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DMM, JAM, CW, PMOB were responsible for the study design and concept. DMM and EBH were responsible for patient recruitment, clinical characterisation and sample acquisition. Sample processing and study assays were performed by AFS, EBH, SL, LJF, JAM and CW. Statistical analysis was performed by the authors in conjunction with biostatistical support from The Clinical Research Facility University College Cork. Analysis and interpretation of data, manuscript preparation and approval was performed by all authors.

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The authors have no conflict of interest in relation to this work.

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Abstract (249)

Background: It is suggested that airway fungi, in particular *Aspergillus* may impinge on clinical phenotype in asthma. Indeed the term severe asthma with fungal sensitisation (SAFS) has been coined. We aimed to ascertain whether the presence of fungi, in particular *Aspergillus fumigatus*, in the airway correlated with asthma severity and control. Furthermore, we aimed to determine if traditional markers of *Aspergillus* sensitisation related to the presence of *Aspergillus* within the airway.

Methods: Sixty-nine patients characterised by asthma severity (GINA) and level of control (ACQ-7) underwent bronchoscopy and bronchoalveolar lavage (BAL). Serum was assessed for *A. fumigatus* specific IgE and total IgE. Galactomannan and relevant cytokine levels were assessed in serum, plasma and BAL. BAL was analysed for the presence of *A. fumigatus.*

Results: In BAL, fungi were visible by microscopy in 70% and present by qPCR in 86% of patients, while *A. fumigatus* was detectable by qPCR in 46%. Plasma and BAL IL-4, IL-6, IL-10, IL-13 and TNF-α correlated with BAL fungal presence, while plasma IL-17 correlated with BAL fungal presence. *Aspergillus* positive BAL correlated with increased plasma and BAL IL-6 and BAL IL-13. There was no relationship between fungal airway presence and steroid dose, asthma severity or control. The presence of *Aspergillus* within the airway did not relate to serum IgE positivity for *Aspergillus*.

Conclusions: Fungi were present in a large proportion of our asthmatic patients' airways, but their presence was not predicted by traditional markers of sensitisation, nor did it appear to be related to measures of disease severity or control.

Keywords: Allergy, *Aspergillus fumigatus*, Asthma, Bronchoscopy, Fungi

Introduction

The term severe asthma groups patients with varying pathophysiological and phenotypic characteristics[1]. Studies have suggested roles for fungi in severe asthma and the term severe asthma with fungal sensitisation (SAFS) has been coined; the current definition of which is based

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on demonstrating sensitisation to fungus in the absence of allergic bronchopulmonary aspergillosis (ABPA)[2]. Therapies aimed at fungal elimination have afforded mixed results[3,4].

The respiratory tract is constantly exposed to hyphal fragments, fungal spores and yeasts[5-7]. Thermotolerant fungi, including *Candida, Aspergillus* and *Penicillium* are sources of allergens, commensals and opportunistic pathogens and can colonise airways with potential clinical ramifications[5].

Aspergillus fumigatus, a ubiquitous fungus can elicit a destructive response in a susceptible host[8]. Due to the spherical shape of *A. fumigatus* conidia, typically 2.0-3.0µm in diameter, the conidia can evade mucociliary clearance and reach the lower airways[9]. Pathogen recognition receptors and innate immune cells can subsequently initiate a cascading anti-fungal response, culminating in phagocytosis, removal of the fungal insult and down-regulation of inflammatory cytokine signalling[10,11]. In a healthy host these innate mechanisms ensure that fungal spores are cleared[11]. In a host with a pre-existing disorder, such as asthma, there is a risk that fungal exposure will lead to airway colonisation, and disorders such as Severe Asthma with Fungal Sensitisation (SAFS) or ABPA[10,11]. It has been suggested that fungal sensitisation and asthma severity may be related, and a temporal relationship between fungal exposure and clinical outcome has been shown[2,12]. While interest remains in relation to *Aspergillus* colonisation, attention to other fungi is also of interest and a wider group of sensitisation to genera such as *Candida* and *Penicillium* exists and is encompassed within the diagnosis Allergic Bronchopulmonary Mycosis[5].

In this study, we sought to ascertain whether fungi, and in particular *Aspergillus* were present in the airways of asthmatics across the spectrum of disease severity and determine whether their presence was associated with airway and systemic cytokines, levels of control, disease severity or treatment with steroids. We additionally wished to determine whether or not systemic sensitisation with *Aspergillus* as currently utilised to define SAFS was related to *Aspergillus* presence within the airway.

Methods

Patient recruitment

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The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Sixty-nine patients with a history of asthma (either documented change in FEV1 ≥12%/200ml with bronchodilator or a positive inhalation challenge) were recruited through a dedicated asthma clinic in a tertiary referral centre. Patients with ABPA were excluded. Patients were scheduled for bronchoscopy, with spirometry, ACQ-7 and blood sampling performed on the day of bronchoscopy. Written informed consent was obtained from patients on the day of bronchoscopy. Spirometry was performed according to ATS/ERS guidelines[13]. The cohort was stratified by both severity [mild (GINA 1+2), moderate (GINA 3) and severe (GINA 4+5)] and asthma control (ACQ-7)[14,15] (Table 1). Serum was assessed for total IgE and *A.fumigatus* specific IgE and plasma was assessed for cytokines. Patients underwent bronchoscopy according to standard guidelines[16]. Bronchoalveolar lavage (BAL) was obtained as a standardised 180ml (3X60ml) sample from either the right middle lobe or lingula as we have previously described[17].

Microscopy

A differential cell count was performed on BAL cytospin slides, stained with the Kwik-Diff kit (Thermo-Fisher) using light microscopy and Olympus DP software. Immune cell percentage counts were calculated and slides scored for fungal presence by 3 independent observers and microscopic observation of fungus was determined by size and presence of hyphae and/or spores. Samples received a positive or negative fungal score (0/1). A representative positive sample is shown in figure 1 B-C.

DNA extraction

To remove host cell DNA, BAL was centrifuged at 500g for 10 minutes, supernatant collected and DNA extraction performed. DNA extraction took place using a QIAamp UPC Pathogen Mini Kit (Qiagen, Manchester, UK) with DNA quantification performed using a NanoDrop 2000 Spectophotometer (Fisher Scientific, Dublin, Ireland).

Fungal quantitative PCR

Primers were designed and validated. qPCR was performed on extracted BAL DNA to determine presence of fungi and *A. fumigatus* using a total fungal primer (Fungi Quant)[18] and an *A. fumigatus* ITS specific primer[19] on a LightCycler 480 machine (Roche) (Table S1 in online data supplement).

