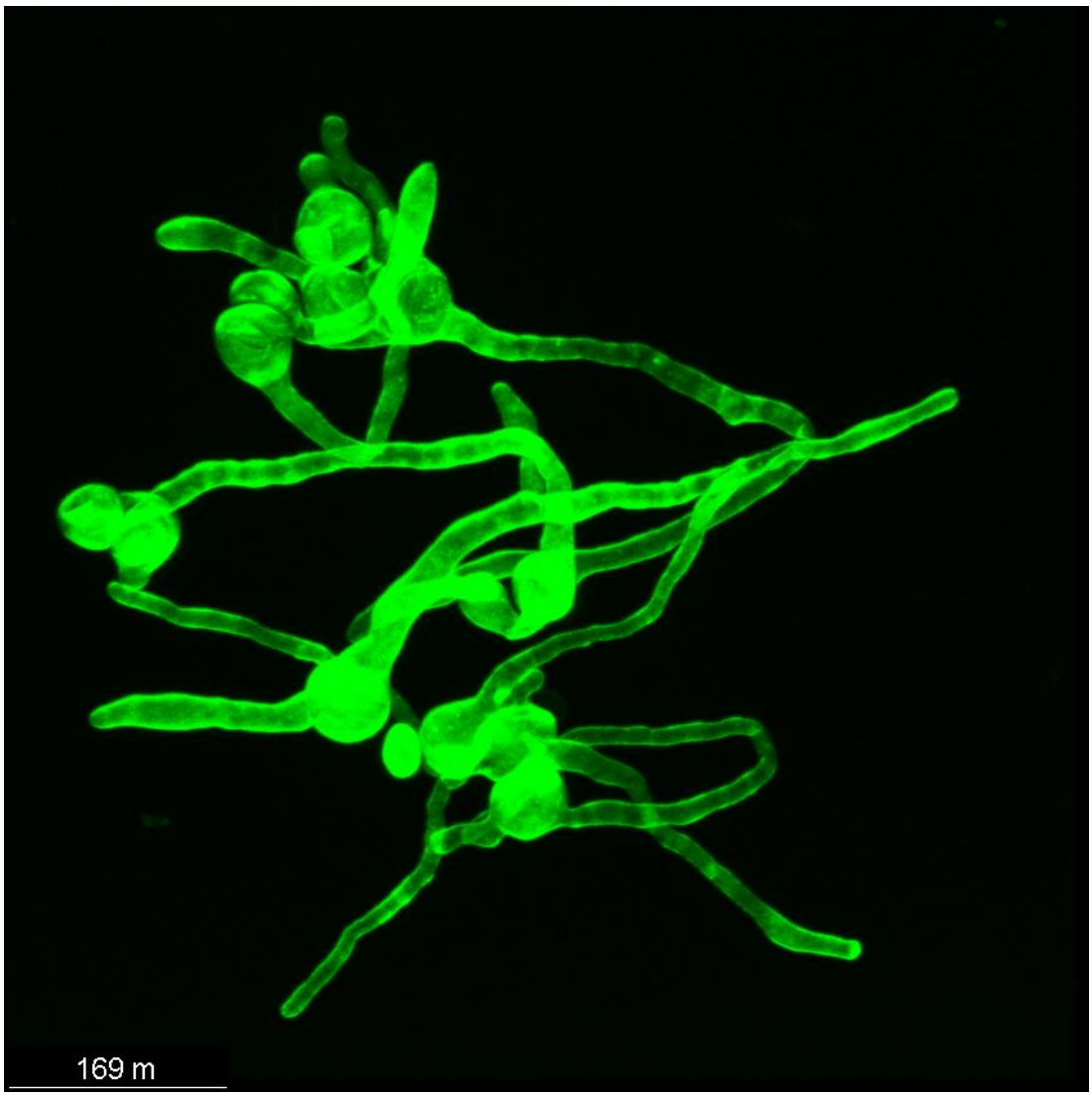
Exploring molecular mechanisms of antifungal activity of human serum



MASTER THESIS

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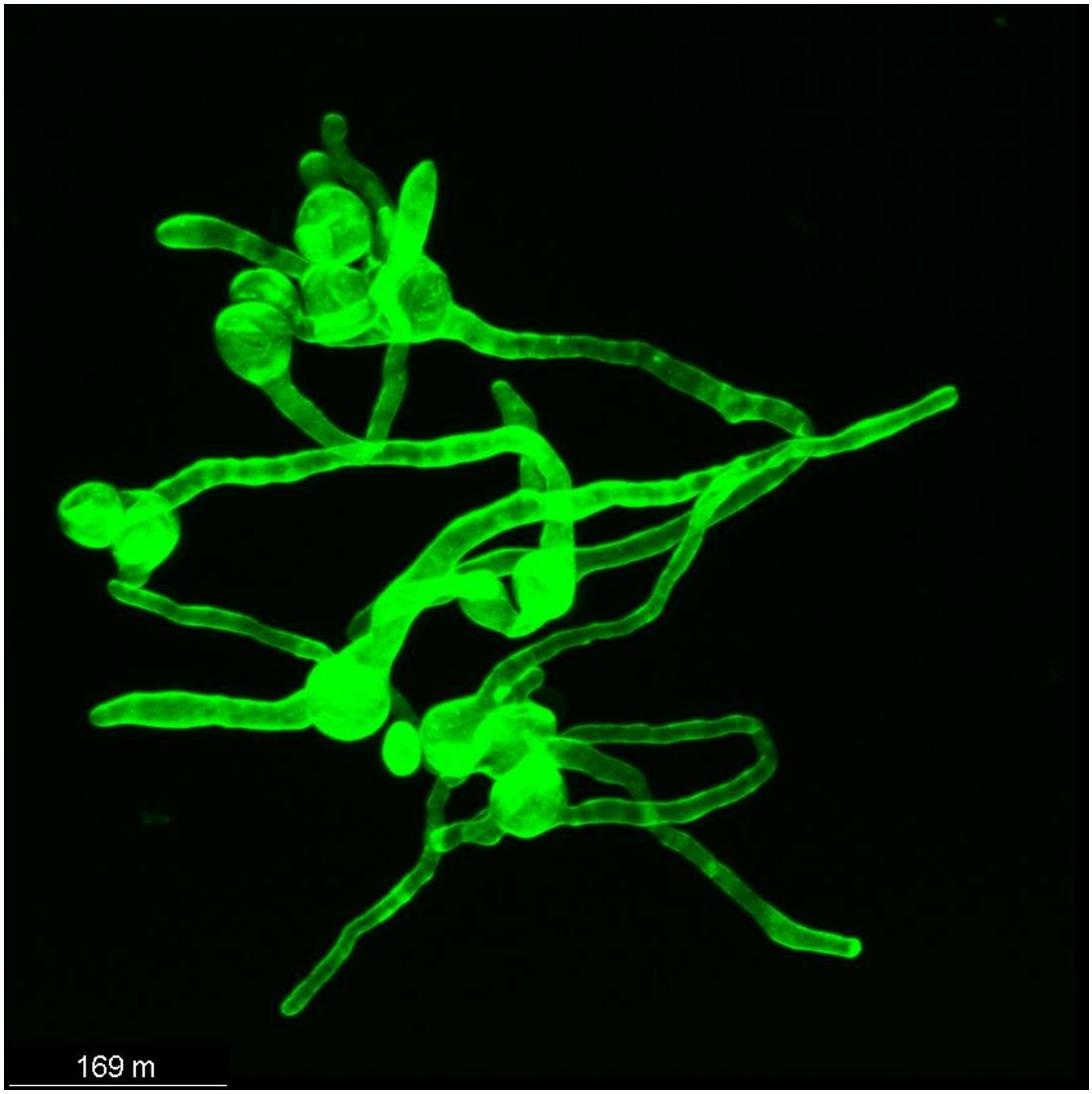
Εξερεύνηση των μοριακών μηχανισμών της καταπολέμησης μυκήτων από τον ανθρώπινο ορό



ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

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Abstract in Greek

Οι μύκητες είναι σαπροφυτικοί οργανισμοί οι οποίοι σπάνια προκαλούν λοιμώξεις σε ανθρώπους. Ωστόσο, ασθενείς με σοβαρή ανοσοκαταστολή μπορούν να προβληθούν από διηθητικές λοιμώξεις που προκαλούν κυρίως οι υφομύκητες *Aspergillus* και *Mucorales.* Η Μουκορμύκωση είναι μια ιδιαίτερα επικίνδυνη μυκητίαση με υψηλά ποσοστά θνησιμότητας τα οποία μπορούν να ξεπεράσουν το 80% σε κάποιες περιπτώσεις. Μεταβολικές διαταραχές, κυρίως η διαβητική κετοοξέωση και καταστάσεις υπερφόρτωσης σιδήρου, αποτελούν κύριους παράγοντες κινδύνου εμφάνισης της Μουκορμήκωσης. Παράλληλα, η μουκορμύκωση εμφανίζεται σε ανοσοκατασταλμένους ασθενείς με αιματολογικές κακοήθειες και μετά από μεταμόσχευση. Μέχρι στιγμής έχει δειχτεί ότι για την αντιμετώπιση της Μουκορμήκωσης από τον οργανισμό, είναι ζωτικής σημασίας η αναστολή της ανάπτυξης των σπορίων του μύκητα. Διαταραχές των φυσιολογικών αυτών μηχανισμών μπορούν να οδηγήσουν στην εκδήλωση της νόσου προκαλώντας καταστροφικές συνέπειες στον οργανισμό. Προηγούμενες μελέτες έχουν δείξει ότι ο ανθρώπινος ορός μπορεί να καταστέλλει την ανάπτυξη σπορίων διαφόρων μυκήτων. Αδημοσίευτα αποτελέσματα έχουν δείξει ότι χαμηλά επίπεδα αλβουμίνης του ορού σχετίζονται με την ανάπτυξη Μουκορμήκωσης σε αιματολογικούς ασθενείς. Σκοπός της παρούσας μεταπτυχιακής εργασίας ήταν μελετήσει αν η αλβουμίνη έχει άμεση αντιμυκητιακή δράση έναντι των μυκήτων *Mucorales* καθώς τους υποκείμενους μοριακούς μηχανισμούς. Δείξαμε λοιπόν ότι τα λιπαρά οξέα της αλβουμίνης αναστέλλουν την ανάπτυξη των μυκήτων και μειώνουν τη μολυσματικότητα τους. Τα αποτελέσματα της μελέτης μας υποδηλώνουν ότι η αλβουμίνη διαθέτει σημαντικές ανοσολογικές ιδιότητες με θεραπευτικές εφαρμογές στην αντιμετώπιση της μουκορμύκωσης.

