *A. fumigatus*

The airborne spores of *A. fumigatus*, which form its infectious particles, are called conidia[22]. These conidia are of such a small size that they are able to circumvent mucociliary clearance and penetrate lung alveoli (Figure 2)[23]. If these conidia are not cleared by pulmonary phagocytic cells, they undergo multiple morphological shifts over time. When not halted by an adequate immune response a period of conidial swelling (isotropic growth) occurs, followed by the outgrowth of elongated, multicellular, filamentous structures, called hyphae[24,25]. During this germination, several pulmonary structures, including airway epithelial cells (AECs), extracellular Matrix (ECM) and any uncovered basement membrane components, are exposed to numerous fungal cell surface-associated and secreted factors (Figure 2). This causes adhesion of fungal structures to host cell components, which further enables fungal growth, fungal persistence and pathogenesis[25–29].

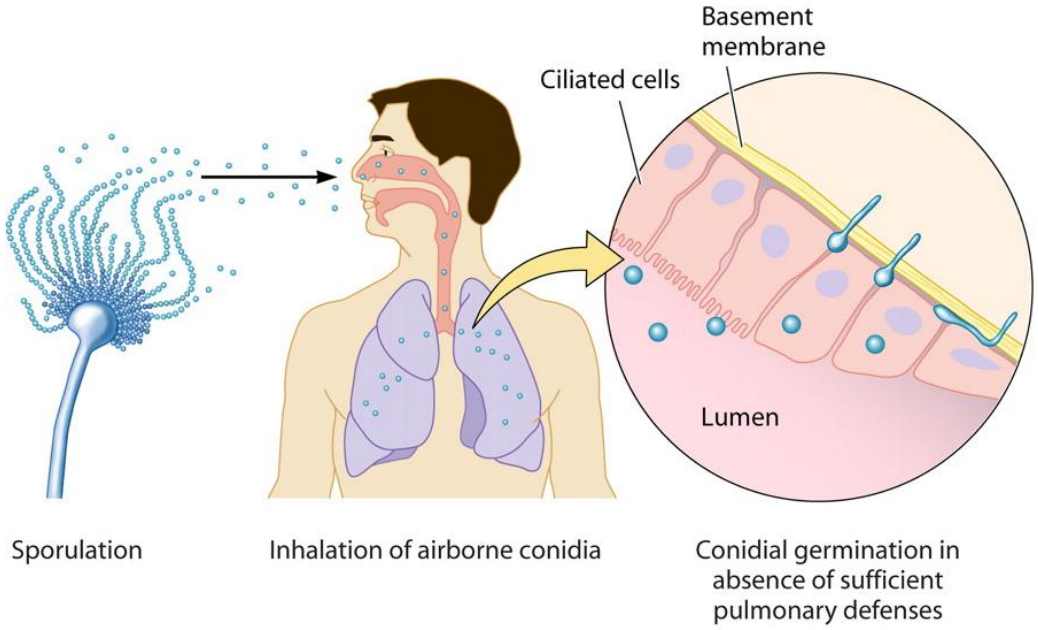


Figure 2: Interactions between *Aspergillus* (*A.*) *fumigatus* and Airway epithelial cells (AECs).

A. fumigatus spreads via its airborne spores called conidia. Resting Conidia (RC) survive extreme conditions, and can be inhaled by the human host. Due to their small diameter, RC can reach the lower respiratory tract. If not halted by a functional immune response, RC are allowed to germinate in pulmonary lumen. Morphological changes during fungal swelling are causing diverse interactions with pulmonary structures, such as epithelial cells and basement membrane components.
Illustration obtained from *Dagenais and Keller (2009)*[30]

Since basement membrane components are not accessible in healthy lungs and type I AECs cover up to 95% of the pulmonal surface[31], it is most likely that conidia first encounter type I AECs. After binding to AECs, approximately 30%-50% of conidia are internalised leading to fungal intraphagosomal occupation and generally resulting in conidial degradation via phagolysosomal acidification[32–34]. Nevertheless, recent studies have shown that 3% of internalised conidia remain viable after 24 hours, which allows 34% of these surviving conidia to germinate into extracellular space[32,33,35]. Within this context, epithelial cells serve as a crucial defence to neutralise *A. fumigatus* exposure in healthy people. Yet, in immunocompromised individuals, internalisation into epithelial cells permits fungal entry and capacity to elicit fungal damage. A similar trend has been reported for other harmful pathogens such as the Herpes Simplex Viruses (HSVs)[36], HIV[37], the malaria parasite *Plasmodium (P.) falciparum*[38,39] and bacteria such as *Listeria Monocytes*[40]. A primary goal of this thesis is to study the molecular mechanisms underlying internalisation and AEC behaviour during fungal infection, thereby discovering possibilities to block this pathway and prevent these hazardous microbes from spreading throughout the body.

cyclic adenosine 3'-5' monophosphate (cAMP)

In order to survive, all organisms must sense and respond to changes in environmental circumstances. An important instrument to translate these external signals into cellular processes are second messengers such as cAMP[41]. cAMP is highly conserved in diverse lifeforms, including mammals, fungi, protozoa, bacteria and viruses, indicating its importance[42]. Accordingly, cAMP plays a critical role in essential biological processes, such as cell differentiation and proliferation, via interactions with its downstream receptors including protein kinase A (PKA) and the more recently identified exchange protein directly activated by cAMP (EPAC)[41].

In the human lungs, cAMP is well-known for its role in smooth muscle relaxation after stimulation with β_2-adrenoreceptor agonists. This stimulation effectuates airway smooth muscle contraction and relieves dyspnoea in asthma and to a lesser degree in chronic obstructive pulmonary disease (COPD) patients[43]. However, cAMP is also crucial in the pathogenesis of pathogen-host interactions[42,44]. Besides the fact that *A. fumigatus* relies on cAMP for its virulence and resistance to drugs[42,45,46], cAMP is essential in the host's mechanisms of fungal clearance, including innate immune responses and autophagy[44,47]. Additionally, recent research has shown that extracellular microbes utilise host's dependence on cAMP by elevating it, causing PKA and EPAC to suppress the innate immune functions of phagocytes, including the generation of inflammatory mediators, phagocytosis and intracellular elimination of ingested pathogen (Figure 3)[44]. The inhibition on phagocytosis is established via downregulation of actin assembly in cells, preventing them from making cytoskeletal changes essential in cellular microbial uptake[48].

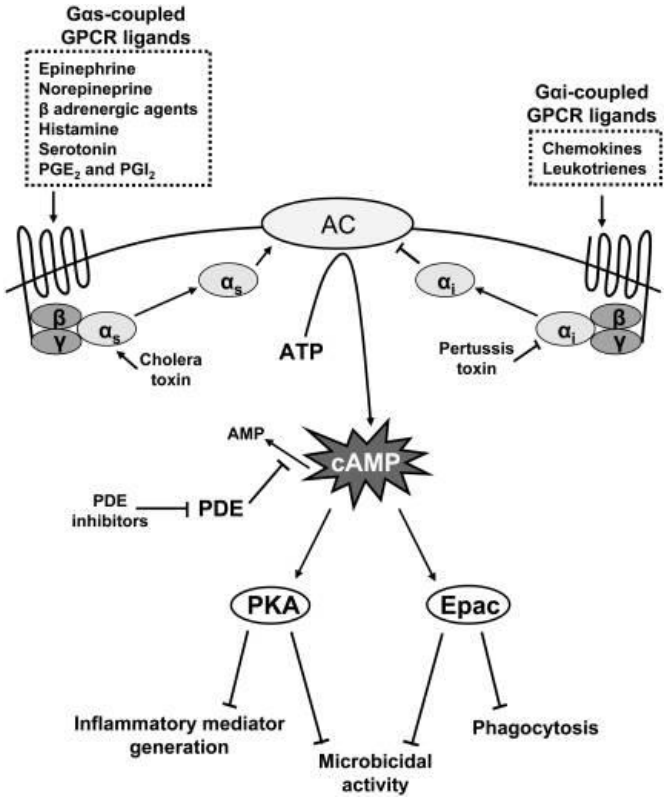


Figure 3: Influences on regulation of cyclic adenosine Monophosphate (cAMP) by antimicrobial actions[44]

AdenylylCyclase (AC) is responsible for converting Adenosine Triphosphate (ATP) to cAMP. This process is influenced by two G Protein–Coupled Receptors (GPCR). After receptor activation by a binding agonist, a conformational change allows liberation of the Ga subunit from the βγ-subunits. Depending on the type of GPCR, the Ga subunit can either stimulate (Gas) or inhibit (Gai) AC. cAMP has two downstream effectors: Protein Kinase A (PKA) and Exchange Protein Directly Activated by cAMP (EPAC), with their own effector functions. Phosphodiesterases (PDE) are responsible for degrading cAMP and can be influenced by PDE inhibitors.