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Platelia *Aspergillus* antigen (Galactomannan assay)

Platelia *Aspergillus* antigen immunoenzymatic sandwich microplate assay (Bio-Rad, Dublin, Ireland) was performed on patient serum and BAL to determine presence/absence of *Aspergillus* galactomannan antigen. The optical density of each well was read at 450nm (reference filter 620/630nm).

Cytokine measurement

Interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IFNγ and tumour necrosis factor (TNF)-α were measured in plasma and BAL using an electro-chemiluminescence QuickPlex SQ 120 imager from Meso Scale Discovery (Gaithersburg, MD, USA).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism Software 6. Appropriate tests such as T-test, Spearman and Pearson Correlations were performed, with corrections for parametric/non-parametric data. Results were considered statistically significant when $P<0.05$.

Results

Patient demographics

Of the sixty-nine patients recruited, 61% were female. The cohort was stratified by asthma severity into 35% mild (GINA 1+2), 22% moderate (GINA 3), 43% severe (GINA 4+5) and level of control (ACQ-7) with 38% controlled (ACQ-7<1.5) and 62% uncontrolled (ACQ-7≥1.5). Seven patients (10%) were on maintenance oral steroids at the time of testing (7.5mg median; range 5-30 mg daily). Ten asthmatics were newly diagnosed and ICS naïve at the time of sampling. Results and patient characteristics are summarised in table 1.

Serological detection of fungal sensitisation and presence

IgE levels were elevated in 38/69 (55%) of patients (mean 299.5; SD 512.1) (Figure S2). 6/69 patients (9%) demonstrated positive IgE reactivity to *A. fumigatus.* The Platelia *Aspergillus* assay

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for detection of *Aspergillus* galactomannan antigen afforded a positive result in one of the sixty-nine patient's serum. This was not related to elevated IgE.

Fungal detection by microscopy

BAL cell differential revealed macrophages to be the most dominant immune cell type with varying levels of neutrophils, eosinophils and lymphocytes present (Figure 1A). Fungal presence was detectable by microscopy in 48/69 (70%) of cytospins (Figure1B-C). Presence of fungi in cytospin slides correlated with increased neutrophils ($P<0.05$; rs = 0.275). There was no observed relationship between microscopic fungal presence in BAL and serum IgE levels or BAL eosinophils.

Molecular detection of fungal presence

59/69 patients (86%) had detectable presence of fungi (Fungi Quant) and 32/69 (46%) had detectable *A. fumigatus* in BAL (Figure 2A). BAL *A. fumigatus* qPCR levels negatively correlated with macrophage counts ($P<0.01$; rs = -0.418) (Figure 2B). *A. fumigatus* did not correlate with BAL neutrophil or eosinophil counts. Total fungi, Fungi Quant qPCR, correlated with increased BAL neutrophils ($P<0.05$). Interestingly, patients who smoked had lower levels of *A. fumigatus* but not fungi by qPCR ($P<0.05$; rs=0.171). Severe asthmatic patients (GINA 4/5) also had lower levels of detectable fungi ($P<0.05$; rs=0.084) but asthma severity had no relationship with levels of detectable *A. fumigatus.* There was no significant difference in detection of fungi or *A. fumigatus* in patients on high or low dose inhaled corticosteroids (ICS) or on maintenance oral steroids. When grouped by allergic (total IgE>80 KU/L) and non-allergic asthma 34/38 allergic asthma patients had detectable fungi and 17/38 had detectable *A. fumigatus* by qPCR. For non-allergic asthma patients 25/31 had detectable fungi and 15/31 had detectable *A. fumigatus*. When grouped by eosinophilic and non-eosinophilic asthma 21/23 patients with eosinophilia (peripheral eosinophil count > 0.3) had detectable fungi and 10/23 had detectable *A. fumigatus* by qPCR. In non-eosinophilic asthma patients 38/46 had detectable fungi and 22/46 had detectable *A. fumigatus* by qPCR. There were no statistical differences between detectable fungi or *A. fumigatus* between allergic and non-allergic asthma. There were no statistical differences between detectable fungi and eosinophilic or non-eosinophilic asthma, however there was a statistically significant difference between *A, fumigatus* and eosinophilic and non-eosinophilic asthmatics ($P<0.05$)

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30 patients had detectable fungi by both qPCR assays (Fungi Quant and *A. fumigatus*) and 22 patients had corresponding positive results for *A. fumigatus* detection by qPCR and detectable fungal presence by microscopy [20]. 40 patients had corresponding positive total fungal qPCR and detectable fungi by microscopy of which 21 patients had fungi detectable by microscopy and both qPCR assays (Fungi Quant and *A. fumigatus)*. As expected fungi other than *A. fumigatus* are detectable in BAL, however the identities of these were not investigated further in this study (Figure S3).

5 patients had a positive Galactomannan result for presence of *Aspergillus* in BAL. Only one patient was positive for *A. fumigatus* qPCR, microscopy, BAL Galactomannan and Fungi Quant qPCR. (Figure S5). FEV1 negatively correlated with BAL galactomannan (P<0.05).

Plasma and BAL cytokine levels correlate with BAL fungal presence

Cytokines Interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IFNγ and TNF-α were measured in plasma and BAL (Figure 3). BAL qPCR with the total fungi primer, Fungi Quant, correlated with plasma IL-4 (P<0.001), IL-6 (P<0.05) IL-10 (P<0.01) IL-13 (P<0.05), IL-17 (P<0.01) and TNFα (P<0.001). A correlation between total fungi and IL-1β, IL-5, IL-8 and IFN-γ was not observed (Figure S6). Microscopic detection of fungi in BAL also correlated with plasma IL-4 (P<0.05), IL-6 (P<0.01) and TNFα (P<0.01).

BAL Fungi qPCR correlated with increased BAL IL-4 (P<0.05), IL-6 (P<0.05), IL-10 (P<0.01), IL-13 (P< 0.05) and TNFα (P<0.05) but not IL-1β, IL-5, IL-8 and IL-17 (Figure S7). Microscopic detection of fungi in BAL correlated with BAL IL-4 (P<0.01) and IL-10 (P<0.01).

A. fumigatus positive BAL*,* by qPCR, correlated with increased plasma IL-6 (P<0.001) and increased BAL IL-6 (P< 0.01) and IL-13 (P<0.05) but not BAL and plasma IL-1β, IL-4, IL-5, IL-8, IL-10, IL-17, TNFα or plasma IL-13 and IFNγ (Figure S8).

The presence of fungi by microscopy and qPCR also correlated with changes in BAL cell counts, particularly with increased neutrophils (P<0.05; rs=0.296).