Abstract in English

Fungi are saprophytic organisms, which rarely cause infections in humans. However, immunocompromised patients can be affected by invasive diseases caused by filamentous fungi (molds), mainly *Aspergillus spp.* and the Mucorales. Mucormycosis is a devastating disease with high mortality rates that can exceed 80% in disseminated disease. When compared to other fungal diseases mucormycosis has unique epidemiological and pathogenetic features. Metabolic abnormalities, including poorly controlled diabetes mellitus, acidosis, and iron overload syndromes, are unique risk factor for Mucormycosis development. Immunosuppression associated with treatment of hematologic malignancies and transplantation is also a major risk factor for Mucormycosis. Inhibition of intracellular and extracellular fungal growth is an essential host defense strategy against Mucorales. It has been shown that human serum can inhibit the growth of fungi spores via incompletely understood mechanisms. Unpublished data have demonstrate that low serum albumin levels are associated with development of Mucormycosis. In our work we explored whether albumin has direct antifungal activity against Mucorales spores. Herein, we found that free fatty acids bound to albumin inhibit germination of Mucorales spores, block the expression of major virulence factors and attenuate fungal pathogenicity in vivo. Our findings reveal a previously uncharacterized immunological function of albumin with potential therapeutic implications in management of mucormycosis.

Table of context

Introduction

Fungi are ubiquitous, saprophytic organisms of major ecological importance. The fungal kingdom consists of four phyla Zygomycota, Chytridiomycota, Ascomycota and the Basdiomycota. These phyla are composed of approximately 1.5 million fungal species. Importantly, the vast majority of fungi do not pose any threat to human health[1,2]. In fact, only 300 fungal species are pathogenic in humans, with most of them causing superficial infections (e.g., skin and nail disease). Tissue invasive fungal diseases are the most life-threatening fungal diseases and are caused by a handful of species that belong in one of four genera: *Cryptococcus*, *Candida*, *Aspergillus* and *Pneumonocystis* **(Table 1)**[3]. Invasive fungal diseases are associated with high mortality rates and enormous economic impact[4]. More specifically, human fungal pathogens kill as many people as tuberculosis[5] and malaria[6] affecting approximately one and a half million people every year. Airborne filamentous fungi (molds), including *Aspergillus* spp. and less frequently Mucorales, are emerging pathogens in an expanding group of patients with acquired immunodeficiencies.

Disease (most common species)	Location	Estimated life-threatening infections/ year at that location*	Mortality rates (% in infected populations)*
Opportunistic invasive mycoses			
Aspergillosis (*Aspergillus fumigatus*)	Worldwide	>200,000	30–95
Candidiasis (*Candida albicans*)	Worldwide	>400,000	46–75
Cryptococcosis (*Cryptococcus neoformans*)	Worldwide	>1,000,000	20–70
Mucormycosis (*Rhizopus oryzae*)	Worldwide	>10,000	30–90
Pneumocystis (*Pneumocystis jirovecii*)	Worldwide	>400,000	20–80
Endemic dimorphic mycoses*†			
Blastomycosis (*Blastomyces dermatitidis*)	Midwestern and Atlantic United States	~3,000	<2–68
Coccidioidomycosis (*Coccidioides immitis*)	Southwestern United States	~25,000	<1–70
Histoplasmosis (*Histoplasma capsulatum*)	Midwestern United States	~25,000	28–50
Paracoccidioidomycosis (*Paracoccidioides brasiliensis*)	Brazil	~4,000	5–27
Penicilliosis (*Penicillium marneffei*)	Southeast Asia	>8,000	2–75

Table 1: Statistics of the most significant invasive fungal infections.[3]

1.1 Invasive Aspergillosis

The most common invasive fungal infection in immunocompromised patients is aspergillosis. Invasive aspergillosis can exhibit mortality rates that exceed 50% despite antifungal therapy[7]. The most common risk factors for invasive aspergillosis include quantitative and/or qualitative defects in phagocytes. This is typically encountered in patients with acute leukemia who are on myeloablative chemotherapy or recipients

of hematopoietic stem cell transplantation in the setting of graft versus host disease[3,8].

Interestingly, over the last few years there is an increase in incidence of invasive aspergillosis (IA) in new groups of immunocompromised patients, traditionally considered as low risk for opportunistic infections[9] **(Figure1)**. Specifically, IA and other invasive molds infections are increasingly observed in patients with malignancies, autoimmune and inflammatory diseases who receive targeted therapies with biologicals and patients recovering from severe bacterial or viral sepsis. The mechanisms of pathogenesis of mold infections in these immunocompromised patients remain unknown.

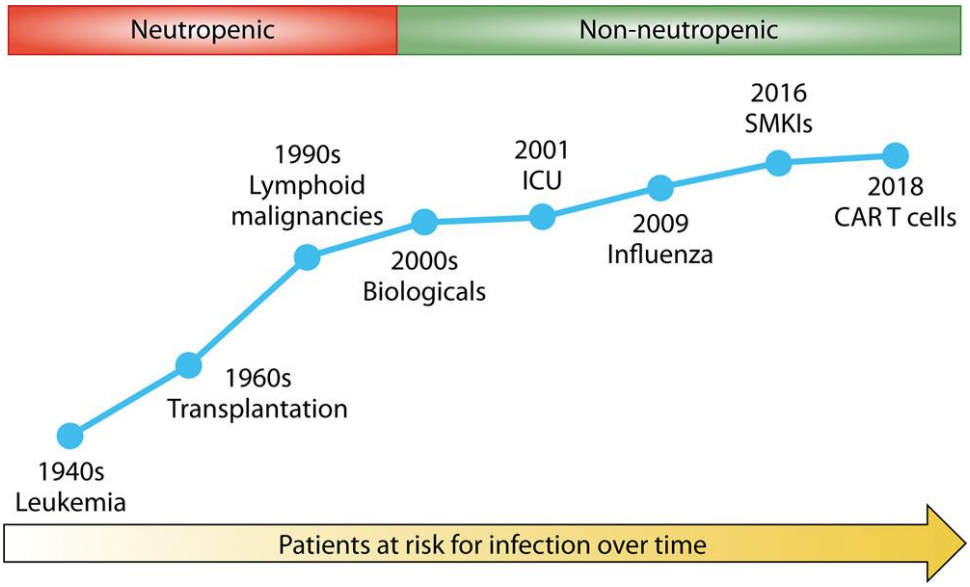

***Figure** 1:* **Epidemiological trends of invasive mold diseases.** Evolving groups of neutropenic patients at risk for aspergillosis development are those who are under immunosuppressive therapies for malignancies, autoimmune and inflammatory diseases. Moreover, other high risk patients are those with complex immunometabolic abnormalities and patients in ICU[10]. The past few years, the new high-risk groups that have emerged belong patients that are recovering from severe sepsis or are under small kinase inhibitors therapy or chimeric antigen receptor T-cells (CAR-T cells).

Treatment of invasive mold infections includes timely initiation of systemic antifungal therapy and surgical debridement of necrotic lesions when feasible. The therapeutic approach against aspergillosis now includes the use of the newer triazoles including voriconazole, posaconazole and isavuconazole accompanied with earlier initiation of the treatment mainly due to better diagnostic techniques.

1.2 Mucormycosis: an emerging fungal disease with unique epidemiology and pathogenesis

Mucorales belong to the phylum of Zygomycota and can reproduce both asexually and sexually in nature. However, during infection, asexual reproduction is the dominant form of fungal growth in tissue[11]. Specifically, inhaled conidia germinate to elongating forms (hyphae) that invade tissues. Sexual reproduction has not been observed in histopathological sections of patients with Mucormycosis so far.

Mucorales, like all the fungi, have also cell wall, which protects them from environmental stress. Moreover, apart from protecting the fungi from exogenous conditions, their cell wall is associated with their pathogenesis. Cell wall molecules are involved in tissue adherence, immune escape strategies and stimulation of host defenses including phagocytosis [12]. Compared to other fungi, Mucorales cell wall components and structure remain unknown. The outer layer of Mucorales cell wall is composed of melanin, whereas in the inner layer the polysaccharides that have been identified, so far, are chitin, manan and glucans.

The fungal cell wall has unique structural and biosynthetic components that make them excellent targets for antifungal drugs. Nevertheless, Mucorales exhibit increased resistance in antifungal drugs targeting their cell wall. This is probably due to a gene duplication at ergosterol synthesis pathway and other cell wall synthase enzymes, which pose common targets for antifungal drugs. Furthermore, Mucorales have an evolutionarily conserved amino-acid substitution in lanosterol 14a-demethylase, the enzyme targeted by azole drugs. Finally, Mucorales can exhibit resistance to drugs targeting their Calcinurin pathway by forming epimutants via an RNA-interference (RNAi)-based mechanism. This mechanism exploits RNAi-pathways and specifically silences the genes targeted by the dugs[13,14] . It is clear that Mucorales are highly resistant to drug-induced stress, which makes treatment of mucormycosis challenging.

1.3 Epidemiology of Mucormycosis

The last two decades Mucormycosis has emerged as the third most common fungal invasive disease in severely immunocompromised patients with hematological malignancy and transplantation, after *Candida* and *Aspergillus*. Mucormycosis is a life threatening disease with unacceptably high mortality rates of almost 50% and is caused by fungi in the order of Mucorales, predominately by *Rhizopus* species [15,16]. Specifically, inhaled Mucorales conidia that escape from immune surveillance germinate to elongating forms (hyphae) that invade epithelia and endothelial layers in tissue causing necrotizing disease, angioinvasion and hematogenous dissemination.

As opposite to Aspergillosis and other mold infections, poorly controlled diabetes mellitus is a unique risk factor for development of Mucormycosis. In particular, diabetic ketoacidosis (DKA) and other forms of acidosis markedly increase the risk for the disease. In addition, other metabolic diseases, including acquired iron overload syndromes, are associated with heightened risk for Mucorales infection. Of interest, patients on deferoxamine iron chelating therapy have unique predisposition for Mucormycosis. Other patients with acute or chronic metabolic abnormalities, including malnutrition and low birth weight, chronic alcoholism, liver cirrhosis, renal failure are also typically associated with Mucorales infections [16–20] .

It has also been observed that Mucormycosis occasionally occurs in immunocompetent patients following immunosuppression-induced by severe trauma, burns, surgery or sepsis [15,17]. Although iron deregulation and other immunometabolic abnormalities affecting myeloid phagocytes likely account for the unique susceptibility of all these patients to Mucormycosis, the underlying molecular mechanisms remain unknown.