In contrast, our group has shown that after long term exposure (hours) dihydroxynaphtalene (DHN)-melanin, a membrane component of *A. fumigatus,* decreases host cAMP expression, while increasing EPAC and leaving PKA unchanged [49]. Interestingly, *Almeida et al.* demonstrated that a higher fungal expression of DHN-melanin correlates with more virulent fungal strains as melanin is associated with fungal survival under harsh environmental and parasitic conditions[50]. Furthermore, DHN-melanin is associated with promotion of *A. fumigatus* viability in phagolysosomes by preventing acidification[51,52]. Interestingly, it also implies that host cAMP signalling facilitates elusion of the immune system by promoting cellular uptake of *A. fumigatus* in AECs, potentially followed by intraphagosomal escape. This hypothesis is further supported by *Shen et al.*, who showed that elevation of intracellular cAMP prevented invasion of *Escherichia (E.) coli* in bladder epithelial cells via cAMP-induced inhibition of actin polymerisation, a crucial step required for internalisation and invasion[53]. Moreover, increased intracellular cAMP not only protects AECs from propagation of Respiratory Syncytial Virus (RSV), but also prevents entry of RSV in AECs by PKA related effects on actin dynamics[54].

These findings make cAMP signalling pathways an interesting target for preventing invasive microbial infections, but before we can establish this, more information about these dynamic pathogen-host interactions causing cAMP alterations during fungal infection is required.

Research aims

Several lines of research indicate that in order to bypass the innate immune system, host cAMP is influenced by different microbial factors, including DHN-melanin. Although these studies are a breakthrough in the research of internalisation, the effect of *A. fumigatus* resting conidia (RC) on AEC cAMP expression has yet to be validated under more realistic exposure conditions and, moreover, confirmed by functional studies to unravel exact mechanisms. Furthermore, what is not yet clear is the impact of different morphological fungal structures on the change in cAMP levels, caused by morphologic-specific virulence factors during germination and inter-fungal variances. This information might provide insight into the timeframe of internalisation and IA after fungal infection, but might also increase our understanding of the highly varied individual pathogenesis and disposition of fungal syndromes. Therefore, the research question of this thesis is: 'What is the influence of morphological fungal structures and inter-fungal variances on alterations in host cAMP expression during infection with *A. fumigatus*?'

We hypothesise that *Aspergillus fumigatus* conidia inhibits cAMP production in AECs to promote internalisation. Moreover, we expect that more virulent strains have a greater inhibitory effect, and RC are more potent than swollen conidia (SC). To investigate this, the focus of this project will be measuring cAMP expression in an alveolar epithelial cell line after fungal infection. Then, to extend our knowledge on a functional level, this infection process will be repeated with SC. Finally, the infection model will be iterated with different clinical isolates from *A. fumigatus,* derived from COPD and IAA patients.

This thesis will contribute to a more detailed understanding of the influence of cAMP signalling on clinical aspects of fungal infection, with the intention of confirming cAMP signalling as a potential target in treating and diagnosing Aspergillosis. Thus, this study may also provide insight into host processes during fungal interactions, thereby addressing a neglected part of aspergillosis research, which may be crucial for developing new diagnostic strategies.

2. Methods

A. fumigatus strains and AEC cell line

Characteristics of the four different *A. fumigatus* strains used within this research are summarised in Table 1. All strains were inoculated on Sabouraud Dextrose Agar (SDA) plates, supplemented with Chloramphenicol and Gentamicin (ThermoFisher, PP2029).

A. fumigatus strain B5233, a hypervirulent and frequently-studied clinical isolate from a patient with fatal IA, was kindly provided by the Institute for Disease Control & Prevention of the Academy of Military Medical Sciences (AMMS), China. Its susceptibility to anti-fungal drugs was confirmed by agar-based gradient technique for quantitative antifungal susceptibility E-test (AB BIODISK, Solna, Sweden).

The University Medical Centre Groningen (UMCG) obtained three clinical isolates from sputum of IA patients with known co-morbidities. Fungal identification was confirmed by microscopic morphological description and Sanger sequencing of the internal transcribed spacer (ITS) region. P1ms and P1mr were extracted from a patient with Influenza A H1N1 and no other relevant underlying disease. Although these mixed infection isolates were obtained from the same patient, unpublished studies by Miss. F. Puértolas Balint illustrated through a comparative genomic analysis that both isolates are genetically dissimilar, leading to differences in susceptibility to the following antifungal agents: Itraconazole, Isaconazole, Voriconazole and Posaconazole. The third clinical isolate (P2cs) was derived from a patient diagnosed with HIV in 2002 and COPD in 2005. This patient was treated with both antiviral therapy (emtricitabine/tenofovir alafenamide/rilpitvirine) and standard COPD treatments (Fluticason, cotrimoxazol, formeterol and ipratropium). Despite positive *A. fumigatus* cultivation during COPD exacerbations in 2016, a diagnosis of chronic pulmonary aspergillosis was discarded due to the absence of fungal load in a chest imaging study.

Table 1: Characteristics of Aspergillus fumigatus strains utilised.
** Resistance was measured via agar-based gradient technique for quantitative antifungal susceptibility E-test (AB BIODISK, Solna, Sweden) and expressed according to in-vitro EUCAST broth microdi lution reference cut-off values.*
*** tested with the agar-based method VIPcheckTM test (Nijmegen, The Netherlands).*
ODEFSEY: anti-viral treatment with emtricitabine/tenofovir and alafenamide/rilpitvirine

Origin	*Strain*	*Co-morbidities*	*Susceptibility*	*Additional information*
Disease Control & Prevention of the Academy of Military Medical Sciences (AMMS), China	B5233		Susceptible*	Clinical reference isolate
University Medical Centre Groningen (UMCG), The Netherlands	P1ms	Influenza A H1N1	Susceptible*	Obtained 2 days after hospitalisation (before anti-fungal treatment).
	P1mr	Influenza A H1N1	Resistant: Itraconazole, Posaconazole, Isavuconazole * Intermediate: Voriconazole *	Obtained 9 days later than P1.ms (during anti-fungal treatment).
	P2cs	HIV, COPD	Susceptible **	Treated with ODEFSEY, Fluticason, cotrimoxazol, formeterol and ipratropium.

Preparation of conidial suspension

After five days of fungal growth at 37°C, *A. fumigatus* conidia were harvested from SDA-plates by using sterile Dulbecco's Phosphate-Buffered Saline (PBS) (Merck, D8537-500ML) supplemented with 0.1% Tween20 (Sigma, P9416-50ML). Subsequently, hyphae were extracted from fungal suspensions by filtering twice through a 40µm nylon cell strainer (Sigma, CLS431750). The remaining supernatant was washed once with PBS. Dulbecco's Modified Eagle's Medium (DMEM) low glucose GlutaMAX from Gibco (Thermo Fisher, 10566016) supplemented with 20mM HydroxyEthyl PiperazineEthaneSulfonic acid (HEPES), 2% Penicillin/Streptomycin (P/S) and 0.4% Fetal Bovine Serum (FBS) was used to enumerate conidia to a concentration of $1x10^8$ conidia/mL. Unless otherwise indicated, the cells were frozen down to -80°C with the aid of a Mr. Frosty™ Freezing Container containing Isopropanol before storage at -80°C for a maximum period of three months.

For each infection, an inactivated control of the same conidia was prepared 24 hours prior to infection via five hours of ultraviolet (UV) irradiation. These control samples were stored at 4°C for a maximum period of 24 hours, plated out on SDA-plates and incubated overnight to check for the absence of fungal growth.