BAL eosinophil percentage counts correlated with plasma IL-5 (P<0.005; rs=0.349) and BAL IL-5 (P<0.05; rs=0.263). Neutrophil cell percentage counts correlated with plasma IL-4 (P<0.05; rs=-0.248) and TNFα (P<0.05; rs=-0.276) and BAL IL-8 (P<0.05; rs=0.278). Macrophages correlated

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with BAL IL-6 (P<0.01; rs=-0.296), IL-10 (P<0.01; rs=-0.302) and TNFα (P<0.05; rs=-0.250). Monocytes correlated with plasma IL-17 (P<0.001; rs=0.381), TNFα (P<0.01; rs=0.372) and IFNγ (P<0.005; rs=0.372) and BAL IL-4 (P<0.01; rs=0.320). FEV1 negatively correlated with BAL IL-5 (P<0.005; rs=-0.352).

Discussion

Our study has demonstrated presence of fungi within the airways of adult asthmatic patients with varying asthma severity and levels of control. Microscopic and nucleic analysis revealed that fungi, including *A.fumigatus*, were detectable in BAL. Microscopy alone revealed spores and hyphae within 70% of the cohort and qPCR analysis demonstrated that 86% of patients have fungal presence within their BAL. Almost half (46%) of the cohort had detectable *A.fumigatus* in their airways. Despite the high frequency of fungi and specifically *A. fumigatus*, we were unable to find any association between its presence and measures of control, severity, or steroid use, in contrast to previous reports[21]. In fact, severe asthmatic patients had less detectable fungi in comparison to mild and moderate GINA grouped patients but there was no relationship between severity and levels of *A. fumigatus*. Asthma severity was associated with BAL neutrophils, however the presence of *A. fumigatus* did not correlate with BAL neutrophil or eosinophils but was associated with reduced BAL macrophages. The detection of fungal DNA (Fungi Quant) in BAL did however correlate with BAL neutrophil counts. This observation of BAL fungal presence associated with BAL neutrophilia, is in keeping with other studies in which *A. fumigatus* alkaline protease 1 levels in sputum correlated with sputum neutrophilia but not eosinophil levels[22].

Elevated IgE levels were observed in 38/69 patients suggesting that 55% of the cohort has atopic asthma. While qPCR revealed fungi in BAL of 86% and *A. fumigatus* in 46% of patients, serum antibody titres for *A. fumigatus* specific IgE were only positive in 9%. *A. fumigatus* IgE positivity did not indicate a *A. fumigatus* qPCR positive result in 3 patients (Figure S4). Antigen testing for fungi involves detection of specific antigen components present in the fungal cell wall which have been shed into blood or other body fluids. Several of these antigens have been utilised recently as an aid in detection of fungi, for example, galactomannan antigens in serum or BAL to diagnose invasive *Aspergillus* infection[23]. In our study, galactomannan was detectable in serum of 1 patient and BAL of 5 patients. However, when these results were analysed for concordance in detection

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methods, no patient tested positive for all parameters (Figure S4). Antigen testing has restrictions which must be considered when used to diagnose fungal infections. False positive serum *Aspergillus* galactomannan assays may exist in patients presenting with other fungal infections, in those taking β-lactam antibiotics or in presence of penicillium infections because of the possibility of cross-reactivity[23]. However, none of our patients were exacerbating or on antibiotics at the time of sample acquisition.

Nucleic analysis was revealed to be a more robust method of detecting presence of fungi and *A. fumigatus* within BAL of our asthmatic cohort. It was discovered that 21 patients tested positive for all criteria; total fungi by qPCR (Fungi Quant), *A. fumigatus* by qPCR and fungal detection by microscopy. 40 patients diplayed a positive total fungi qPCR result and fungal presence by microscopy, while 10 displayed fungal presence by qPCR alone. This suggests that while *A. fumigatus* is present within BAL of our asthmatics it is not the only fungal species present, as elucidated by our total fungi and microscopy results (Figure S3).

The results suggest, at present, the best indicator of fungal and *A. fumigatus* presence in BAL is qPCR (Figure S3-S5). Previous studies have reported *A. fumigatus* detection in sputum was associated with IgE sensitisation, airway neutrophilia and reduction in lung function. This study utilised fungal culture of sputum. Furthermore, *Aspergillus* was cultured in 63% of IgE sensitised patients, but also 31% of non sensitised patients. In this study of 79 patients, 89% were classified as GINA 4/5[24]. Our study included less severe asthmatics but higher numbers of mild and moderate asthmatics.

The presence of *A. fumigatus* in BAL, by qPCR, negatively correlated with macrophage counts. Alveolar macrophages are responsible for uptake and removal of inhaled conidia[25]. The ability of macrophages to kill *Aspergillus* species is dependent on LC3-associated phagocytosis via NADPH oxidase function. In human macrophages *A. fumigatus* has the ability to germinate in the late phagosome resulting in macrophage necrosis. Macrophages play a key role in protection of the host and their alteration or destruction can cause deleterious effects in the host[11]. We are uncertain as to whether the associated reduction in percentage BAL macrophages we observed is reflective of destruction by *Aspergillus* or simply a reciprocal decrease due to increase in neutrophils and fluxes in other cell percentages, but believe this warrents further investigation.

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Cytokines, intracellular signalling proteins, target specific cells causing consequences such as cell mediated immunity and allergic responses[26-28]. It was generally believed disequilibrium between pro-inflammatory Th1 and anti-inflammatory Th2 groups plays a role in the pathogenesis of allergic airway responses. However regulatory T cells (Tregs) producing cytokines such as inhibitory IL-10 and IL-17 producing Th17 cells make elucidating the pathogenesis of allergic disorders more complex[27-32]. Cytokines were chosen in order to investigate known cytokines of interest in asthma and to also examine the complex groups of Th1, Th2, Tregs and Th17 representative cytokines. Within our cohort it was seen that presence of fungi within BAL, as detected by qPCR, correlated positively with levels of a number of cytokines. Fungal presence within the lungs positively correlated with plasma IL-4, IL-6, IL-10, IL-13, IL-17 and TNFα and BAL IL-4, IL-6, IL-10, IL-13 and TNFα. It is therefore plausible that presence of fungi within the lungs of these patients has triggered an immune response resulting in upregulation of these cytokines[33-35]. Our study focused on detection of fungi rather than specific viable culture. Further studies to culture specific fungal species may be attempted in the future, however there are many difficulties in culturing fungi in clinical laboratories. It has been documented that culture identification can be significantly affected by the starting volume of sample and qPCR has been observed to be the most sensitive method of detection[36]

It is notable within our cohort, that presence of *A.fumigatus* in BAL, as detected by qPCR, positively correlated with BAL IL-6 and IL-13 levels and plasma IL-6, suggesting a systemic response.

Neutrophils are key players implicated in removal of fungal components within the lung through the use of phagocytic oxidative and non-oxidative mechanisms and through release of granules which mediate extracellular killing[34]. Patient BAL neutrophils correlated negatively with plasma IL-4 and TNFα and positively with BAL levels of the neutrophil chemoattractant IL-8 .Neutrophil presence also correlated with the presence of fungi by microscopy.