1.4) Clinical manifestations of Mucormycosis

Acute respiratory infection manifesting as necrotizing sinusitis or pneumonia is the typical clinical form of Mucormycosis [15]. Angioinvasion, vascular thrombosis, and extensive tissue necrosis are cardinal features of the disease when compare to other fungal infections. Pulmonary Mucormycosis can remain localized to the lungs or extend to other tissues via either hematogenous dissemination or direct tissue invasion. Rhino-orbito-cerebral form usually originates from paranasal sinuses and is manifested with bone destruction and subsequent invasion of the orbit, eye and the brain[21]. Cutaneous Mucormycosis is the most typical manifestation of the disease following trauma, burn or other type of skin injury[22]. Patients with severe cachexia due to malnutrition and low birth-weight infants can develop gastrointestinal infections, which are associated with atypical symptoms (e.g., abdominal distention, bloody diarrhea) due to ischemia and intestinal necrosis and extremely high mortality rates.[23,24] . The overall mortality in Mucormycosis infections can be from 40% to higher than 80% (in case of disseminated disease), depending on the underlying condition and the site of infection[15]. Improved survival is related to earlier diagnosis, aggressive surgical debridement, reversal of the underlying host predisposing conditions and timely administration of antifungal therapy. Liposomal Amphotericin B is the best therapeutic option for Mucormycosis, whereas the newer triazoles have mediocre activity against Mucorales. Importantly, reversal of the underlying host factors, such as prompt correction diabetic ketoacidosis, is essential for disease outcome.

1.5) Physiological immune response against Mucorales

Mucorales are saprophytic organisms for humans. Despite constant interactions of fungal conidia with the host, physiological immunity in the respiratory tract prevents the development of disease. Lung resident macrophages comprise the first and most

important line of host defense against Mucorales. Furthermore, poorly understood extracellular host effectors mechanisms in the serum and brochoalveolar fluid inhibits germination of fungal conidia and invasive disease. In healthy individuals, phagocytes have differential immune response, depending on fungal state of growth. AMs can suppress the germination of *Ro* spores and thus preventing the formation of invasive hyphae[25]. However, they are unable to kill them. On the contrary, monocytes, macrophages and neutrophils can kill fungal hyphae by means of oxidative mechansisms[26]. Human neutrophils after encountering Mucorales hyphae trigger activation of Toll-like receptor 2 and Nf-kB pathways promoting a pro-inflammatory response[27]. Moreover, dendritic cells induce pro-inflammatory T_H17 responses by producing interleukin 23 through Dectin-1 pathway[28]. Physiologically, when Mucorales spores are phagocytosed by AMs, they remain dormant and exhibit prolonged persistence inside nascent phagosomes. Mucorales spores have the ability to retain their cell wall melanin, blocking the phagosome biogenesis and LAP and thus impeding their killing[29]. In addition, from the host's aspect AMs accomplish to inhibit the germination of Mucorales spores by restricting its available iron **(figure 2)**. Failure of nutritional immunity mechanisms in patients with abnormalities in iron metabolism allow the germination of the intracellular and extracellular spores promoting invasive tissue growth. However, the exact molecular mechanisms behind these abnormalities and how they promote Mucormycosis, have not been elucidated yet.

1.6) Virulent factors of Mucorales

Mucorales-host interactions are characterized by the endothelial tropism of the fungus and its ability to induce vascular thrombosis and necrotizing disease. *R. oryzae* spores and germ tubes can adhere to human umbilical vein endothelial cells and cause tissue damage in a manner related to phagocytosis[30]. The invasion of Mucorales is dependent to the glucose-regulated protein GRP78 that acts as a receptor[31] binding the Mucorales specific protein CotH[32] . Furthermore, hyperglycemia and increased iron levels in the serum have been found to increase the expression of GRP78, partially explaining why diabetes poses a major risk factor for Mucormycosis[31]. Moreover, another way that Mucorales can exploit their hosts and promote their virulence is through actively acquiring iron from them. Fungi can obtain iron using high-affinity iron permease (*FTR1*)[33,34]. Indeed, the use of antibodies against Ftrp-1 protected DKA mice from Mucormycosis. Thus, *FTR1* immunotherapy may be a promising strategy against this invasive fungal disease[35]. Furthermore, it has been observed that nonviable spores can induce damage to endothelial cells, suggesting that secondary fungal metabolites with toxin like properties play a role in Mucormycosis pathogenesis[30]. Indeed, rhizotoxin, which has been found to be produced by the Rhizopus symbiont bacterium *Burkholderia*, has a major role in virulence during plant infection but is indispensable for mammalian pathogenicity[36]. Recently, a ricin-like toxin with essential role in pathogenicity and tissue necrosis induced by the fungus was identified in Mucorales.

1.7) Disease immunopathogenesis

The exact mechanisms of Mucorales immunopathogenesis have not fully been elucidated yet. Studies in animal models and in patients showed that defects in number and function of phagocytes results in invasive disease. On the contrary, HIV patients do not seem to develop invasive Mucormycosis. This indicates that neutrophils but not necessarily T lymphocytes, are critical for inhibiting fungal spore proliferation[37,38]. Corticosteroids, diabetes mellitus, iron overload syndromes, sepsis and many other conditions that compromise the phagocytic and effector capacity of innate immune cells are risk factors for mucormycosis. All these immunometabolic defects have been traditionally linked to increased iron availability to the fungus. For example, during acidosis the capacity of transferrin to bind iron is reduced leading to an increase in free iron levels in the serum, which is essential for Mucorales growth[39]. Nonetheless, the effector mechanisms that inhibit the fungal growth in serum remain incompletely understood.

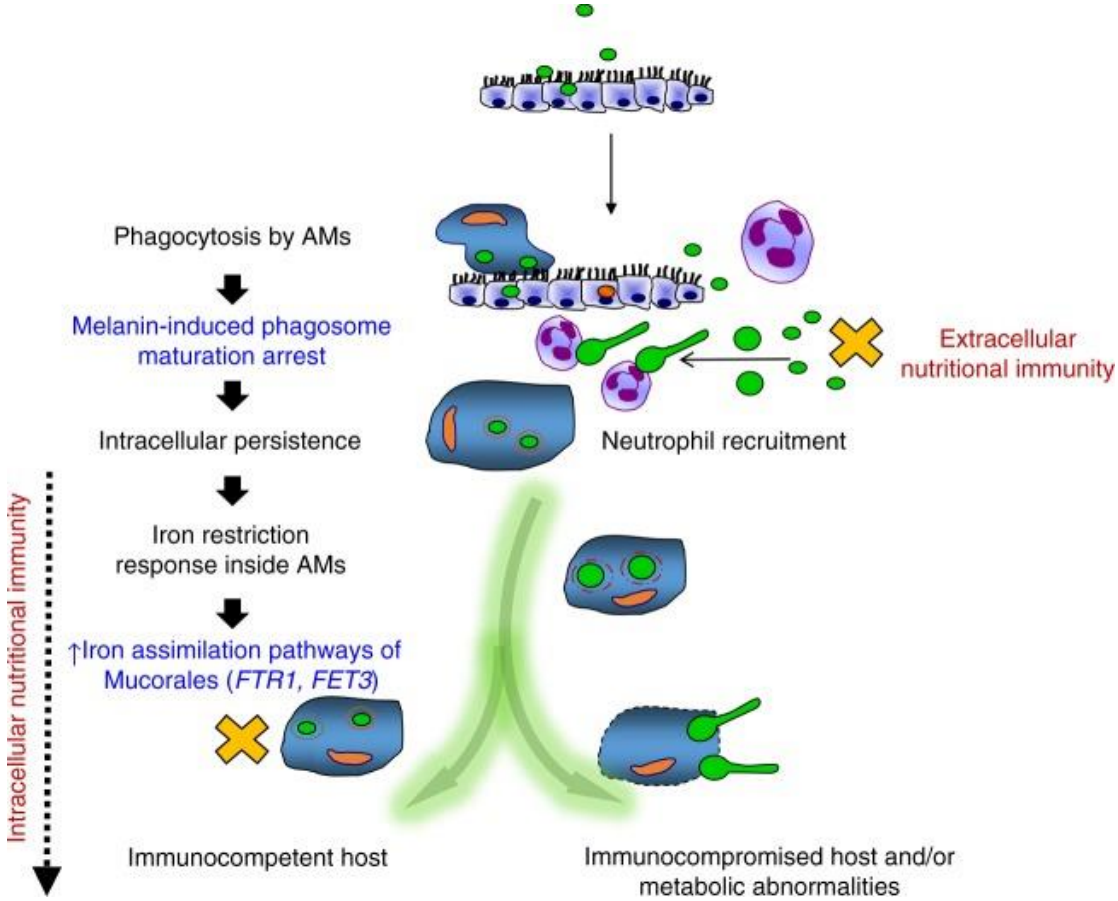

***Figure 2**:* **Proposed model of nutritional immunity inside macrophages against Mucorales**[29]**:** Intracellular conidia block phagosome formation and LAP by retaining their cell wall melanin. On the contrary, alveolar macrophages are able to inhibit the germination of the spores by restricting the available iron. Failure of nutritional immunity in patients with abnormalities on iron metabolism (DKA) allow the germination of the spores.

1.8) Antifungal effects of human serum

Mucorales spores that overcome the host-defense mechanisms, can germinate and promote invasive growth and potentially lethal disease. Thus, the most essential host defense strategy against saprophytic organisms, like Mucorales, is the inhibition of their growth. Studies in healthy mice revealed that conidia pre-incubated for a few hours comprises all antifungal mechanisms leading to death[29]. Therefore, understanding the physiological factors that restrict in vivo growth of saprophytic fungi and how their malfunctions lead to disease is of major clinical importance.

Several studies have shown that, human serum from healthy individuals exhibits antifungal effects[40]. For example, human serum can inhibit the growth of *Candida Spp.* and Mucorales and this effect is diminished in patients with hematologic malignancies[41]. Antifungal activity of serum has been largely attributed to nutritional

immunity via iron limitation mediated by transferrin. Transferrin can act as a chelator removing the available iron and thus inhibiting the fungi growth[42]. Moreover, this effect is diminished in sera from DKA patients or other forms of acidosis. Specifically, at the acidotic stage, transferrin has low affinity for iron binding, due to protonation and iron release at low PH levels, which promotes fungal growth[43].

However, several studies have indicated that human serum can exhibit fungistatic effects with mechanism other than iron deprivation [44]. Of interest, albumin is the most abundant protein found in human serum. Epidemiological studies for Mucormycosis imply that low serum albumin levels (2.3g/dL) correlate with worse disease outcomes[45]. Whether low albumin levels reflect broad metabolic abnormalities in patients with malnutrition and/or critical illness or have a causative link with development of mucormycosis is unknown. Importantly, albumin has been shown to possess antifungal activity against certain fungi via unknown mechanisms.