Cell culture and infection

The human alveolar type II epithelial adenocarcinoma cell line, A549, at passage number 20–40 was used to study the effects of *A. fumigatus* infection on AECs. A549 cells were seeded in 90mm petri dishes in the DMEM medium described above supplemented with 10% FBS. Unless otherwise stated, a cell quantity of 3×10^6 cells per petri dish was used. After 24 hours cells reached a confluence of >80% and were incubated in DMEM supplemented with 0.4% FBS (minimal medium) overnight. Where indicated, the phosphodiesterase inhibitor (PDEi) 3-isobutyl-1-methylxanthine (IBMX) was used to prevent degradation of cAMP during infection and cell lysis. To establish this effect, 30 minutes incubation with 0.5mM IBMX in 0.1% dimethylsulfoxide (DMSO)/minimal medium was used before infection and cell lysis. Controls for the influence of solvents were included.

Prior to infection, conidial suspensions were incubated at 37°C either for one hour to obtain RC or for four hours to obtain SC. Subsequently, A549 cells were infected for one hour with conidial suspension at a multiplicity of infection (MOI) ranging from 1-100, and incubated at 37°C 5% CO_2. After washing the cells once with PBS, cell lysates was prepared with the aid of the cAMP Parameter Assay Kit (In vitro technologies, KGE002B).

Measurement of Intracellular cAMP

Intracellular cAMP levels of cell lysates were measured via a competitive assay, utilising the cAMP Parameter Enzyme-Linked Immuno Sorbent Assay (ELISA) Assay Kit. Samples were treated according to manufacturer's protocol and supplemented with high, medium and low cAMP controls from the parameter Immunoassay Control Set 764 for cAMP (In vitro technologies, QC52). Briefly, a microplate was coated with goat anti-mouse polyclonal antibody, before incubated with primary antibody solution for one hour at 500rpm. After washing, cAMP conjugated to horseradish peroxidase with red dye and preservatives was added. Standard, samples and controls were then pipetted on the plate within fifteen minutes of adding cAMP conjugate and the plate was incubated at room temperature on a shaker set at 500rpm. Afterwards, an equal mixture of stabilised hydrogen peroxide with stabilised chromogen (tetramethylbenzidine) was added and the plate kept in the dark for 30 minutes, before 2N sulfuric acid was added to stop the reaction. Absorption was measured at 450nm, and corrected for absorption at 570nm and non-specific binding.

Statistical Analysis

Unless otherwise indicated, the data shown is based on 3–5 independent experiments performed in duplicate. For analysis of multiple comparison experiments a non-parametric one-way ANOVA test is performed (Kruskal-Wallis analysis). The Mann-Whitney U-test was used to test single comparisons. Values of $P < 0.05$ were considered statistically significant.

3. Results

Several lines of research indicate that microorganisms, such as *A. fumigatus,* influence host cAMP signalling pathways to control autophagic processes. This study set out to assess the influence of morphological fungal structures and inter-fungal variances on alterations in host AEC intracellular cAMP expression during infection with *A. fumigatus.* In order to test this, cAMP expression in A549 cells was measured after infection with conidia in different morphological stages and conidia originating from different strains.

Optimisation and validation of cAMP assay method

To obtain measurable and stable amounts of cAMP, the first set of experiments was designed to optimise procedures for the competitive intracellular cAMP ELISA assay. This included validation of cell lysate storage conditions, together with optimising baseline cAMP levels in A549 cells via variation of cell numbers and pre-incubation with IBMX.

Initially, cAMP yield was measured after seeding different cell numbers of A549 cells. The sensitivity of this method showed a cell number-dependent effect (Figure 4). Accumulation of cAMP in 5 x 10^5 cells was insufficient, whereas when a cell number of 3 x 10^6 was used, the mean cAMP recovery was 3.03 ± 0.33pmol/mL (Figure 4).

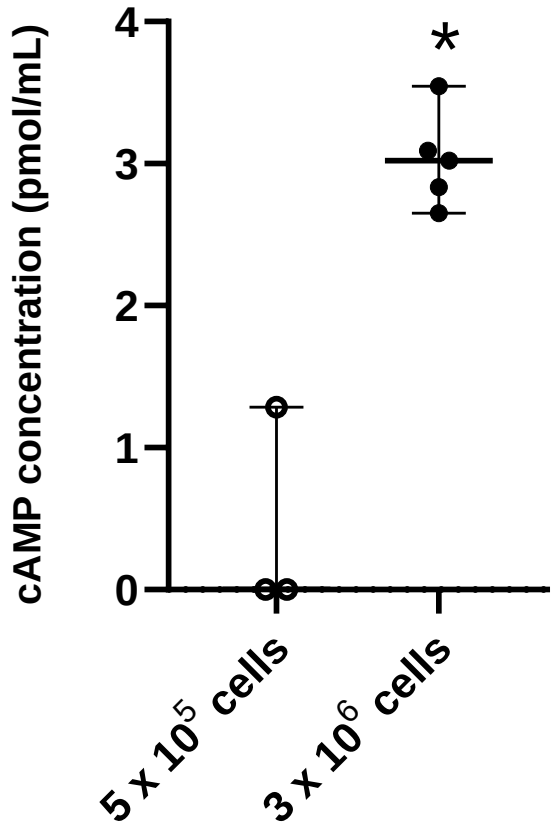


Figure 4: A549 cell number optimisation of cyclic Adenosine Monophosphate (cAMP) competitive ELISA assay.

*A549 cells were seeded in the stated quantity, and were grown to confluence for 24 hours, before medium was changed to minimal medium. After overnight incubation, cells were lysed and intracellular cAMP was detected by a competitive ELISA assay. Data is displayed as median and 95% confidence (n = 3–5). *P < 0.05 (Mann-Whitney U-test).*

However, recoveries smaller than 3.75pmol/mL cAMP have to be interpreted with caution, as the kit requires extrapolation of the standard curve for these values. Therefore, in order to further optimise the method, IBMX was utilised to increase and stabilise baseline cAMP levels. As shown in Figure 5, no differences between blank and vehicle conditions were found, and statistics did not possess the power to prove significant differences between blank and IBMX. However, pre-incubation with 0.5mM IBMX significantly increased cAMP expression to 6.45pmol cAMP/mL compared to the vehicle control. Moreover, inter-experimental variation decreased as a result of IBMX treatment, as shown by the narrower error bars. Similar trends were observed in samples that were stored for one day or seven days at -20°C. Due to higher cAMP baseline levels and increased assay precision, 0.5mM IBMX pre-incubation is used in future experiments.

Furthermore, storage of A549 cell lysate at -20°C was tested and validated for up to seven days. Interestingly, no significant differences in cAMP levels after one day and seven days of storage at -20°C were identified when compared to cAMP levels of post lysis samples (Figure 5). The addition of 0.1% DMSO and 0.5mM IBMX did not influence this trend. Hence, all storage conditions were used during the remainder of the experiment.