Macrophage cell percentage counts negatively correlated with BAL IL-10, IL-6 and TNF-α, and monocytes correlated positively with plasma IL-17, TNF-α, IFN-γ, and BAL IL-4. It is of interest that in our cohort the percentage of macrophages negatively correlated with BAL IL-10. IL-10, an anti-inflammatory cytokine, regulates lung inflammation and a decrease in IL-10 might be expected to be associated with potential worsening in clinical outcome[37].

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As in previous studies we found BAL eosinophil levels correlated with plasma and BAL IL-5 levels. IL-5 is essential for maturation of eosinophils in the bone marrow and subsequent release into the blood. Furthermore, IL-5 inhibition through monoclonal antibody therapy has demonstrated clinical effectiveness in treating patients with severe, eosinophilic asthma[38]. IL-17 has been highlighted as a key component of fungal immunity[35]. It is known that pulmonary epithelial cells possess IL-17 receptors which induce antimicrobial peptide and chemokine production and resulting Th17 cell induction and neutrophil recruitment[35]. Members of our group have previously demonstrated ability of IL-17 to upregulate release of several cytokines and chemokines from primary bronchial epithelial cells but in particular those associated with neutrophilia[39]. In our current study, we observed that BAL fungal presence by qPCR correlated with plasma IL-17. Patient BAL monocyte precentage counts positively correlated with plasma IL-17.

It has been recently highlighted by Hernández-Santos *et al*. that ability of innate immune cells to kill fungi is dependent on presence of NF-κB mediated production of chemokines by pulmonary epithelia, which is IL-1R dependant. This chemokine cascade orchestrates Th17 derived IL-17A and GM-CSF signalling which are known to aid phagocytic destruction of fungi[35].

Our results have demonstrated through both rapid microscopy and qPCR methods, that fungi and to a lesser extent *A. fumigatus* are present in lower airways of a large proportion of an adult asthmatic cohort regardless of their disease severity. Serological and antigen testing alone revealed a far lower level of positivity for *Aspergillus.* The presence of serum *A. fumigatus* specific IgE did not always predict presence of *A. fumigatus* by qPCR in patients BAL. Positive *A. fumigatus*-specific IgE was not linked to disease severity or control. It is therefore possible that while there are detectable fungal and *A. fumigatus* communities within the lungs of our cohort, perhaps this presence is not always provoking an active immune response. Furthermore, positivity for serum *A. fumigatus* specific IgE was not associated with a reduction in FEV1. Our findings appear to differ from previously published results suggesting that asthma patients sensitised to *Aspergillus* had lower FEV1[24].

FEV1 negatively correlated with BAL IL-5 and BAL galactomannan, however it should be noted that only 5/69 patients showed BAL galactomannan positivity. Of the 5 patients who tested positive for galactomannan only one was *A. fumigatus* qPCR positive. This appears to suggest that

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BAL Galactomannan may relate to airway fungal presence but not necessarily presence of *A.fumigatus*.

One of the weaknesses of the current study is the lack of control group which we aim to address in future work. This considerable undertaking is suggested by the results of our present study and will require a careful ethics case to be made, that will allows a generally safe but invasive procedure in volunteers. A strength of our study however was that it did include patients of all asthma severity and indeed patients on maintenance oral steroids and patients newly diagnosed and yet to receive any asthma-directed therapy. The decision to exclude patients with ABPA was made in an effort to specifically interrogate the current definition of SAFS which excludes this group. Further studies aimed at addressing this group are planned for the future. Fungal sensitisation is the sudden cutaneous hyperreactivity to fungal antigen or elevation of fungal specific IgE[40]. SAFS is a subphenotype of severe asthma[41]. Fungi are associated with severe asthma through fungal spore inhalation, sensitisation or the causation of Allergic Bronchopulmonary Mycosis[40].. Previous studies revealed numerous fungal species can cause sensitisation in asthmatics, however it is not yet known which is implicated to the greatest degree in SAFS and if it is prevalent within other subgroups of asthma[40]. It would therefore be of interest to investigate this further in order to interrogate the current definition of SAFS.

Further studies are required to assess whether or not *Aspergillus* within the asthmatic airway has a pathological role in the disease. Our study has shown an almost ubiquitous presence of fungi within the asthmatic airway regardless of disease severity, treatment taken and presence of systemic sensitisation. It has become apparent from this study that other fungal species are present within the lungs of our asthmatic cohort. Whether or not these fungi play a pathophysiological role or are mere bystanders has yet to be determined.

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Table

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Table 1: Patient demographics broken into groupings of mild, moderate and severe asthma as defined by GINA groupings Mild (GINA 1+2), Moderate (GINA 3) and Severe (GINA 4+5).

		Mild (GINA 1-2)	Moderate (GINA 3)	Severe (GINA 4-5)
Patients	n=69 (%)	24 (35%)	15 (22%)	30 (43%)
Male		11 (46%)	5 (33%)	11 (37%)
Female		13 (54%)	10 (67%)	19 (63%)
Smoker	Current	2	1	4
	Ex-	17	2	9
	Never	15	12	17
IgE (KU/L)	[Mean (SD)]	[354 (654.1)]	[178 (252.3)]	[317 (484.1)]
***A. fumigatus* IgE**	Positive	2	1	3
ACQ-7 †	[Mean (SD)]	(1 (0.6)]	[2 (1.0)]	[3 (1.1)]
FEV-1 (Percent Predicted) ‡	[Mean (SD)]	[86 (13.7)]	[88 (21.7)]	[66 (22.3)]
FeNO (ppb) §	[Mean (SD)]	[25 (24.6)]	[20 (14.0)]	[17 (25.3)]
BMI (kg/m²) ¶	[Mean (SD)]	[26 (4.5)]	[29 (6.8)]	[31 (6.7)]
BAL Galactomannan	Positive	3	0	2
Serum Galactomannan	Positive	0	0	1
BAL Fungal Microscopy	Positive	18	12	18
BAL Total Fungi (Cp)	Positive	20	14	25
	[Mean (SD)]	[34 (2.2)]	[34 (2.6)]	[36 (2.2)]
BAL *A. fumigatus* (Cp)	Positive	13	7	12
	[Mean (SD)]	[34 (3.4)]	[36 (1.4)]	[34 (2.6)]
BAL Monocyte % count	[Mean (SD)]	[8 (7.6)]	[6 (8.3)]	[4 (4.6)]
BAL Macrophage % count	[Mean (SD)]	[80 (10.20)]	[62 (27.4)]	[75 (20.1)]
BAL Lymphocyte % count	[Mean (SD)]	[7 (5.1)]	[9 (11.4)]	[9 (10.6)]
BAL Eosinophil % count	[Mean (SD)]	[2 (2.1)]	[3 (5.5)]	[3 (8.2)]
BAL Neutrophil % count	[Mean (SD)]	[3 (7.4)]	[14 (20.7)]	[5 (6.9)]
BAL IL-4 (pg/ml)	[Mean (SD)]	[0.5 (1.9)]	[0.1 (0.1)]	[1 (1.9)]
BAL IL-5 (pg/ml)	[Mean (SD)]	[0.2 (0.4)]	[0.7 (2.4)]	[2 (7.4)]
BAL IL-6 (pg/ml)	[Mean (SD)]	[2 (2.9)]	[4 (5.6)]	[10 (20.4)]