2) Aim of the study

The mechanism of antifungal activity of serum are incompletely understood. In unpublished work, we found an association between low albumin levels and development of Mucormycosis. Accordingly, we opted to investigate whether serum albumin has a direct antifungal activity against Mucorales and delineate the underlying molecular mechanism. A significant part of this study is related to the PhD thesis of Antonis Pikoulas. During my master thesis, I have performed an independent set of experiments to dissect the mechanisms of antifungal activity of serum albumin against Mucorales. In particular, we tested the role of albumin bound FFAs in antifungal activity of albumin in vitro, in human and bovine serum albumin. Next, we have tested the antifungal activity of purified FFAs of various length and chemical composition (saturated, unsaturated, and esterified). In addition, I was involved in experiments exploring the mechanism of modulation of fungal pathogenicity by albumin. Specifically, I have tested the effect of albumin on expression of major virulent factors during germination of Mucorales conidia.

3) Results

3.1) Selective depletion of albumin from human serum significantly reduces its inhibitory activity against *Rhizopus Oryzae*

Low albumin levels correlate with increased risk for Mucormycosis development (unpublished work). Therefore, we designed a set of experiments to evaluate whether albumin has direct antifungal activity against Mucorales. First, we depleted albumin in sera obtained from healthy volunteers and tested the effect on antifungal activity against *R. oryzae*. In order to selectively deplete albumin, we used affinity column chromatography with blue sepharose (see materials and methods) according to previously established protocols [46]. Importantly, we performed Coomassie blue staining on poly-acrylamide gel to validate specificity and efficacy of albumin depletion in sera **(Figure 4C)**.

Next, we tested the activity of human serum before and after albumin depletion following incubation with a standardized inoculum of *Rhizopus Oryzae* (10^4 conidia). Fungal growth was assessed at different time points visually and representative microphotographs were obtained at representative time points (5h) to assess the length of hyphae (**Figure 3 A-E).** We found that albumin depleted serum exhibits significant decrease in inhibitory activity against *R. Oryzae (hereafter RO)* compared to the untreated serum **(Figure 3 A-E)**.

Notably, albumin depletion might result in unspecific reduction of other serum antifungal effectors. In order to address this possibility, we purified human serum albumin from healthy volunteers and tested its antifungal activity. Specifically, we eluted fractions by affinity chromatography, validated the purity of isolated albumin and performed fungal growth assays with or without the presence of purified albumin in minimal growth medium that supports fungal growth[47]. *Rhizopus Oryzae (RO)* conidia that were incubated in the presence of the isolated albumin, had significantly less ability to grow compared to the control conidia **(figure 4)**. In order to be further ensure that this inhibitory effect of the albumin-containing media against *RO* was not related to the presence of an undetermined contaminant, we tested the ability of commercially available albumin of various sources to inhibit the growth of *RO* in vitro. We found that both bovine serum albumin (BSA) and human serum albumin (HSA) have the ability to inhibit *RO* at physiologically relevant concentrations present in serum (**Figure 7 D&E)**. Specifically, BSA exhibits IC50 at 17mg/ml and HSA at 28 mg/ml and these concentrations are much lower than the physiologically relevant

concentration of (35-45mg/ml. As far as *Aspergillus fumigatus* concerned, BSA exhibited some inhibitory effects but there were no evidence of complete inhibition of their growth at physiological concentrations. **(Figure 4 D&E)**.

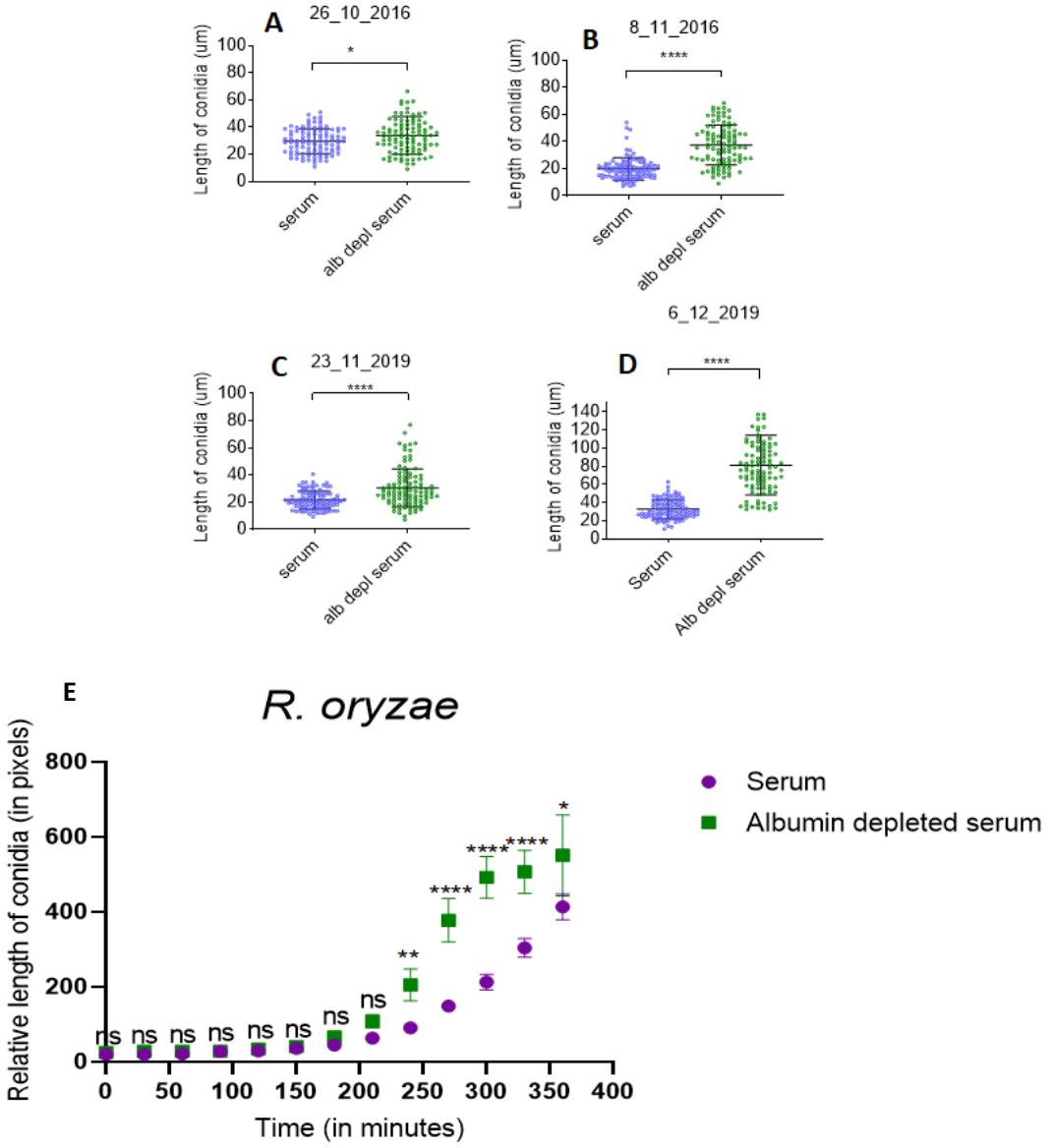

Figure 3: ***<u>Albumin depleted human serum exhibits reduced ability to inhibit growth of Rhizopus Oryzae conidia</u>*** ***A-D****). Human serum was collected from 4 donors and albumin was depleted via affinity column chromatography. Standardized incoculum of RO (10⁴ conidia/well) were grown in microtitare plates with serum or albumin depleted serum for 5 hours. Representative photos from different optical fields were obtained and the length of the germinating conidia from at least 3 was measured with Fujji Image J. Differences in fungal growth were compared statistically with the ± SD. **** P < 0.0001, *p<0.5,* Parametric Student's *t test.* ***E)*** *Representative photos from time-lapse videos of RO grown in media with or without purified albumin is shown. The relative length of RO conidia were measured with Fujji Image J amd are plotted in each condition as mean ± SEM. **** P < 0.0001, *p<0.5, ns=no significant,* Two-way anova multiple comparisons