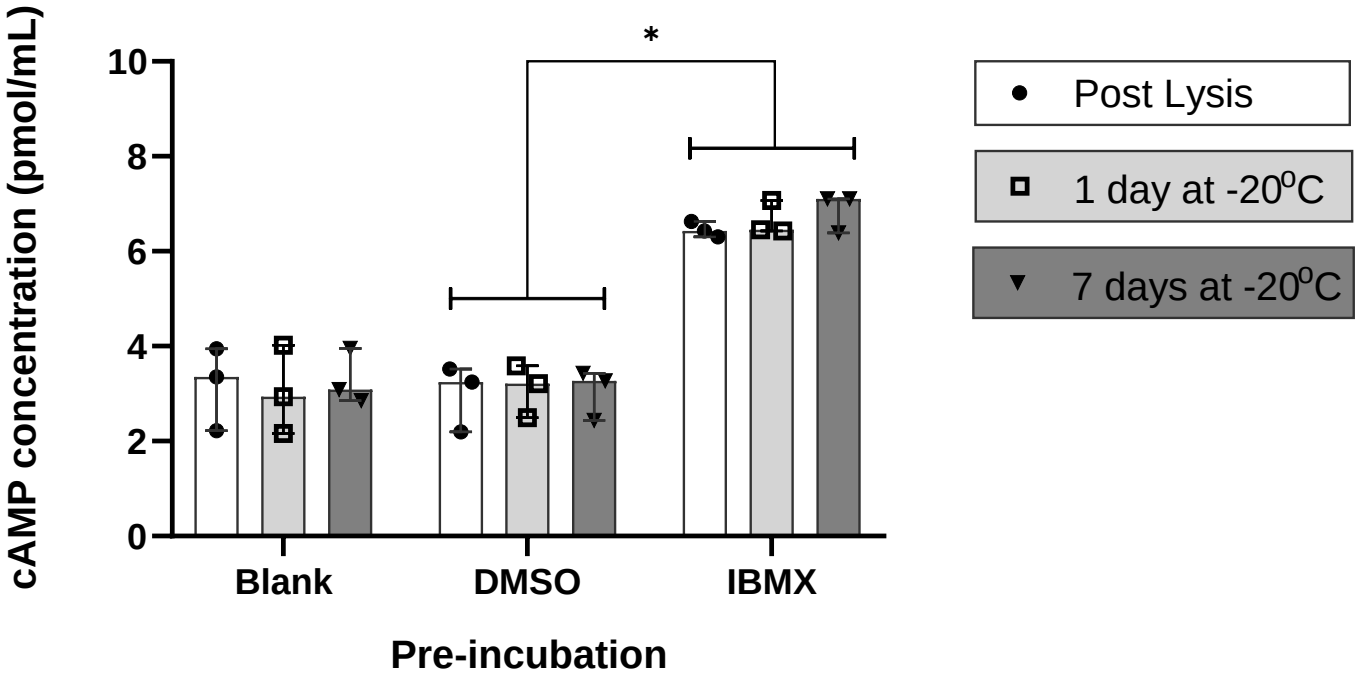


Figure 5: Optimisation of storage conditions and preparation of cell lysate for cyclic Adenosine Monophosphate (cAMP) competitive ELISA assay.

blank = minimal medium, DMSO = 0.1% Dimethylsulfoxide (DMSO) / minimal medium, IBMX = 0.5 mM 3-isobutyl-1-methylxanthine in 0.1% DMSO / minimal medium.

*3 x 10^6 A549 cells were grown to confluence in 24 hours, before medium was changed to minimal medium for overnight incubation. After 30 minute pre-incubation with stated compound, cells were lysed and stored at -20°C for the indicated period. Intracellular cAMP was detected by a competitive ELISA assay. Data is displayed as median and 95% confidence interval (n = 3). *P < 0.05 (Kruskal-Wallis).*

Taken together, these optimisation experiments show that while 3 x 10^6 cells produced measurable baseline cAMP levels, the addition of IBMX produced significantly more stabilised cAMP recovery. Furthermore, this data showed that cell lysate cAMP levels were not significantly affected by storage at -20°C for up to seven days.

The effect of A. *fumigatus* Resting Conidia on A549 intracellular cAMP

Having designed and validated the optimal conditions for obtaining constant and adequate baseline cAMP expression, the next section of this thesis addresses alterations in AEC intracellular cAMP during *A. fumigatus* infection. Accordingly, A549 cells were incubated with either RC or UV-inactivated conidia *A. fumigatus* wild-strain B5233 at different MOIs (conidia:cells).

A549 cAMP levels appeared to be unaffected by incubation with B5233 RC at a MOI of 1, but B5233 RC at a MOI of 10 decreased A549 cAMP levels significantly (Figure 6). Surprisingly, a higher fungal load was not able to further decrease cAMP levels (Figure 6).

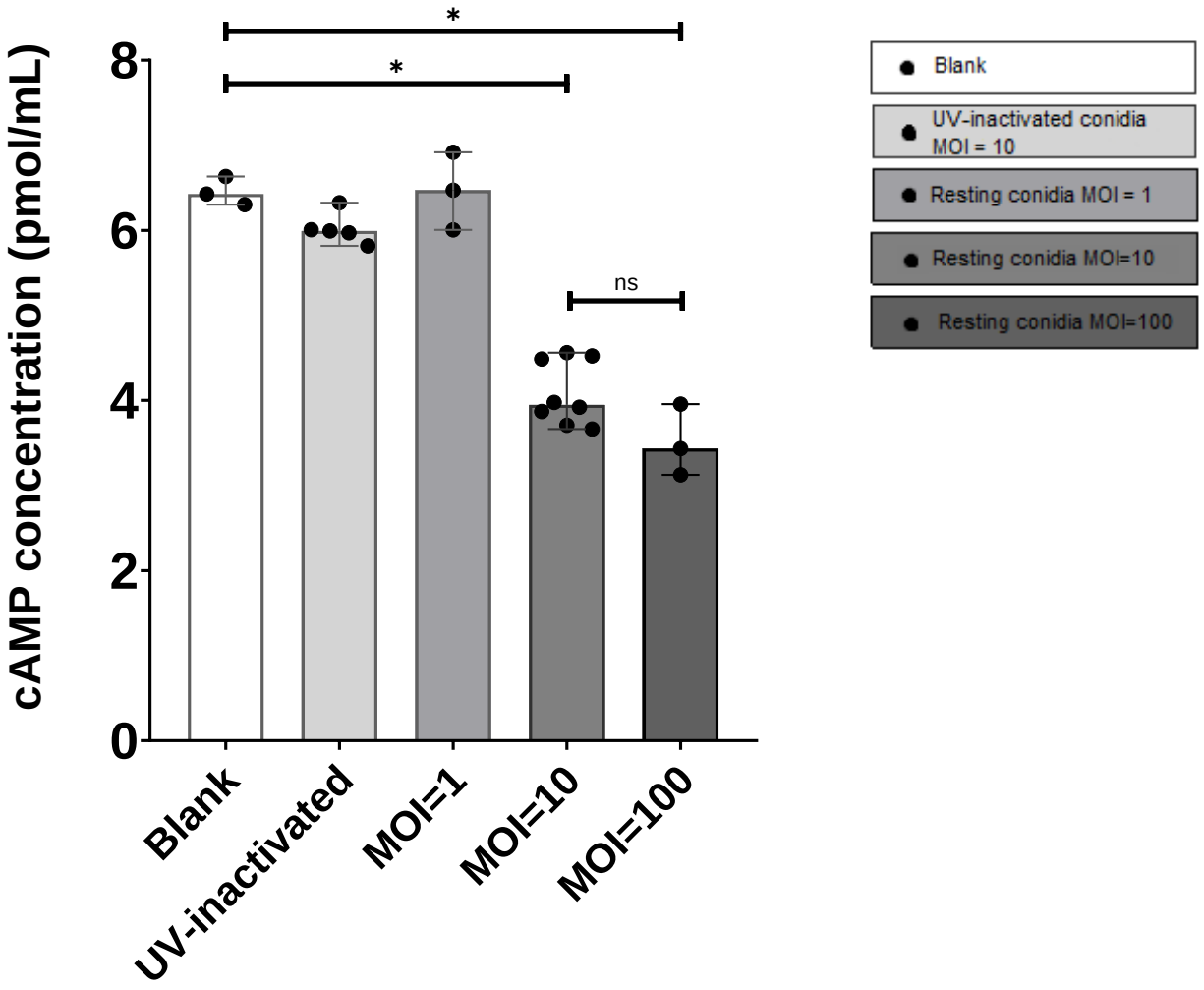


Figure 6: Influence of *Aspergillus (A.) Fumigatus* B5233 conidia on cyclic Adenosine Monophosphate (cAMP) expression of A549 cells at different multiplicities of infection.

*3 x 10^6 A549 cells were grown to confluence in 24 hours, before medium was changed to minimal medium for overnight incubation. After 30 minute pre-incubation with 0.5mM 3-isobutyl-1-methylxanthine (IBMX), cells were infected with ultraviolet (UV) inactivated conidia or Resting Conidia (RC) of A. fumigatus B5233 at the stated multiplicity of infection (MOI) for one hour. Cell lysate was produced and intracellular cAMP was detected by a competitive ELISA assay. Data is displayed as the median and 95% confidence interval. *P < 0.05 (Kruskal-Wallis test).*

Overall, these results indicate that *A. fumigatus* RC of B5233 wild-strain decreased A549 intracellular cAMP expression in a dose-dependent manner, with a MOI of 10 causing the greatest change in cAMP levels.

Validation of conidial suspension preparation

Thus far, our results indicated that host cAMP is affected by infection with *A. fumigatus* conidia at a MOI>10. Next, we wished to validate storage of conidial suspensions, in order to facilitate advanced logistics in future experiments. To evaluate this, conidia of *A. fumigatus* strain B5233 were either stored at 4°C for a period of 48 hours, or were frozen and stored at -80°C for up to two months. Subsequently, the effect of these conidial suspensions on cAMP levels of A549 cells was assessed and compared with the effect of UV-inactivated B5233.