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BAL IL-8 (pg/ml)	[Mean (SD)]	[397 (843.2)]	[740 (1846)]	[236 (318.1)]
BAL IL-10 (pg/ml)	[Mean (SD)]	[0.7 (2.3)]	[0.2 (0.3)]	[1 (1.4)]
BAL IL-13 (pg/ml)	[Mean (SD)]	[4 (10.7)]	[3 (1.7)]	[6 (6.9)]
BAL IL-1β(pg/ml)	[Mean (SD)]	[8 (30.8)]	[5 (16.2)]	[5 (10.1)]
BAL TNF-α(pg/ml)	[Mean (SD)]	[1 (2.4)]	[0.4 (0.5)]	[2 (2.3)]
Plasma IL-4 (pg/ml)	[Mean (SD)]	[0.6 (1.6)]	[0.2 (0.1)]	[3 (5.1)]
Plasma IL-5 (pg/ml)	[Mean (SD)]	[0.6 (0.8)]	[0.8 (1.6)]	[0.9 (2.0)]
Plasma IL-6 (pg/ml)	[Mean (SD)]	[1 (1.8)]	[0.8 (0.9)]	[3 (4.3)]
Plasma IL-8 (pg/ml)	[Mean (SD)]	[5 (2.4)]	[8 (14.1)]	[13 (28.1)]
Plasma IL-10 (pg/ml)	[Mean (SD)]	[1 (3.0)]	[0.8 (1.2)]	[5(10.0)]
Plasma IL-13 (pg/ml)	[Mean (SD)]	[8 (16.6)]	[5 (4.3)]	[25 (35.9)]
Plasma IL-1β(pg/ml)	[Mean (SD)]	[0.6 (3.0)]	[0.1 (0.2)]	[1 (2.3)]
Plasma TNF-α(pg/ml)	[Mean (SD)]	[5 (11.8)]	[2 (1.3)]	[16 (24.9)]
Plasma IFN-γ(pg/ml)	[Mean (SD)]	[5 (2.4)]	[6 (4.4)]	[7 (8.9)]

Note † ACQ-7, Asthma Control Questionnaire; ‡ FEV-1, Forced Expiratory Volume in the first second; § FeNO, Fractional exhaled Nitric Oxide; ¶ BMI, Body Mass Index.

Table 1 Patient Demographics. Breakdown of patient demographics based on patient groupings of mild, moderate and severe asthmatics as defined by GINA groupings of GINA 1+2 = mild, GINA 3 = moderate and GINA 4+5 = severe with our results for each level of asthma severity shown.

Figure Legends

Figure 1 Microscopic evidence of fungal presence in asthmatic patient bronchoalveolar lavage (BAL). (A) BAL immune cell differential counts revealed macrophages to be the most dominant immune cell type present with varying levels of neutrophils and eosinophils present within the patient BAL. (B-C) Representative microscope images of patient BAL fluid stained with a commercial Kwik-Diff stain displaying fungal presence. Fungal hyphae (B) and conidia (C) detected at 40x. n=69