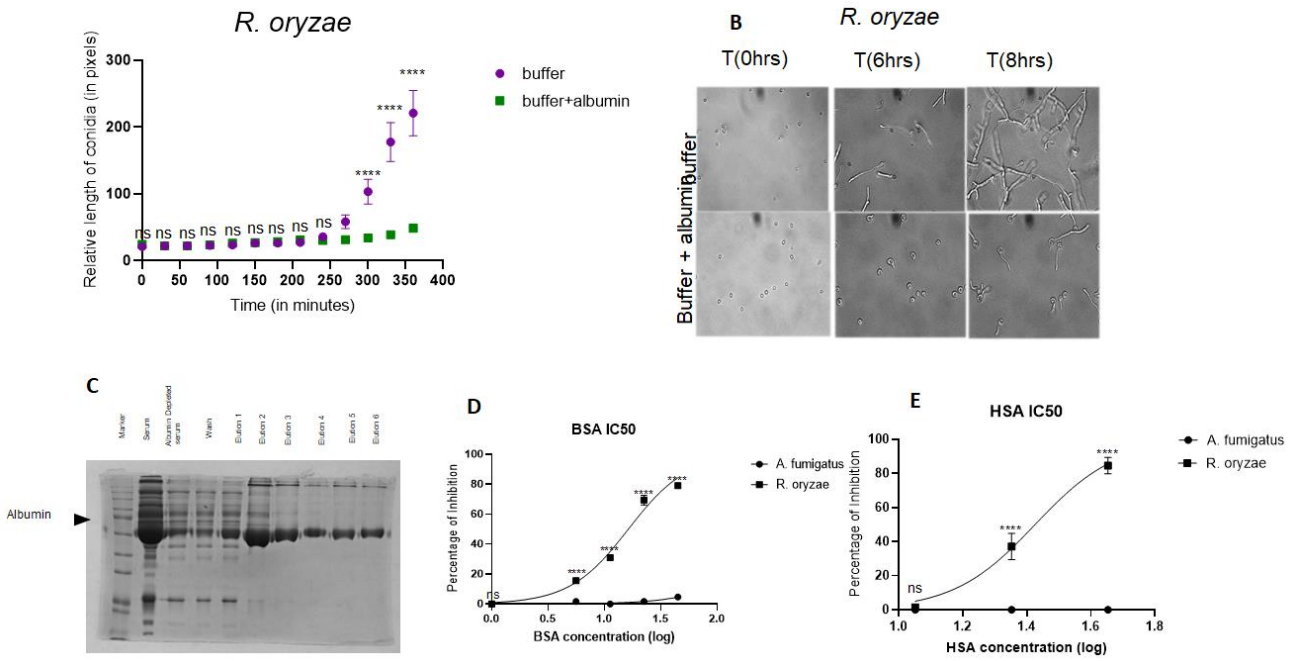

Figure 4: <u>Isolated albumin exhibits inhibitory capacity against Rhizopus Oryzae.</u> A). Human serum albumin was isolated with affinity chromatography using blue sepharose. 10^4 RO conidia were incubated in media containing either the minimal growth medium with or without human albumin(42mg/ml). The growth of conidia was assessed with live imaging and the relative length of conidia every 30 minutes , with Fujji image J. In each condition the differences are plotted as mean ± SEM. Differences in fungal growth were statistically compared with two-way anova multiple comparisons, **** P < 0.0001 ns=no significant. **B)** Images for three representative time points (0hr, 6hrs, and 8hrs) is shown. In the minimal requirement media RO conidia have grown until 8 hours. On contrary, in the albumin containing media their growth has been retained. **C)** Coomassie blue staining of an acrylamide gel containing the different experimental condition showing the purity ,as far as albumin concerned, in elution 2-6 which used in the experiments. **D&E)** Rhizopus and Aspergillus conidia were grown in RPMI 1640 media supplemented with different concentrations of either BSA or HSA. The IC50 for BSA was at 17 mg/ml and for HSA at 28 mg/ml as far as RO concerned. No IC50 could be calculated for Aspergillus Fumigatus. Represenative photos were taken and the germination of conidia is plotted as ± SEM and were statistically compared with Parametric unpaired Student's t test. **** P < 0.0001, ns=no significant

3.2) Albumin exhibits a specific fungistatic effect for Mucorales Spp.

Next, we evaluated whether albumin exhibits fungicidal or fungistatic effects against RO by testing viability of conidia following albumin removal from the medium. Thus, we cultured *RO* spores overnight either in the presence of RPMI or RPMI supplemented with 45mg/ml BSA. The next day we replaced the existing media with fresh RPMI and let them grow again overnight in order to assess their germination. Moreover, we had also spores that grew in the presence of RPMI supplemented with BSA for 48 hours. We saw that the spores grown at the presence of RPMI plus BSA,

could not germinate. On the contrary, the spores that grew in BSA supplemented media, which afterwards was removed, could exhibit normal growth. **(Figure 5)**. Concluding, we showed that albumin exhibits a significant fungistatic inhibitory activity against Mucorales. Notably, albumin activity was selective against Mucorales as it was not observed against other fungal or bacterial human pathogens (the same set of experiments were done with other fungal and bacteria species by Adonis Pikoulas, data not shown).

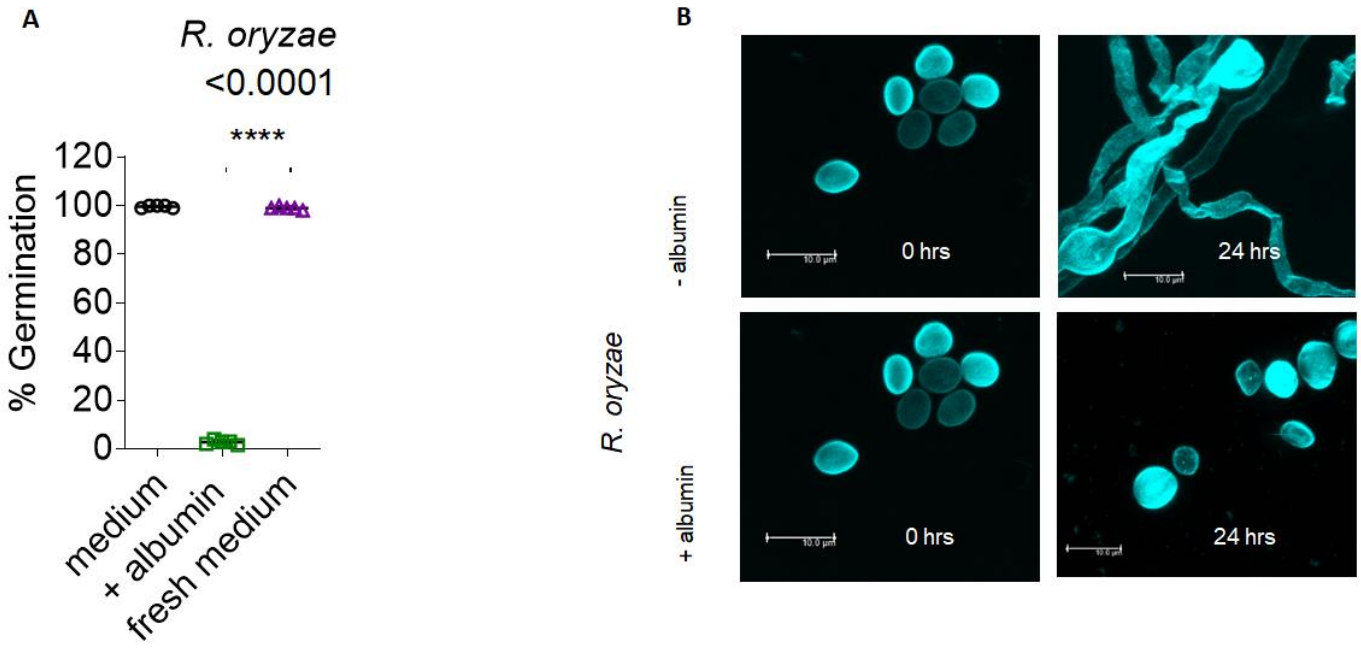

Figure 5: *Albumin has a selective inhibitory activity against Mucorales Spp.* ***A)*** *Rhizopus conidia were grown overnight in the presence of either RPMI or RPMI supplemented with 45 mg/ml BSA. The next day the fungi were washed twice with PBS, new medium without BSA was added, they again cultured for another day, and then representative photos from different optical fields were obtained and the germination ability of Rhizopus was plotted at* ± SD and statistically compared with unpaired Student's t-test ****p-value<0.0001,. ***B)*** *Rhizopus pre-stained conidia with fluorescent brightener 28 were cultured overnight with either RPMI or BSA and then they were examined under Leica SP8 inverted confocal microscopy.*

3.3 Albumin renders *Rhizopus Oryzae* avirulent in vivo

Next, we evaluated if albumin antifungal activity has relevance in vivo; thus we challenged 8-12 week-old C57BL/6(B6) mice with $2.5X10^6$ swollen conidia via intra-tracheal installation. Conidia were grown until the swelling stage, either in RPMI medium supplemented with 2% glucose or in the same medium supplemented with 45mg/ml BSA (see materials and methods). Surprisingly, mice challenged with the conidia that were swollen in the presence of BSA, showed significantly less mortality compared to mice that were infected with control spores. Moreover dormant conidia, could not induce mortality in mice [29]. (**Figure 6A)**. This reduction in the mortality upon albumin pre-exposure was accompanied with decreased inflammation and the

absence of invasive fungal growth; in contrast, infection with swollen conidia grown in media without BSA resulted inflammatory infiltrates and invasive fungal disease **(Figure 6B),** findings consistent with previous work of our group[29]. Therefore, albumin has the ability to render conidia much less virulent than they normally are. Based on this, we (experiments performed by A.P. for his ongoing PhD thesis) did RNA sequence analysis in the conidia grown in the presence of albumin in order to compare their transcriptome with that of control-conidia grown in regular culture medium without albumin. From the RNA-seq analysis he found that albumin down regulates the expression of several virulence factors related with the iron acquisition from the host (FTR proteins and siderophores)[48]. Moreover, in the conidia swelled in the presence of albumin we found reduced expression of proteins directly related with *RO* virulence like **Cot-H** and **Ricin**. Cot-H is a protein that binds specifically in the GRP78 receptor and helps in the invasion of Rhizopus through the endothelium [31,32,49]. Moreover, Ricin is a exotoxin that is found in the plants and fungi and is related with endothelial cells necrosis by inhibiting protein synthesis through ribosomal inactivation [50]. Then we tried to confirm the downregulation of Ricin expression levels with staining with rabbit anti-Ricin antibody by quantification of mean fluorescent intensity (MFI) **(Figure 6C&D)**. We also did staining for Cot-H3 protein in order to validate its expression levels but we had inconsistent results, as the range of expression was too wide **(data not shown)**. Both Ricin and Cot-H3 stainings were conducted with the BSA filtration flow-through, acquired by using Amicon Ultra Centrifugal filters (see materials and methods) with molecular weight cut-off 50 kDa, to avoid the high background signal acquired in BSA treated conidia.

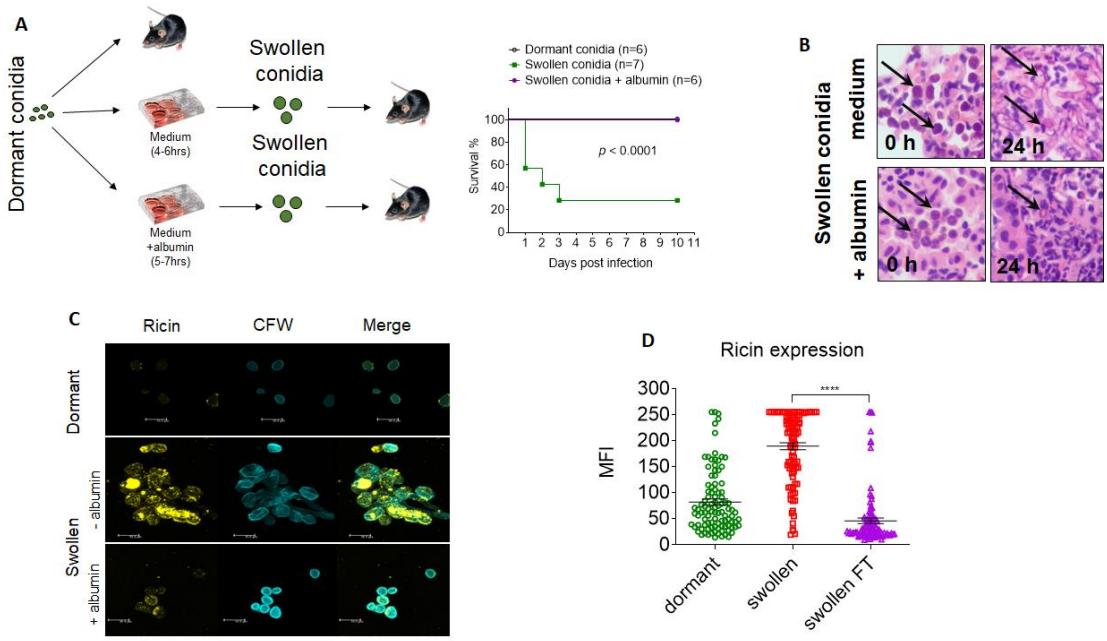

***Figure 6*: Albumin reduces the pathogenicity of Rhizopus Oryzae in vivo A)** 8-12 week-old female and male C57BL/6(B6) mice were infected with $2.5X10^6$ conidia which were grown till the swollen stage in either RPMI medium or in RPMI medium supplemented with 45 mg/ml BSA, The nonparametric log-rank test was used to determine differences in survival times . **B)** Sections from paraffin embedded lungs stained for hematoxylin and eosin plus PAS staining for the fungus. In the lungs from mice which were infected with BSA treated conidia much less inflammation and filamentation could be observed, the Arrowhead shows an area with characteristic tissue invasion by *R.o.* **C&D)** Confocal microscopy for quantification of the expression of Ricin. Representative photos from different optical fields were obtained and the MFIs measurements were plotted as *mean ± SEM and compared statistically with unpaired students T-test. **** P-value <0.0001,*