As demonstrated in Figure 7, both conidial suspensions caused a significant decrease in cAMP level compared to UV-inactivated conidia, but no significant changes in the effect of differently stored conidial suspensions were detected.

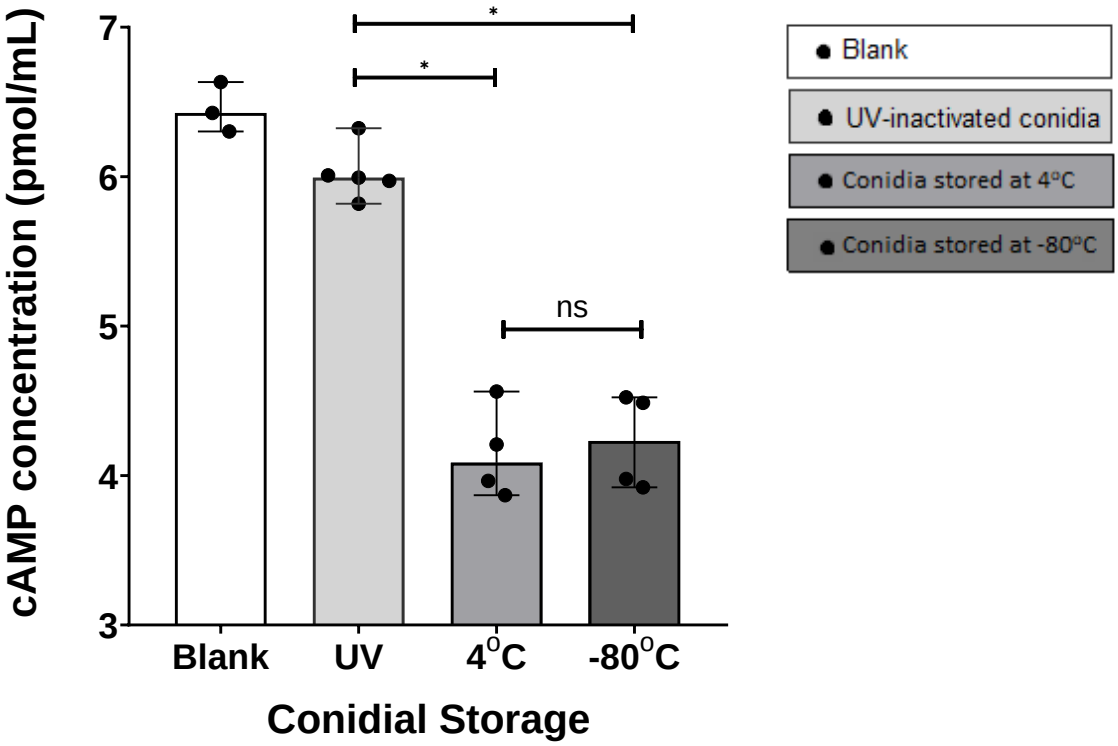


Figure 7: Influence of different conidial suspensions of *Aspergillus (A.) Fumigatus* B5233 on cyclic Adenosine Monophosphate (cAMP) expression of A549 cells.

*3 x 10^6 A549 cells were grown to confluence in 24 hours, before medium was changed to minimal medium for overnight incubation. After 30 minute pre-incubation with 0.5mM 3-isobutyl-1-methylxanthine (IBMX), cells were infected with ultraviolet (UV) inactivated conidia or Resting Conidia (RC) of A. fumigatus B5233 at a multiplicity of infection (MOI) of ten for one hour. As indicated, conidial suspensions were either stored at 4°C for 48 hours or at -80°C for a period up to two months. Cell lysate was obtained and intracellular cAMP was detected by a competitive ELISA assay. Data is displayed as the median and 95% confidence interval. *P < 0.05 (Kruskal-Wallis test).*

This result is further supported by the finding of similar growth of both suspensions after 24 hours of incubation at 37°C on SDA-plates (Figure 8 B,C and E,F). Moreover, after inoculation, strain B5233 exhibited light grey colonies, with a white mycelium, whereas conidial suspension caused a green-centred white growth pattern. This is indicative of a successful hyphal filtration process, that lead to conidial suspension in the early growth phase of swelling, which is demonstrated by increased DHN-melanin expression. Finally, there was no visible growth after inoculating SDA plates with 1 x 10^8 UV-inactivated B52233 conidia/mL, indicating a successful inactivation process (Figure 8 D,G). Consequently, during the remainder of this study, both storage conditions

of conidial suspensions were applied and UV-inactivated conidia stored for a maximum time of 24 hours were used as a control condition.

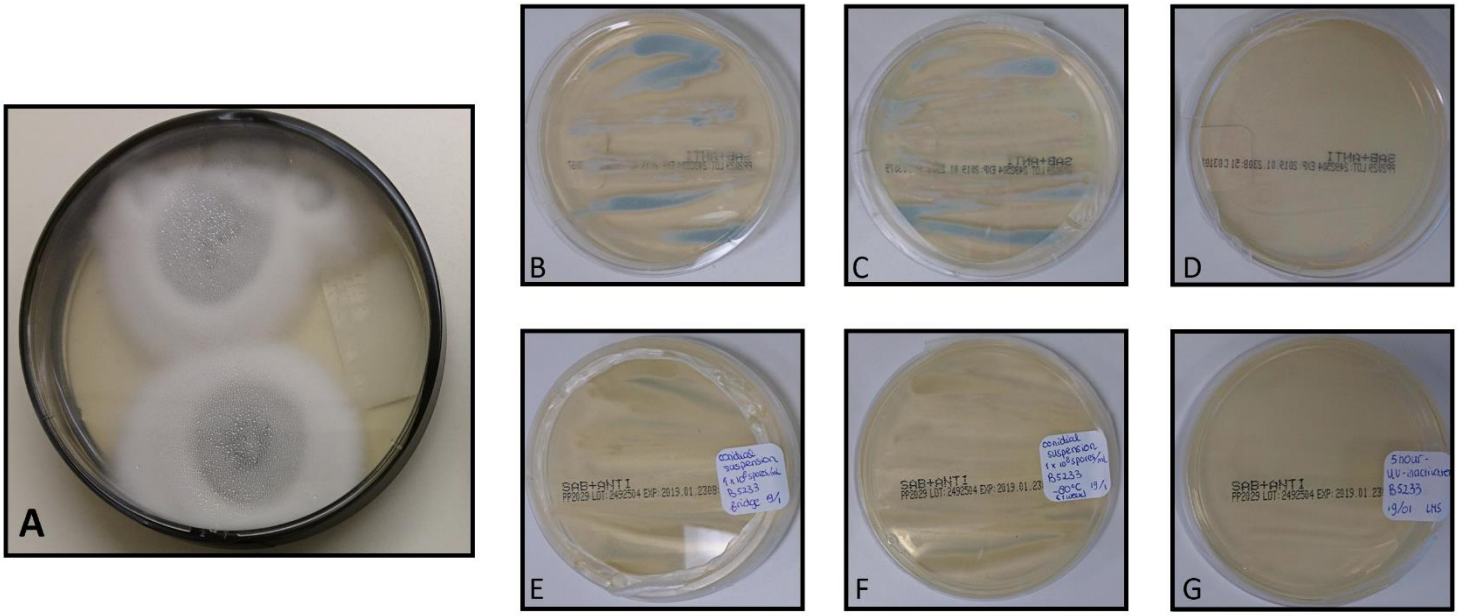


Figure 8: Macroscopic view of *Aspergillus Fumigatus* B5233 growth.

A) Parenteral tribe; B) top view conidial suspension stored at 4ºC for 48 hours; C) top view conidial suspension stored at -80ºC for 48 hours; D) top view UV-inactivated conidial suspension; E) bottom view conidial suspension stored at 4ºC for 48 hours; F) bottom view conidial suspension stored at -80ºC for 48 hours; G) bottom view UV-inactivated conidial suspension.

Sabouraud Dextrose Agar plates were inoculated with a B5233 fungal plug (A) or a fungal suspension of 1 x 10^8 B5233 conidia/mL (B-G) with a sterile inoculation loop. Fungal growth was examined after 24 hours of incubation at 37ºC.