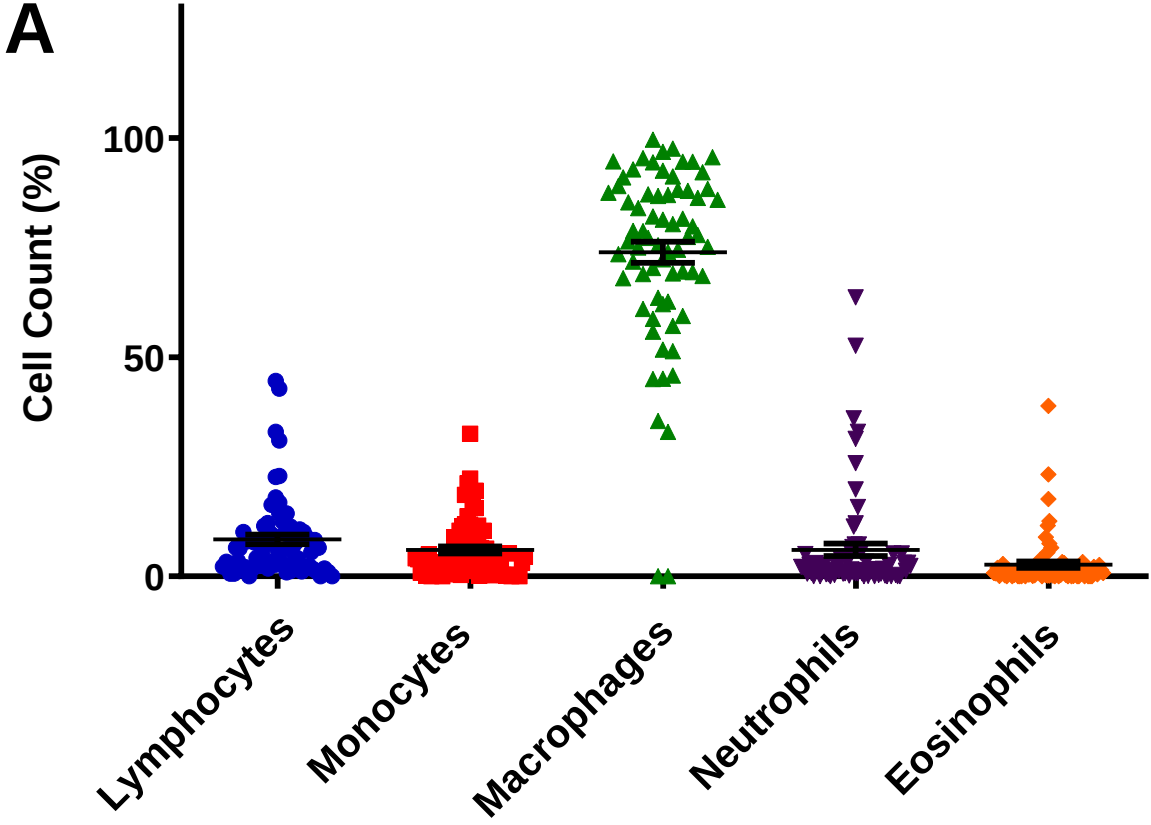
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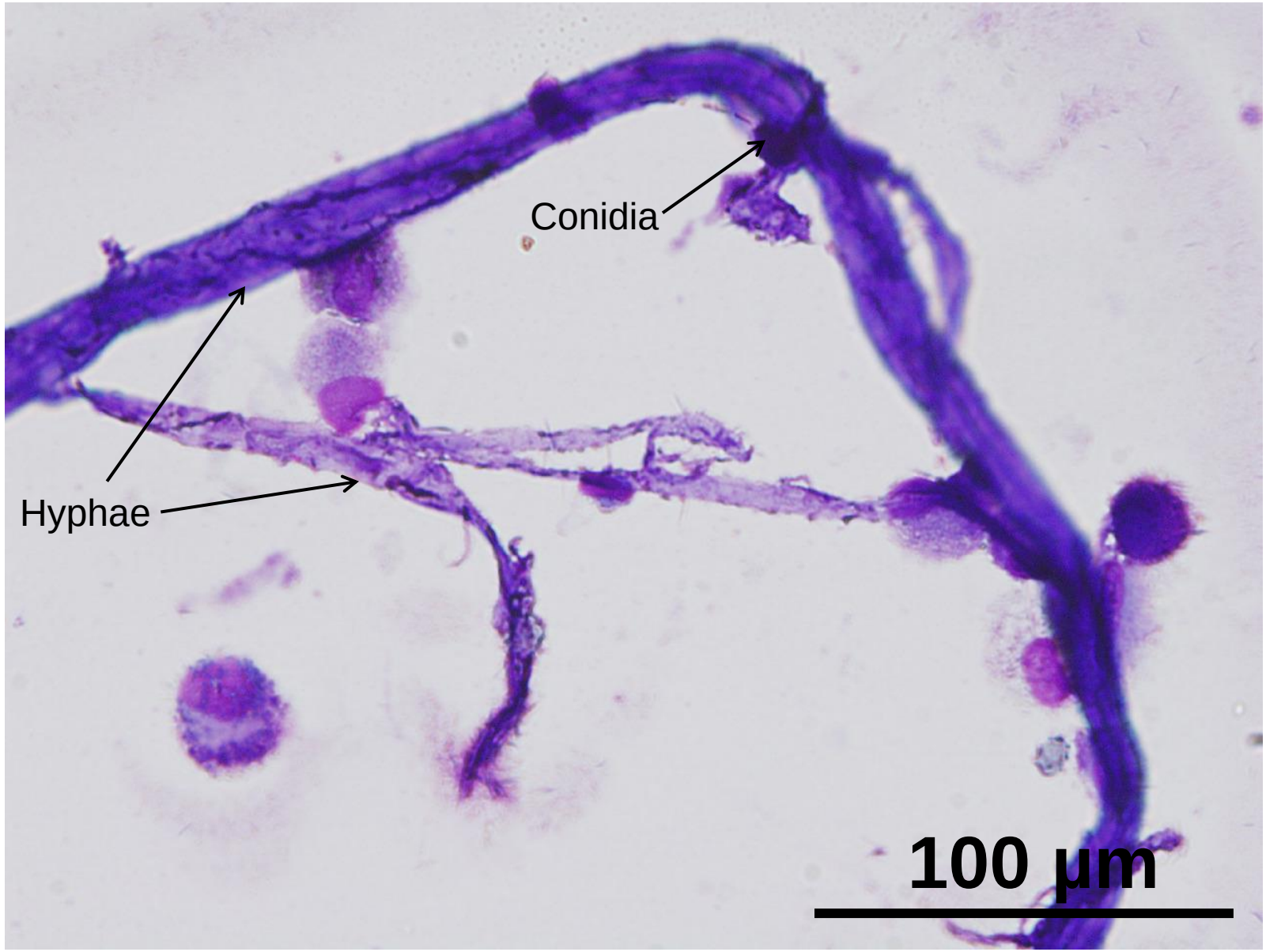
Figure 2 qPCR detection of Fungi in asthmatic BAL. (A) qPCR was performed on the extracted BAL DNA using the Fungi Quant, and *Aspergillus fumigatus* ITS specific primer; 59 patients (86%) had detectable fungi within their lungs and 32 patients (46%) had detectable *Aspergillus fumigatus* within their lungs. (B) BAL *Aspergillus fumigatus* qPCR levels were seen to correlate with macrophage immune cell percentage count levels ($P<0.05$; Spearman r = -0.4176). n=69

Figure 3 ***Cytokine Levels in the Patient Plasma and BAL*** Measurement of secreted human cytokines interleukin- 1β (IL-1β), IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IFNγ and tumour necrosis factor-α (TNF-α) were measured in patient serum and BAL using an electro-chemiluminescence QuickPlex SQ 120 imager from Meso Scale Discovery (Gaithersburg, MD, USA) where antibodies labelled with SULFO-TAG™ reagents emitted light upon electrochemical stimulation. Cytokines were detectable above the lower limit of detection set by the assay guidelines for each cytokine.