3.4) Fatty acids bound to albumin mediate antifungal activity against Rhizopus Oryzae

Next, we wanted to investigate the mechanism of antifungal activity of albumin against Mucorales. Albumin is the sole carrier of free fatty acids (FFAs) in the human serum[51,52]. FFAs are well known antimicrobial effectors [53]. Thus, we tested if FFA bound to BSA are responsible for its fungostatic activity. Firstly, we treated 10^4 *RO* conidia with different types of BSA **(figure 7A)**. We found out that BSA without free fatty acids could not retain the growth of conidia. Moreover, diabetes is a unique predisposing factor for the development of Mucormycosis. The elevated levels of glucose in the diabetic patients' serum have been shown to induce glycosylation of serum proteins, such as hemoglobin and albumin. Glycosylation of albumin in diabetic patients has been shown to reduce its capacity for FFA binding [54]. Thus, we treated *RO* 10^4 inocula with glycosylated albumin and we showed that the albumin glycosylation reduced its ability to inhibit the growth of *RO* conidia in vitro. Moreover, we wanted to see if oxidation of the albumin could also exhibit the same effect. Surprisingly, we showed that oxidized albumin could retain the ability to inhibit the growth of *RO* conidia compared to vehicle treated albumin **(figure 7A)**. Next, we observed that apart from albumin, its flow through could also inhibit the growth of *Rhizopus Oryzae* **(Figure 7B)**. Albumin flow-through was obtained using using Amicon Ultra Centrifugal filters (see materials and methods). Thus, we speculated that some of the free fatty acids bound in the BSA could be released upon filtration. In order to investigate this hypothesis we performed fractionation of the BSA flow through (see materials and methods) in order to see which cluster possesses the fungistatic effect. We found out that only Fraction 2 could inhibit the fungal growth in vitro **(Figure 7C)**. GC-MS analysis of the fraction 2 revealed that it is mainly composed of Caprilic **(figure 7D&E)**. Thus, we concluded that BSA fungistatic effects could be mainly attributed to Caprilic.

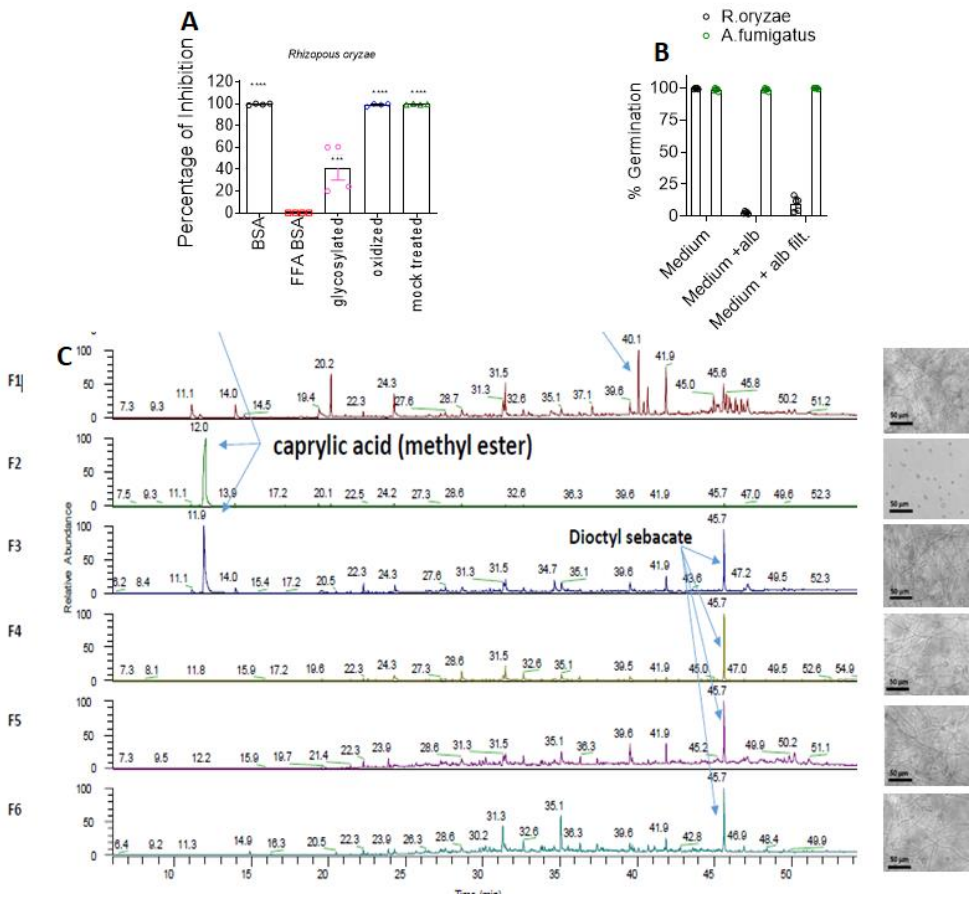

Figure 7: ***<u>Free fatty acids bound to albumin inhibit the growth of Rhizopus Oryzae</u>****. **A)** 10^4 Rhizopus Oryzae conidia were treated in vitro with different types of albumins. FFA albumin and glycosylated albumins exhibited less inhibitory capacity compared to regular BSA and vehicle treated BSA (mock-treated BSA) which serve as their control respectively. Representative photos from different optical fields were obtained and the RO germination was measured after over night incubation and were compared statistically with* Parametric unpaired Student's *t test for the ± SD. **** P < 0.0001, ***p<0.001. **B)** 10^4 conidia of RO and AF were treated with BSA, BSA flow-through or RPMI 1640 and their germination was assessed. Flow-through exhibits the same inhibitory capacity against RO compared to BSA. **C-E)** Fractionation of BSA flow-through revealed that only fraction two has inhibitory effect against RO. According to GC-MS analysis the fraction two is the most abundant in caprilic.*

3.5 Antifungal activity of pure free fatty acids

The transport of FFA in the human plasma is of high importance as it makes lipids from fat depots, like adipose tissue, available for energy production from all cells. FFA exist in plasma primarily as anions and are "free «only in the sense of not being bound by covalent linkages. Less than 0.01% of FFAs are actually free in solution. Only a small

fraction of FFA transported in the serum are bound to lipoproteins and thus the albumin-FFA complex has high biological significance[55,56].

Albumin serves as the main carrier for middle and long-chain free fatty acids. Therefore, we tested the in vitro inhibitory capacity of the main serum FFAs[57]. Moreover, we also tested the fungistatic effects of small-chain FFA which are known for their antimicrobial effects [53]. The free fatty acids were obtained commercially (99% purity) and serially diluted in organic solvent (5% EtOH). Small chain free fatty exhibited high inhibitory activity against *RO*, but as far as the normal range of concentrations found in human serum concerned, we showed that the middle and long-chain free fatty acids also exhibited inhibitory effects **(Figure 8 A&B)**. Palmitic and oleic , the most abundant free fatty acids found in the serum[58] exhibited IC50 at 118μM and 85 μM respectively, which are within the range of physiological concentrations in human serum. Moreover, the complexation of BSA FA free with several free fatty acids also restored the inhibitory effects indicating that the free fatty acids bound on the albumin possess the anti-fungal activity **(Figure 8C)**.

The above-mentioned data demonstrate that, FFAs in the concentrations found normally in human serum are able to inhibit the growth of *RO*. Moreover, the fact that free fatty acids diluted in RPMI without any ethanol to facilitate their dilution, exhibit no inhibitory effect in the same concentration that they normally do, points out that they need a dilution agent. Moreover, when long chain free fatty acids are diluted in the presence of BSA FA free, which facilitates their dilution, their inhibitory effects are restored[59].Thus, we concluded that the free fatty acids found in the serum need albumin as dilution agent in order to exhibit their inhibitory role against *RO*.