Differences in A549 intracellular cAMP caused by *A. fumigatus* inter- and intrastrain variations

We optimised and validated our model and storage conditions, in order to determine a decrease in A549 intracellular cAMP by *A. fumigatus* B5233 RC. However, in reality, pathogen-host interactions are highly dynamic, and influenced by a range of factors, including diversity between fungal strains, together with morphological changes during fungal germination. Therefore, the next part of this thesis contributes to a more detailed understanding of the cross-talk between *A. fumigatus* and AEC cAMP by defining the influence of these two major components of pathogen-host interactions on cAMP alterations.

Differences between clinical isolates were evaluated by observing their macroscopic growth pattern (Figure 9). All isolates created typical circular suede-like surfaces consisting of a dense felt of conidiophores. Interestingly, whereas wild-strain B5233 lacks pigment, all three clinical isolates feature uniquely shaped green-grey colonies with white hyphal growth. Pigment intensifies from P2cs to P1mr and P1ms. These colour differences indicate diversity in expression of DHN-melanin, a compound known to influence host cAMP, therefore, functional effects of these differences were to be determined.

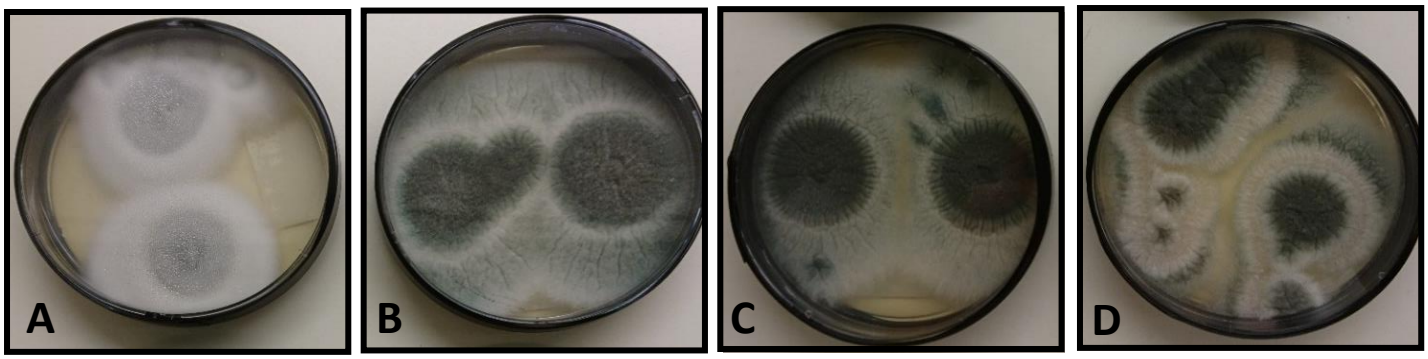


Figure 9: Fungal growth of *Aspergillus Fumigatus* clinical isolates. A) B5233; B) P1ms; C) Plmr; D) P2cs.

Sabouraud Dextrose Agar plates were point-inoculated with a fungal plug of strain named. Fungal growth was examined after 24 hours of incubation at 37°C.

Before examining the functional effects of inter-strain differences, it was important to recognise that besides varieties in pathogen-host responses caused by diversity in fungal strains, pathogen-host interactions are impacted by expression of different *A. fumigatus* morphotypes during fungal germination. Therefore, A549 cells were infected with RC (one hour pre-incubation) or SC (four hours pre-incubation) of four different clinical fungal isolates to assess the influence of both inter- and intrastrain variations on AEC cAMP expression (Figure 10).

Although no significant difference in intracellular cAMP levels was detected after infecting cell lines with UV-inactivated conidia compared to minimal medium only, RC decreased cAMP production in A549 cells with an average of approximately 60%. Surprisingly, when comparing effects between strains, no significant differences were detected (Figure 10). Furthermore, SC of all tested strains decreased cAMP levels significantly when compared to UV-inactivated conidia (Figure 10). However, conidial swelling of B5233 and P2cs was less effective in downregulating cAMP levels than RC of these strains, while conidial swelling did not influence the potency of P1mr and P1ms conidia.

In summary, this section showed that, although *A. fumigatus* inter-strain differences did not influence cAMP expression directly, inter-strain variation alters the effect of swelling on AEC cAMP expression. Therefore, this section further highlights the complexity of pathogen-host interactions in IA.

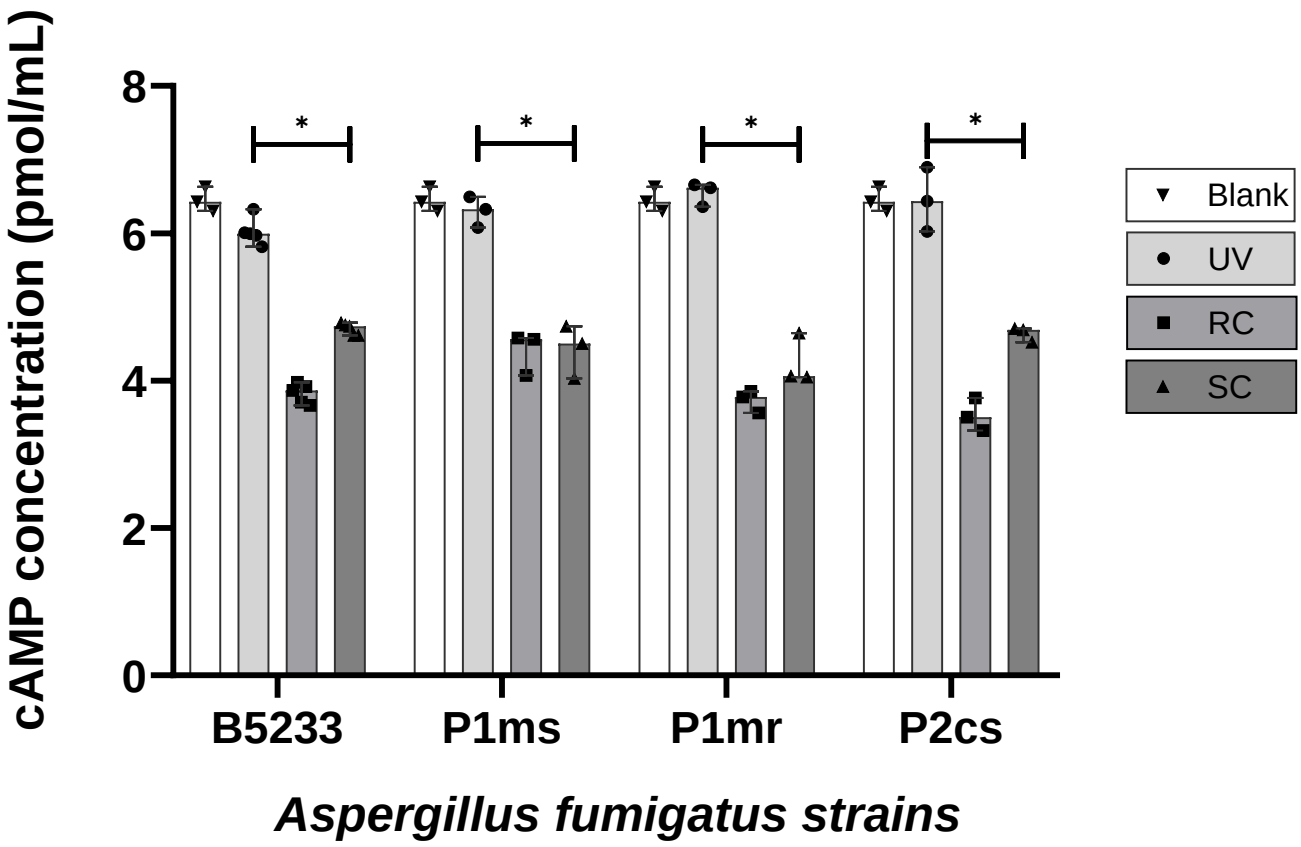


Figure 10: Influence of swelling *Aspergillus Fumigatus* B5233 conidia on cyclic Adenosine Monophosphate (cAMP) expression of A549 cells.