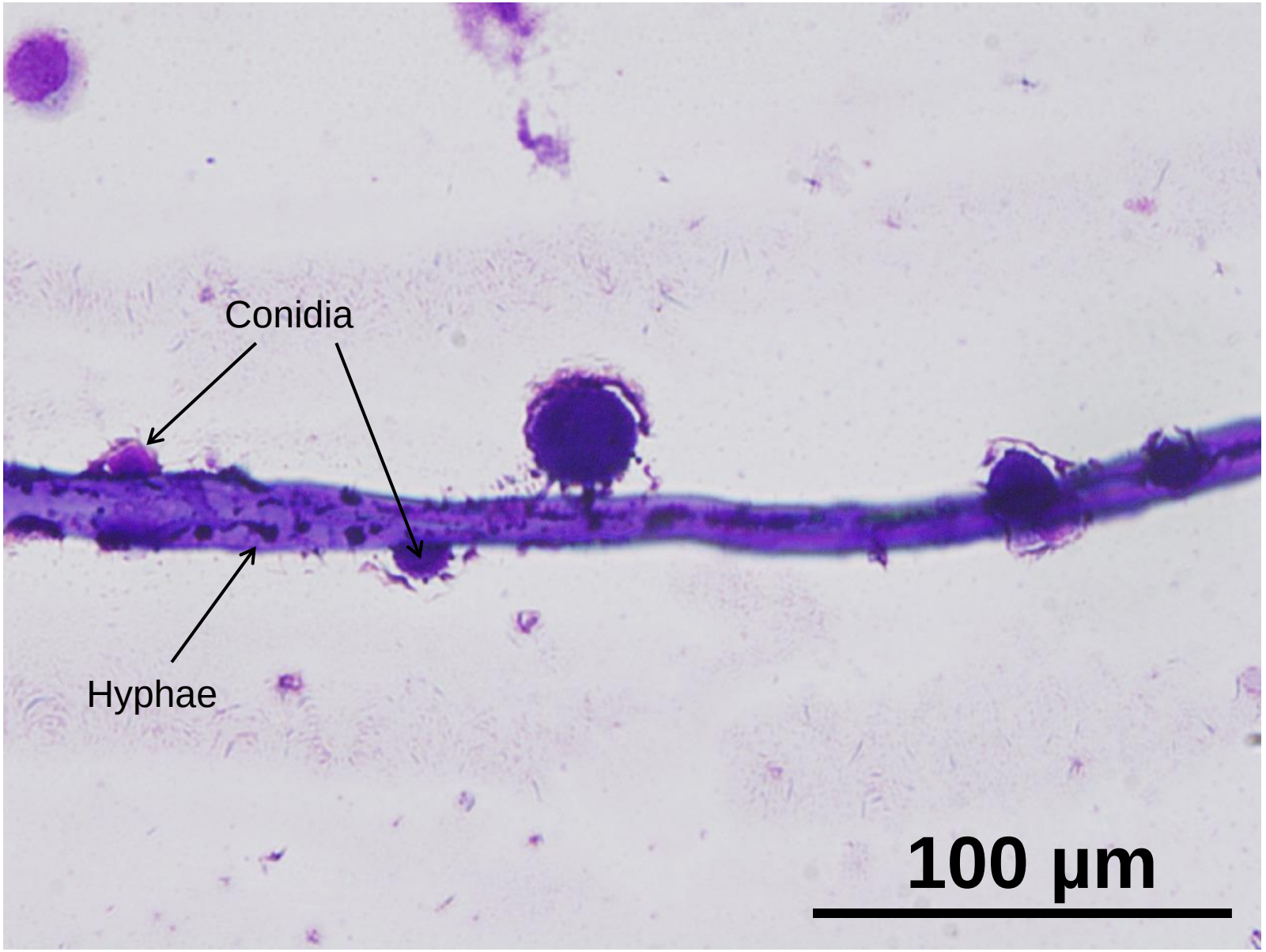
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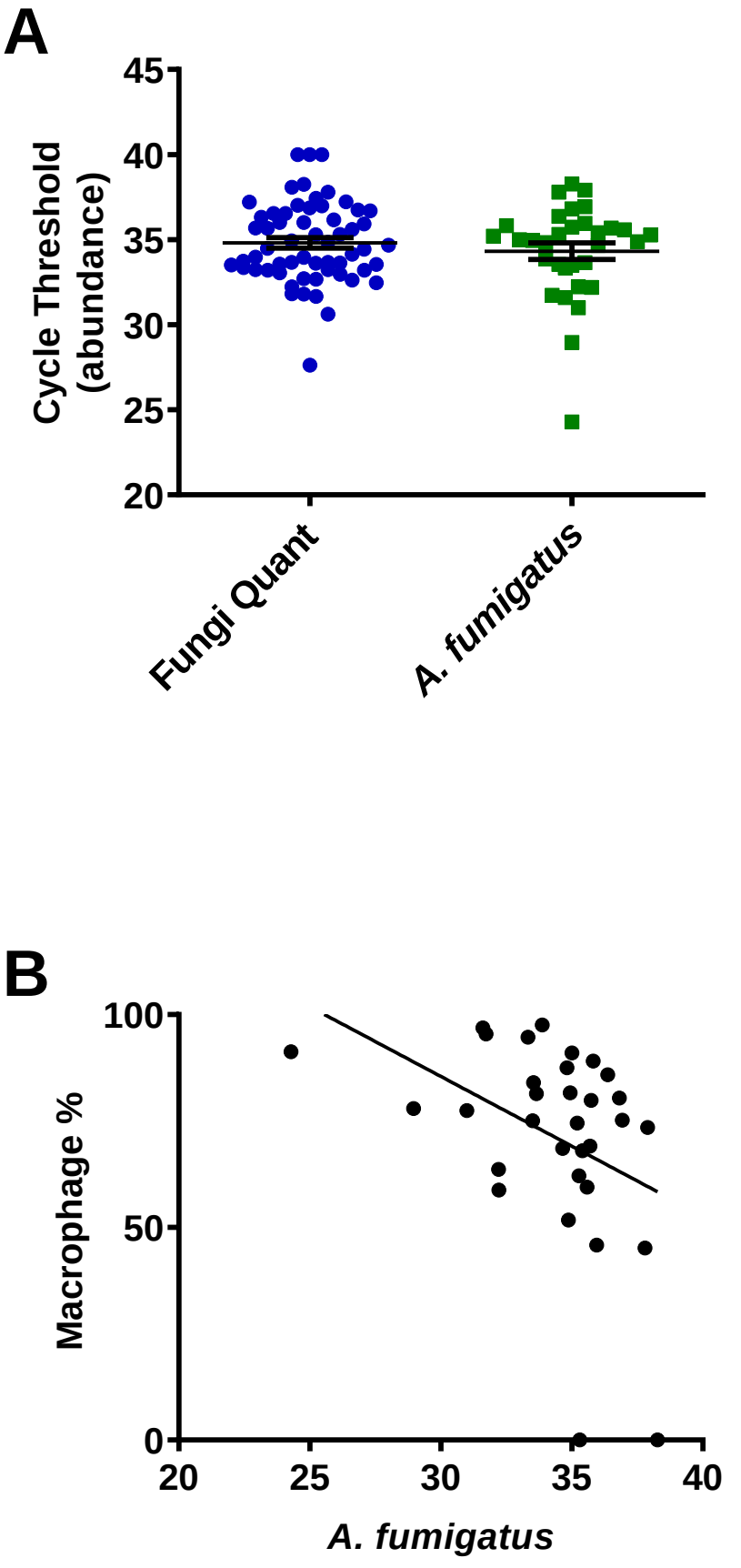
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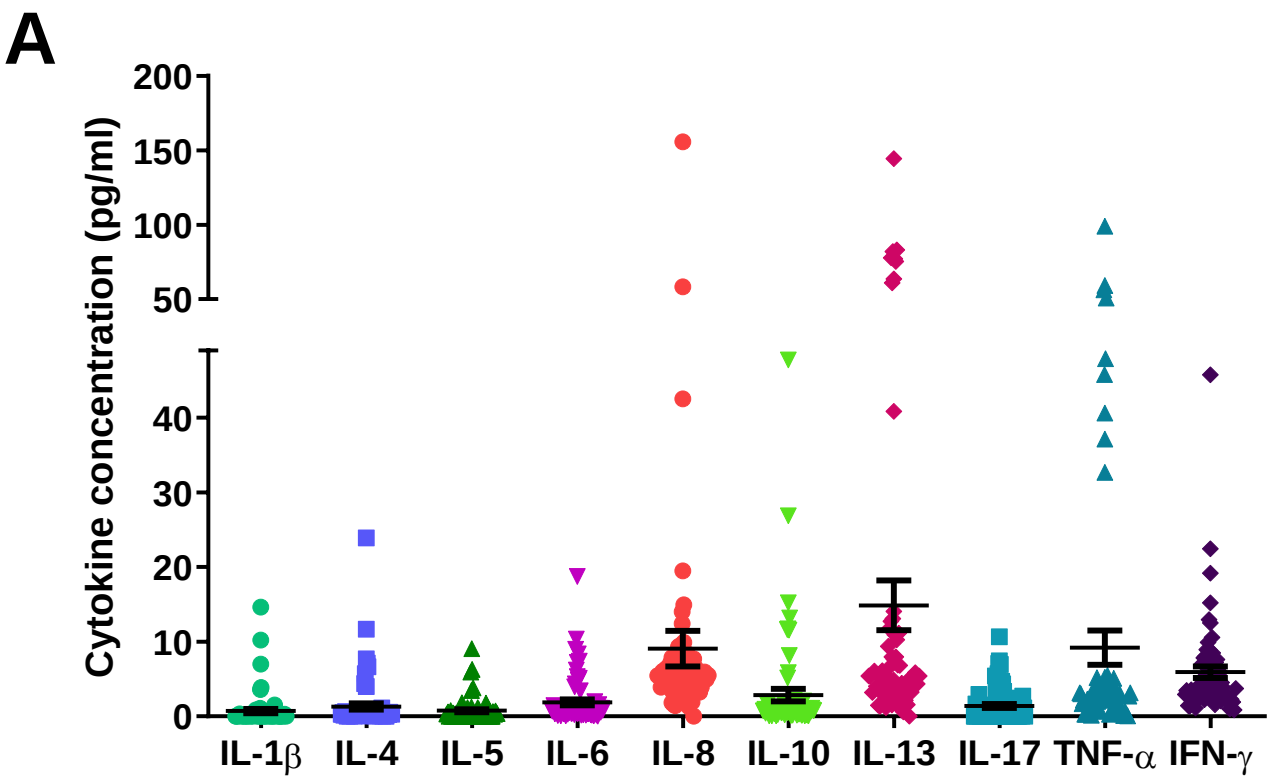