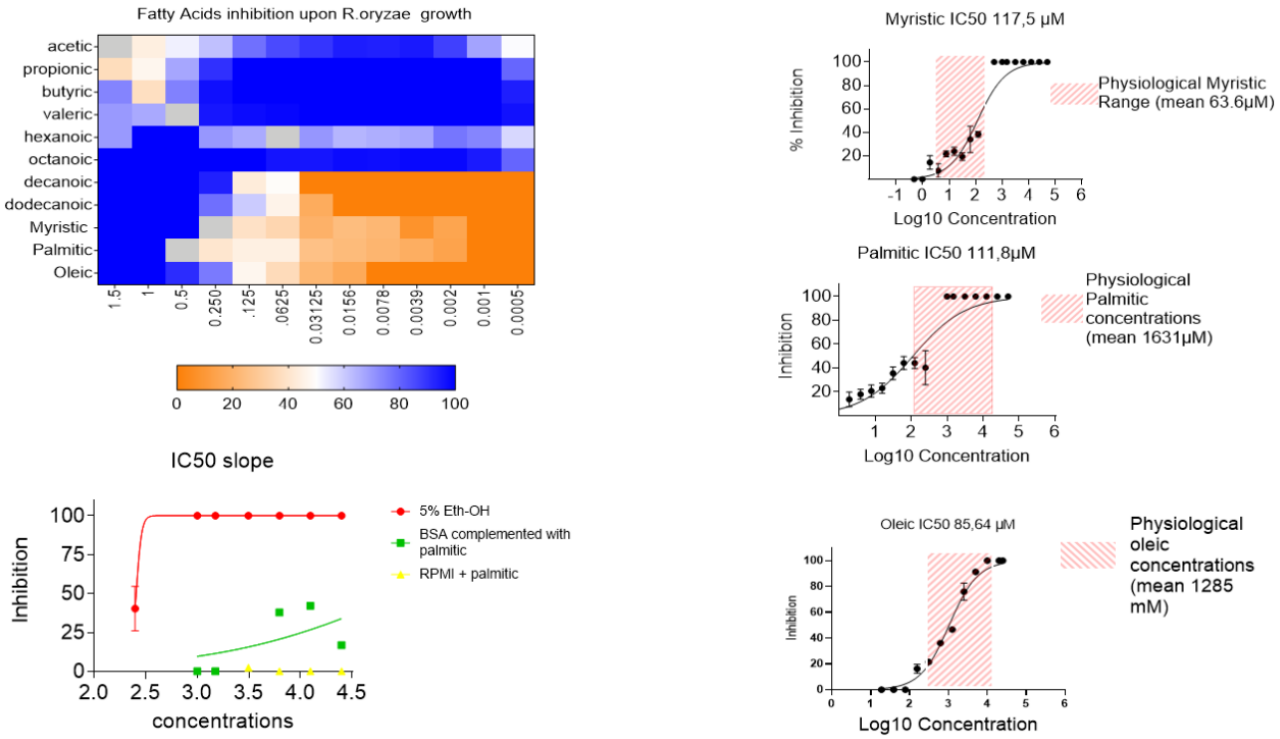

Figure 82: ***<u>Concentration gradient for the pure FFA inhibitory abilities.</u>*** **A)** *Short, middle and long-chain Free Fatty Acids concentration gradient for fungistatic activity was assessed in representative photos. Short chain free fatty acids exhibit good inhibitory activity.* **B)** *Middle and long chain pure free fatty acids have inhibitory activity with IC50 between the concentrations found normally in the human serum*. **C)** BSA FFA free complexation with palmitic. FFA BSA free was complexed with palmitic in 4.5/1 ratio FFA/BSA and its inhibitory capacity was compared with 5% EtOH plus palmitic and RPMI plus palmitic

3.6 Reduced inhibition of *Rhizopus Oryzae* growth in the serum of patients at risk for Mucormycosis

Mucormycosis is an opportunistic infection affecting mainly patients with metabolic abnormalities who have low albumin levels in their serum[45]. In addition, severely immunocompromised patients with hematological malignancy who develop mucormycosis have lower albumin levels. Moreover, in diabetic patients, albumin gets glycosylated losing its capacity for FFA binding[54]. In ongoing experiments (performed by A.P.) he found that glycosylated albumin loses also its inhibitory effects against *Ro* **(Figure 7A)**. Thus, we tested serums from diabetic, cirrhotic and hematologic malignancies patients for their ability to retain the growth of *Rhizopus Oryzae*. 10^4 *RO* conidia were cultured in different patient serum for five hours. Firstly, we compared the ability of *RO* spores to grow in the serum of hematological malignancy (HM) patients with low albumins levels (<3.5mg/dL) compared to those with relative normal levels (≤3.5mg/dL). We showed that the sera of HM patients, which had higher albumins levels, had also better capacity to inhibit *RO* germination compared to those with lower levels **(Figure 9 A).** Moreover, we also tested the inhibitory capacities of sera from decompensated cirrhotic patients and compared them with those from

patients that had compensated cirrhosis (**Figure 9 B)**. Compensated cirrhotic patients have normal albumin levels in their sera. We found out that sera from compensated cirrhotic patients had statistically significantly higher inhibitory activity compared to sera from patients with decompensated cirrhosis **(Figure 9B)**. Moreover, we found an inverse correlation between albumin level and inhibitory activity of serum from all groups of patients tested **(Figure 9B)**. In both cases, we measured the length of the growing conidia as an indicator of their growth status. As far as the diabetic patients concerned, we measured the length of conidia in patients with normal glycosylation versus those who had high glycosylation levels. As an indicator of the extent of albumin glycosylation in the serum, we used the percentage of glycosylated hemoglobin (HbA1c). Patients with less than 6% glycosylated hemoglobin were considered as a control. On the contrary patients who had levels greater than 8.5%, were considered to have uncontrolled diabetes. We saw that, *RO* could grow significantly better in diabetic sera compared to the control sera **(Figure 9 C)**.

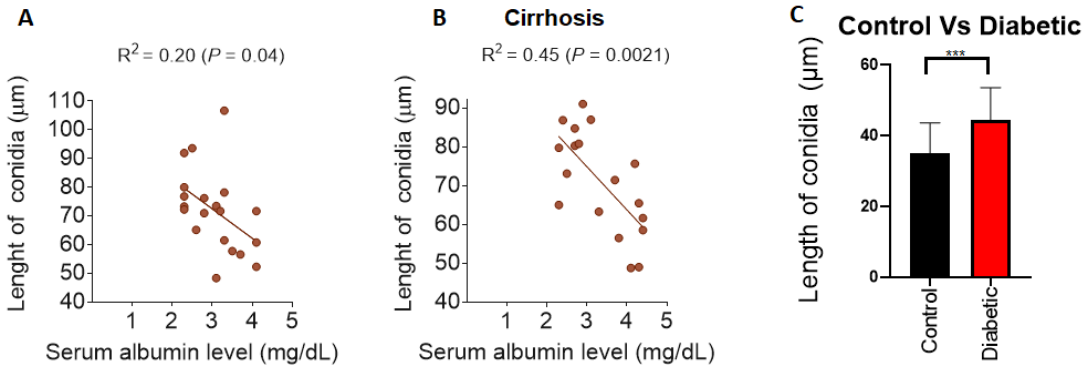

Figure 9: <u>Inhibitory effect of sera from patients at increased risk for Mucormycosis. Sera were obtained from diabetic, hematologic malignancies and cirrhosis patients and the length of conidia was assessed.</u> A) *Length of conidia (10^4 inocula) after 5 hours of incubation, indicative for the stage of growth in the sera of Hematologic malignancies and correlation (linear regression analysis) of the length values with albumin levels.* Representative photos were taken and the length of the conidia was assessed using Fujji Image J. The mean length from each photo is plotted ***B)*** *Length of conidia (10^4 inocula) after 5 hours of incubation, indicative for the stage of growth in sera acquired from compensated and decompensated cirrhotic patients. Sera from compensated cirrhosis patients exhibited increased inhibitory effects compared to those from decompensated cirrhosis. Representative photos were taken C) Comparison of RO growth in diabetic versus control patients. Diabetic sera exhibit reduced inhibitory capacities compared to control*

4) Discussion

Several studies have shown that inhibition of mucorales growth is essential for the disease pathogenicity[29]. Uncontrolled gorwth of *Ro* conidia can lead to dissemination and even death[15]. Human serum has been shown to display important activity against filamentous fungi growth, which remains unexplored at the molecular level. Previous studies suggest that iron starvation is a major antifungal mechanism in serum[44]. However, mucormycosis is a disease that occurs in patients who display metabolic

abnormalities, which are not directly associated with iron deregulation. For example, malnutrition and cirrhosis are associated with diminished antifungal activity of serum and increased risk for mucormycosis[20]. In addition, low albumin levels are strikingly associated with mucormycosis when compared to other fungal diseases[60]. We validated this association of hypo-albumenemia with mucormycosis in hematological malignancy patients who developed mucormycosis and other fungal infections. Therefore, we hypothesized that additional antifungal mechanisms perform in serum against Mucorales. Specifically, we tested the direct antifungal activity of albumin against Mucorales. We found that albumin in the physiological relevant concentration of 45 mg/ml can completely inhibit the growth of *Ro* and reduce the expression of pathogenic factors like ricin.

Our findings provide new evidence on the role of albumin in host defense. Furthermore, our studies provide novel mechanistic link between metabolic deregulation and susceptibility to fungal disease caused by mucorales. Previous work suggest that albumin has antifungal activity against rare fungal pathogens[44]. However, the mechanisms remains obscure.

In our work, we identified the precise mechanism of albumin antifungal activity. In particular, we found that FFAs bound to albumin mediate the antifungal activity against mucorales as free fatty acid deficient albumin cannot inhibit the growth of mucorales. Importantly, albumin is the exclusive carrier of medium and long chain FFAs in serum[57,61]. Evermore, albumin acts both as a carrier and as a provider of FFAs in peripheral tissues[52]. Indeed, we verified that release of FFAs accounts for the antifungal effects of human serum albumin and clarified that albumin is unable to enter inside the fungal cells. FFAs are well known antimicrobial effectors[53]. However, they role of antimicrobial activity of serum FFAs has not been previously reported. Notably, FFAs inhibit Mucorales at physiologically relevant concentrations in serum as opposite to other pathogens. This increased susceptibility of Mucorales to FFAs action explains the major role of albumin in antifungal immunity against Mucorales.

Collectively, our work identifies a new immune effector pathway in serum with major importance in pathogenesis of fungal disease and direct therapeutic implications for the management of invasive fungal infections.

Many important questions emanate from our work. In future studies, we will try to explore the molecular mechanisms underlying this inhibition of growth in *Ro* conidia from albumin. We would like to develop a model for trafficking intracellular fate of FFAs, by labelling them, in order to investigate if they target a specific organelle into the cells. Moreover, because it is well-documented that the primary role of albumin is to dissolve FFAs in the serum. Considering this, we would like to explore the possibility of albumin contributing crucially to the formation of a particular 3D structure of FFAs that enhances their inhibitory capacities. Thus, we will try to compare the structure of FFAs released from albumin in the solvent with those that are dissolved directly in RPMI and do not exhibit any inhibitory effect. In order to examine it, we will acquire TEM microscopy and light scattering technique in order to see if there is any difference in the FFAs 3D structure.

We plan also to perform proof of concept in vivo studies using an albumin knock out humanized mouse model. Experiments on these mice will be conducted in order to check if they exhibit increased susceptibility in *Ro* infections compared to control mice

of the same background that will be treated exogenously with albumin. Finally, we will also try to see if the absence of albumin also affects in vivo the other host's defense mechanism that are essential for Mucorales inhibition.

One of the questions that have been raised is whether FFAs exhibit physiologically relevant inhibitory mechanisms against Mucorales apart from the serum. For instance, is known that when these fungi infect human body through inhalation, interact with brochoalveolar fluid and alveolar macrophages into the lungs. Taking this into consideration, it would be of our interest to clarify the possibility of FFAs in BAL or inside phagosomes exerting some antifungal activities.