*3 x 10^6 A549 cells were grown to confluence in 24 hours, before medium was changed to minimal medium for overnight incubation. Prior to infection, resting conidia (RC) and swollen conidia (SC) were obtained by pre-incubation of one hour or four hours respectively, while A549 cells were pre-incubated with 0.5mM 3-isobutyl-1-methylxanthine (IBMX) for 30 minutes. A549 cells were then infected with ultraviolet (UV) inactivated conidia, RC or SC of the indicated strain, at a multiplicity of infection (MOI) of ten for one hour. Cell lysate was produced and intracellular cAMP was detected by a competitive ELISA assay. Data is displayed as the median and 95% confidence interval. *P < 0.05 (Kruskal-Wallis test).*

4. Discussion

Interactions between pathogens and the host's immune system represents a key factor in understanding pathogenicity of infections. The interplay between virulent factors and host molecular processes is particularly important in fungal infections, where in the case of immunodeficiency, fungal spores are permitted to germinate and grow invasively. Disruption of host signal transduction is one of the most effective virulence strategies to influence intracellular microbial uptake and bypass cellular barriers[25]. Cumulative evidence indicates that internalisation by non-immune cells defends the host against several pathogenic microorganisms, yet this powerful protective phagolysosomal pathway can be utilised by a small percentage of microbes. In this way, intracellular microbes, such as *A. fumigatus*, can prevent phagolysosomal acidification, thereby allowing cellular or systemic entry[51, 55]. Assuming this mechanism is a key feature in IA, this study set out to assess the importance of intracellular cAMP, an important upstream blocker of autophagic processes in the involved pathogen-host interactions.

Assay optimisation and storage validation

In the first set of investigations, storage conditions of conidia and cell lysates were validated and optimised for subsequent experiments. Firstly, the cAMP assay was optimised by increasing A549 cell number, leading to detectable levels of recovered cAMP. Still, cAMP levels did not exceed the lower detection limit of the assay and reproducibility was therefore limited. Consequently, we included pre-incubation with 0.5mM IBMX, and this was found to increase and stabilise cAMP levels of A549 cell lysate within all storage conditions, compared to vehicle control. Finally, we showed cAMP levels were stable for at least one week at -20°C. Presumably, degeneration of cAMP is halted under these conditions, as the degradative enzymes are inactive at this temperature[56]. In summary, this data suggests that, when not strictly managed, PDEs can potentially influence cell lysate cAMP levels during processing, causing low, irreproducible amounts of cAMP. To prevent this, the optimised conditions including pre-incubation with IBMX were used for subsequent investigations.

Next, the quality of conidial suspensions during storage was tested by both morphological representation and functional evaluation of their effect on cAMP levels. No significant differences in cAMP were found between conidia that were stored at 4°C for 48 hours, compared to conidia that were stored at -80°C, thereby facilitating the exchange of both suspensions in following experiments. This was further confirmed by a similar growth pattern of both conidial suspensions on SDA-plates. No fungal growth was observed after inoculating plates with the equivalent UV-inactivated conidia. UV-inactivation was thus successful, and UV-inactivated conidia were used as a control during the remainder of this study. Surprisingly, conidial suspensions slightly differed in morphology from parental tribes. This may be explained by filtration of hyphal fragments from the conidial suspension, thereby speeding up RC growth due to increased RC concentration and more readily available nutrients. The first essential step of the swelling process is dissolution of *A. fumigatus'* outer layer, including DHN-melanin, a fungal cell-wall component known for causing *A. fumigatus'* typical gray-green colour[57]. Therefore, we hypothesise that increased colour expression reflects a higher concentration of RC during the first 24 hours of growth on SDA-plates. This is further supported by an observed colour neutralisation of the SDA plate after 48 hours and 72 hours of incubation (data not shown). Consequently, we conclude that morphologically changing conidia cause a colour deviation during incubation on SDA-plates, a phenomenon that highlights the importance of researching the influences of intra-strain variation on pathogen-host interplay.

cAMP and internalisation

cAMP blocks autophagic processes via activation of EPAC, and host messengers Ip3 and calpain. In this way cAMP controls microbial intracellular uptake[58]. Nevertheless, the effects of microbes on host cAMP signalling are controversial. On one hand, virulent factors upregulating cAMP are widely described, because they can protect extracellular microorganisms from exposure to the host's arsenal of intracellular defence mechanisms[42]. For example, *Bordetella pertussis* overwhelms the cAMP regulatory system of host cells, leading to reduced phagocytosis and granulocyte recruitment[44]. In contrast, downregulation of cAMP, can promote phagocytosis, and in this way allows intruding microbes such as viruses, intracellular bacteria and invasive fungi to breach the epithelial cell barrier[49]. Several microbes have been reported to operate in this fashion, including *E. Coli*, a bacteria found to be less invasive in urinary epithelial cells when cAMP levels were synthetically elevated[53]. Moreover, the intracellular bacteria *Staphylococcus aureus* causes autophagic responses via cAMP downregulation to favour intracellular pathogen survival[58]. Similarly, in the current study we revealed RC of *A. fumigatus* at a MOI of 10 downregulate intracellular cAMP expression of A549 cells compared to UV-inactivated conidia. Further increasing fungal load to a MOI of 100 did not affect the ability of *A. fumigatus* RC to decrease A549 cAMP production. The lack of augmented effect might indicate that after profound reduction of cAMP, compensation mechanisms might be activated. Another finding that highlights the complexity of the dynamic interaction between *A. fumigatus* and AECs is the effect of swelling on this cross-talk. SC of all investigated strains were able to significantly decrease cAMP expression compared to UV-inactivated conidia. Nevertheless, the swelling of B5233 and P2cs conidia influenced their ability to decrease cAMP negatively, whereas no significant differences were detected after conidial swelling of P1mr and P1ms. Early-phase conidia of B5233 and P2cs are more potent in decreasing cAMP than their matured versions, indicating that cAMP alterations are an early onset response in *A. fumigatus* and AEC interactions. Consequently, the interstrain variation in these effects may be explained by differences in their growth rates. This might be related to improved adaptation to the host environment, and therefore higher virulence. However, interstrain variation alone did not affect AEC cAMP levels significantly. It can thus be suggested that differences in pathogenicity of the clinical isolates cannot be entirely explained by interstrain variation affecting cAMP, but that interstrain variation might influence timing and dynamics of pathogen-host interactions by modifying germination, and, in this way, cause differences in patterns of *A. fumigatus*–host cAMP interplay.

This study also assessed the clinical isolates by appearance, and showed interstrain variances in colour and growth patterns. Interestingly, the hypervirulent *A. fumigatus* strain B5233 was found to differ from remaining clinical isolates due to the lack of gray-green pigment. As described earlier, DHN-melanin expression, responsible for this typical colour, distinguishes *A. fumigatus* from other Aspergilli[57]. Increasing evidence shows that DHN-melanin might play a role in pathogenicity by affecting cAMP and downstream effectors. For example, our group showed that DHN-melanin is responsible for a decrease in host cAMP, while being able to increase protein expression of EPAC1 and EPAC2 during *Aspergillus* infection[49]. Moreover, it has been shown that DHN-melanin increases conidial internalisation and promotes spore viability via prevention of phagolysosomal acidification in A549 cells, two processes that seem important in IA[51,52]. Taken together, blockage of this phagolysosomal function may be related to microbial upregulation of cAMP, as demonstrated by a transient decrease in cAMP catalysing phagolysosomal maturation[48]. However, in the case of *A. fumigatus,* AEC cAMP is decreased within one hour of infection, which might drastically interrupt phagolysosomal maturation and acidification. Indeed, blocking cAMP activity prevents phagolysosomal acidification[59]. Additionally, formation of important phagolysosomal oxidative agents depends on transport of chloride via a cAMP-activated chloride channel[60]. Consequently, DHN-melanin knock-out (pksP) mutants were five times more likely to be located in functional, acidic phagolysosomes than their controls[61]. Similarly, RC of wild-strain aspergilli were able to

substantially inhibit phagolysosomal acidification compared to SC of the same strain, possibly due to higher DHN-melanin expression[52].

Accordingly, we hypothesised that RC of all strains would be more potent in decreasing cAMP than SC, and that B5233 would be less capable of altering host cAMP, due to observed differences in pigment (DHN-melanin) expression. Nevertheless, B5233 caused a significant reduction of cAMP levels in AECs, with no differences compared to other studied strains observed. However, intrastrain differences were found for B5233 and P2cs. Whole Genome Sequence analysis of all studied clinical isolates (unpublished) revealed that all strains contain pksP and we observed that increased pigment is expressed when conidial suspensions of B5233 are plated out on SDA plates. In other words, all isolates possess the same ability to produce DHN-melanin, so, theoretically, all isolates are able to alter AEC cAMP production. Due to the reduced potency of SC to decrease AEC cAMP in only two of the clinical isolates, together with our macroscopical observations, we think that DHN-melanin is differently expressed during germination of different fungal strains. This further confirms that timing of AEC cAMP inhibition, and therefore timing of pathogen-host interactions, is strain-dependent.