A
Cell Count (%)
100
50
0
Lymphocytes
Monocytes
Macrophages
Neutrophils
Eosinophils

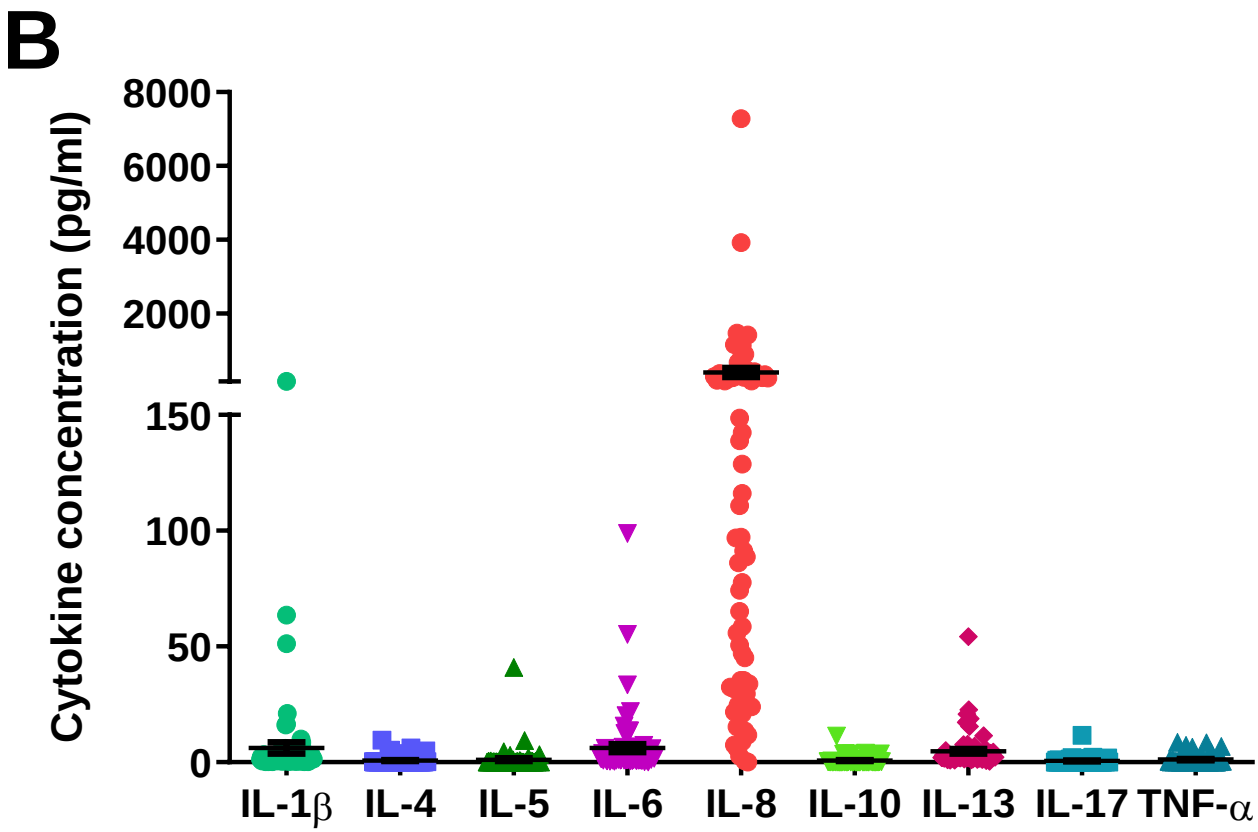

B
Conidia
Hyphae
100 µm

C




A
45
40
35
30
25
20
Cycle Threshold (abundance)
Fungi Quant
A. fumigatus
B
100
50
0
Macrophage %
20
25
30
35
40
A. fumigatus


A
Cytokine concentration (pg/ml)
200
150
100
50
40
30
20
10
0
IL-1β
IL-4
IL-5
IL-6
IL-8
IL-10
IL-13
IL-17
TNF-α
IFN-γ


B
Cytokine concentration (pg/ml)
8000
6000
4000
2000
150
100
50
0
IL-1β
IL-4
IL-5
IL-6
IL-8
IL-10
IL-13
IL-17
TNF-α