Transcriptomic profiles of AECs demonstrated upregulation of critical factors for rearrangement of the cytoskeleton (ABL2, ARC and LST1) after incubation with A. *fumigatus*[62]. The current study suggested that this process might be controlled by influencing host cAMP, a negative regulator of actin assembly. While the mechanism behind this remains mainly unclear, Han et al showed that *A. fumigatus* infection increases EPAC expression, but leaves PKA unchanged[49]. Additionally, their transcriptomic data suggested participation of early growth response 1 (EGR1), as it RNA was rapidly upregulated after infection with *A. fumigatus* RC and silencing of the RNA caused diminished fungal internalisation by approximately 50%[62]. Interestingly, EGR1 contains cAMP response elements (CRE), and is therefore related to cAMP signalling[63]. Consequently, participation of EGRs in AECs during fungal infection may help explain the consequences of the observed decreased cAMP levels in AECs during *A. fumigatus* infection.

Overall, this study proved that *A. fumigatus* infection reduced AEC cAMP production and, for the first time, demonstrated that the fungal germination processes influence this cross-talk. Moreover, this study showed interstrain differences in this cross-talk cannot explain the highly varied individual fungal pathogeneses, but interstrain differences contribute to timing and dynamics of these pathogen-host interactions. In this way, this study provides an important lead on a potential mechanism of fungal-host interactions underlying IA, thereby starting the investigations into targeted treatment strategies.

5. Limitations and future directions

Although this study provides unique information on the sophistication and timing of *A. fumigatus*' effects on AEC cAMP levels, the conclusions of these findings may be restricted by several limitations.

Firstly, the study determined a dose-dependent reduction of AEC cAMP expression by *A. fumigatus* RC. To this end, intracellular cAMP concentration in lysate of infected and non-infected A549 cells was measured with an ELISA assay, but no correction for protein content was included. Therefore, it could be argued that the cAMP reduction is caused by a decrease in cell-number due to cell death, rather than specific processes altering AEC cAMP production. However, given the short duration of infection (one hour) and direct cell lysis afterwards, cell death is an unlikely event, and we assume that the input of this bias is limited.

Secondly, this study determined no interstrain variation on *A. fumigatus'* potential to influence AEC cAMP. However, all tested strains were pathogenic isolates and, therefore, fungal wild-type reference is lacking. Since B5233 is originally a hypervirulent isolate, use of this strain as a control, despite its broad use in research, is not fully justified. This study determined new leads in the potency of fungal virulence on cAMP signalling, but to reflect a complete range of fungal pathogenicity, future research should include less virulent fungal strains and wild-types as well. Strategies to enhance control strains might involve choice of environmental strains over clinical isolates[64], together with assessment of virulence factors, as described by *Raksha et al.*[65]. Another possible bias in this correlation might have been caused by the use of non-parametric statistical tests. Due to the limited, varying sample size, assumption of normal distribution was not possible, hence distribution-free statistics were used. A disadvantage of these methods is their lack of power in small sample sizes, which can cause false negative correlations. We are aware that this might have contributed to the lack of determined interstrain effects, yet, compared to the changes caused by morphological stages, interstrain variations did not show any trend in influencing AEC cAMP expression. Therefore, it is unlikely that this bias might have prevented biologically relevant trends.

For the first time, this study provided an important lead on strain-dependent timing of pathogen-host interactions in IA. Although this lead is a break-through in the field of IA, the clinical implications of the produced data is limited. Due to the well-established role of cAMP in influencing microbial uptake via negatively regulating actin assembly in cells[48], we hypothesise that the decrease of cAMP by *A. fumigatus* conidia contributes to fungal pathogenesis. However, the current research did not take into account downstream effectors of cAMP signalling, such as EPAC and PKA, nor the functional outcomes, including fungal internalisation and phagolysosomal acidification. Moreover, this research only included internalisation as a process underlying IA, yet also other processes such as the disruption of extracellular matrix by proteolytic conidial enzymes may contribute to the pathology of IA. For example, transcriptomic profiles of AECs during *A. fumigatus* infection suggested participation of dual-specificity phosphatases (DUSPs) in internalization, which is not likely to operate via cAMP signalling[62]. Therefore, this current data is subject to certain limitations, and follow-up research is needed to determine the consequences of altered cAMP levels in AECs during fungal infection.

Another objective of future research should include overcoming limitations of the current study set-up. To produce new leads on molecular players in IA, this study was designed around cost effectiveness, an infinite supply of pure cell populations, and limited ethical concerns[66]. For this reason, a submerged monolayer culture of immortalized AECs was used. However, to develop a full picture of cAMP's role in IA, more information regarding pathogen-host interactions in a native pulmonary environment is required. For

example, air-liquid interface (ALI) cultures could provide more information on the complex cellular structures and interactions associated with the alveolar epithelium[67]. Similarly, primary cells can contribute to reflecting a full range of alveolar cellular structures, as type II AECs, such as A549 cells, only make up for ~5% of the alveolar surface[31]. Besides that, due to initial genetical manipulation and possible genetic drift, immortalised A549 cells, cannot be considered normal in their phenotype, native functions and responses to stimuli[66]. Thus, to obtain more in-depth information regarding the newly observed association of fungal influences on AEC cAMP in IA, future work should focus on confirming data in ALI-cultures with primary cells.

This thesis discovered participation of host cAMP in fungal syndromes, yet cAMP is also an important target in the treatment of asthma due to its bronchodilator effects. A problem with this asthma therapy is agonist-induced desensitisation, leading to insufficient cAMP levels in these agonist-resistant asthmatic patients. Another problem in asthma is the atopic sensitisation to *A. fumigatus,* with subgroups of asthmatic patients exhibiting poorly-controllable ABPA as a consequence[68]. Due to the roles of both cAMP and *A. fumigatus* in asthma, it would be interesting to extend our knowledge of this signalling axis during *Aspergillus* infection in asthma patients. In mice, prenatally exposed to cigarette smoke, a single tracheal injection of *A. fumigatus* extract significantly decreased cAMP in the lung[69]. This process was associated with an increase in murine airway hyperresponsiveness[69]. With observations regarding *Aspergillus* infection obtained, this prenatal cigarette smoke exposure might in this way also increase pathogenesis of IA. Consequently, future research should extend our knowledge of the participation of AEC cAMP in the variety of fungal syndromes. An important follow-up step of this research would therefore be determining divergences in cellular signalling in lung tissue obtained from asthmatic and healthy patients suffering from various aspergilloses.

6. Conclusions

Prior studies assigned cAMP as an important, yet controversial, player in autophagic processes such as internalisation. Since internalisation is considered to drive IA, the aim of this thesis was to clarify the role of cAMP in IA pathophysiology, via examining the effects of *A. fumigatus* inter- and intrastrain variations on cAMP levels in A549 cells.

In summary, this thesis demonstrated that, despite cell-number, pre-incubation with IBMX is crucial to measure repeatable amounts of cAMP in A549 cell lysate with a competitive ELISA assay. Furthermore, it demonstrated that intracellular cAMP of A549 monolayers is decreased by *A. fumigatus* RC. Intra-strain differences, with RC presenting more potent than SC, were only found for B5233 and P2cs and not for P1ms and P1mr, whereas no significant differences between the effect of RC from B5233 and the three clinical isolates were determined.

This study points out AEC cAMP as a new lead in IA investigations. It also indicates that the prerequisite for the development of IA is not fundamentally characterised by interstrain differences, but interstrain differences contribute to the timing of morphological changes that presumably influence the interplay between conidia and host cAMP. However, continued efforts are needed to make the complexity of the pathogen-host interactions in IA more accessible for development of targeted treatment strategies.

Important follow-up research should include pathogenic classification of studied isolates, together with measurement of DHN-melanin expression. To further strengthen this discovered lead, repetition of the study in ALI-cultures of primary cells is advised. To deepen our knowledge of cAMP signalling in IA, assessing expression of involved molecular players, such as downstream host cAMP effectors and subsequent functional outcomes (internalisation, prevention of acidifying phagolysosome), together with measuring divergences in tissue derived from asthmatic and healthy patients is essential.

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