Collectively, our work identifies a new physiological role of albumin bound FFAs in immunity against Mucorales. Furthermore, our findings in patients at increased risk for mucormycosis and in the mouse models suggest an important role of defects in albumin-bound FFAs in pathogenesis of disease. Notably, although FFAs possess broad antimicrobial properties they display selective activity against Mucorales at the physiological concentrations present in serum. Therefore, this mechanism partially explains the unique predisposition of patients with metabolic abnormalities for mucormycosis. These findings pave the way for understanding the role of FFAs in antifungal host defense in other sites of interactions with Mucorales, both extracellularly (e.g. epithelia) and inside the phagocytes. Finally, our work has broad therapeutic implications because restoration of low albumin levels could be a therapeutic intervention in mucormycosis.

5) Materials and Methods

Microorganisms and culture conditions

Aspergillus fumigatus ATCC[62] and *Rhizopus* strains used(WT *R. oryzae* ATCC557969 [63]) were grown on Yeast extract agar glucose agar plates for 3 days at 37 °C. Fungal conidia were harvest via gentle shaking with sterile PBS and filtered through a 4 μm pore size cell strainer. For in vitro experiments, RhO spores were incubated at 37 °C in 5% CO_2 in RPMI 1640 media supplemented with 0.2% glucose and 1.7% MOPS, hereafter will be referred as RPMI-MOPS media .

Virulence studies in mice

C57BL/6 mice were maintained in grouped cages in a high-efficiency particulate air-filtered environmentally controlled virus-free facility (24 °C, 12/12-h light/dark cycle), and fed by standard chow diet and water ad libitum. All experiments were approved by the local ethics committee of the University of Crete Medical School, Greece in line with the corresponding National and European Union legislation.

Human serum albumin depletion and isolation experinments

For human serum albumin depletion and isolation experiments, blood sera were isolated from donors who had provided written informed consent. In order to isolate human albumin from sera, affinity column chromatography with blue sepharose **(**GE health care, Cat#GE17-0948-01**)** was used according to manufactures instructions with some modifications. Briefly, 7ml of blue sepharose were washed with double distilled water and dried and then re-hydrated with albumin-free human serum, from the same donor. The albumin from this serum was depleted via Amicon Ultra Centrifugal filters with molecular weight cut-off 50 kDa. The excess albumin-depleted serum was removed and then fresh serum was added. After a gentle mix, sepharose and serum were left in a rotator at 4 ^oC overnight. The following day blue sepharose and serum mix were placed back in the column. The first flow through is serum depleted from albumin.

Moreover, albumin bound in the blue sepharose was also isolated. Firstly, the column was washed with equilibration buffer (20 mM Na_2HPO_4, 20 mM NaH_2PO_4 ,PH8) and then was eluted 6 times with elution buffer (2M NaCl, 20 mM Na_2HPO_4, PH8). Due to high NaCl content the isolated albumin was dialyzed using protein dialysis membrane and equilibration buffer till normal the concentration of NaCl (150 mM) at 4^oC for 4 hours with gently steering. Finally, the isolated albumin was condensed to final volume of 2ml (15X) using amicon ultra centrifugal filters with molecular weight cut-off 50 kDa. The isolated albumin was used immediately for in vitro test.

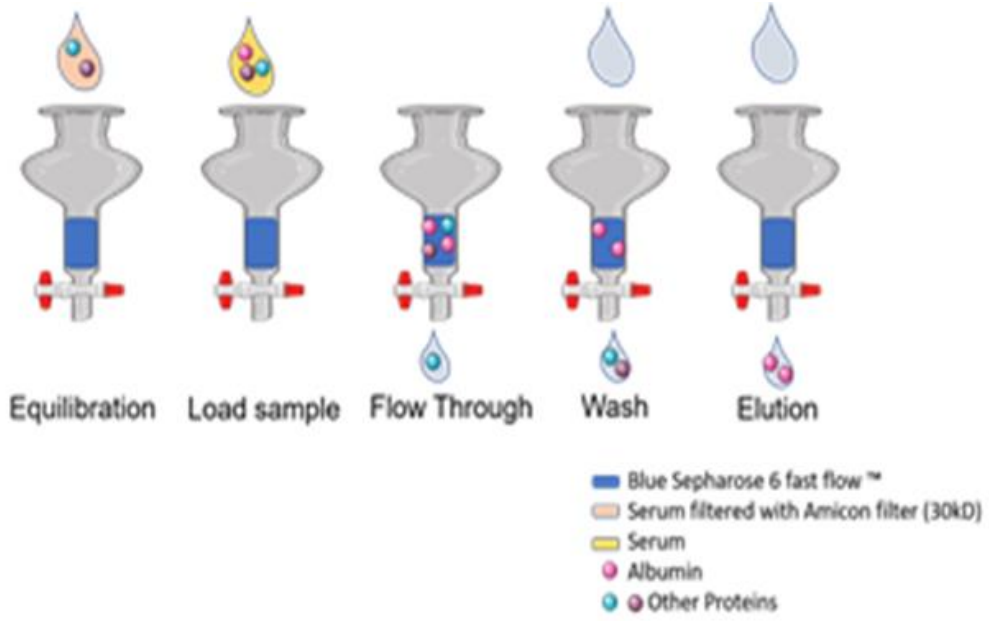

Figure 10: Schematic demonstration of albumin depletion and isolation experiment.

In vitro experiments

All the experiments ,that we tested for antifungal activities, were conducted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference methods with some modifications and the antifungal activities were expressed as inhibitory activity for 50% of the inocula (IC50). More specifically, we used RPMI medium which is considered satisfactory to sustain the growth of filamentous fungi [64]supplemented with 0.2% and 1.7% MOPS PH buffer. The inhibitory activity was assessed by measuring the fungi ability to form germlings.

Measurement of conidia germination

In all the experiments that the germination of the spores was evaluated, conidia with polarized growth that was forming germlings were measured and compared with ungermed conidia from pictures obtained from leica optical microscope (Leica DMIRE2). The measurement of the conidia then was done via Fiji Image J.

In all experiments that the length of the germlings was measured, pictures with germlings were acquired from leica optical microscope (Leica DMIRE 2). The length of each conidia was measured via Fiji image J by setting the appropriate scale.

XTT analysis from fungi growth

XTT measures metabolic activity of the fungus. In order to perform XTT it was diluted in sterile 1X PBS to final concentration of 0.5mg/ml and warmed at 60^{o}C for 30 minutes in order to be diluted. Then it was sterilized through filtration with 0.2 μm pore size

syringe filters. Vitamin K (methadione) is used in 1/80 dilution for XTT. This makes a 2X XTT working solution. Afterwards the 2X XTT solution is diluted with the media containing the fungi whose metabolic activity needs to be measured and placed in 37^{o}C and 5% CO_2 for three hours. The colorimetric change is measured with plate reader. According to ATCC, two absorbance measurements are needed, one at 475 nm (specific absorbance) and one at 660 nm (non-specific absorbance) and the specific absorbance of a sample is expressed mathematically as: Specific Absorbance= A475nm(Test) – A475nm(Blank) – A660nm(Test) [65]

Human serum albumin inhibition test.

10^4 RhO and Af spores were incubated with either isolated albumin or equilibration buffer supplemented with minimal requirements (0.2 % glucose, 0.1% NH_4Cl and 0.15% KH_2PO_4 **)**[47] for 12 hours. Then the germination and the length of the germlings was evaluated.

Human serum albumin depletion experiment

10^4 Rho and Af spores were incubated with either serum or albumin depleted serum at 37^{o}C in 5% CO_2 for 5 hours. Then, the length of the germlings was evaluated.

Staining of conidia cell wall

For conidia cell wall staining fluorescent brightener 28 (Sigma-aldrich, cat#F3543) was used. Freshly harvested conidia in concentration 2x10^7/ml were stained with 500μg/ml for *Rhizopous* and 100μg/ml for *Aspergillus* of fluorescent brightener 28 for 1 hour at room temperature in $NaOHCO_3$. Then the conidia were washed 3-times with 1X PBS and stored at 4^{o}C in 1X PBS till use.

Swelling of Conidia

For the experiments that swelled conidia were used, 5X10^5 conidia were grown in the indicated media for the appropriate time (4-7 hours depending on the media) and then gently harvested with cell scraper. Then the isolated conidia were washed with 1X PBS and used according to experimental needs.

In vitro fungostatic test of Fatty acids.

In order to test the fungostatic activity of fatty acids, 10^4 *Rhizopus* conidia were incubated with purified fatty acids for chromatographic standards and for studies of antimicrobial activity obtained from Sigma-Aldrich, except from Hexanoicand and Acetic acid which were obtained from Alfa Aesar and Scharlau respectively. In the following table are shown the catalogs numbers of its fatty acid.

Table 2: Catalog numbers of different FFAs used in the in vitro experiments

Fatty acid	**Catalog number**
Palmitic acid	P5585 (Sigma-Aldrich)
Decanoic acid	C1875 (Sigma-Aldrich)
Butiric acid	103500 (Sigma-Aldrich)
Oleic acid	O1383 (Sigma-Aldrich)
Dodecanoid acid	L4250 (Sigma-Aldrich)
Acetic acid	0346 (Scharlau)
Propionic acid	402907 (Sigma-Aldrich)
Valeric acid	240370 (Sigma-Aldrich)
Hexanoic acid	A13789 (Alfa Aesar)
Myristic acid	M3128 (Sigma-Aldrich)
1-Octanoyl-rac-glycerol	M2265 (Sigma-Aldrich)

Conidia were challenged overnight with the above commercially available fatty acids and the germination of the spores was evaluated.

BSA FFA free complementation with fatty acids.

In order to make the complexation assay, BSA essentially FA free (Sigma-aldrich, Cat#A-6003) was used. The proportion of FFA to BSA was 4.5/1. Firstly, BSA was diluted in RPMI-MOPS medium and sterilized filter. Then heated at 57 for 30min. Moreover, 80mM of palmitic were diluted in 100% EtOH and then were heated at 57 for 30 minutes. Then BSA and palmitic in proportion 4.5/1 were mixed well and incubated at 37C for an hour. Finally, this BSA-palmitic medium was used for serial dilution.

Virulence studies in mice

For virulence studies both 8-12 week-old female and male C57BL/6(B6) mice were challenged by intratracheal installation with a $2.5X10^6$ swelled *Rhizopus* conidia. In the survival experiments infected mice were let until the indicated time point and then the survival was plotted with Kaplan-Meier plots in graph-pad prism. For cryosection and histopathology studies, mice were euthanized at the indicated time points. Then in the lungs was added intratrachealy ,through a catheter, 1:1 PBS-OCT in order to preserve the structure of the lungs and preserved in OCT in -80^oC till immunofluorescence analysis.

Immunoflourescent experiments

Ricin and Cot-H staining

For ricin and Cot-h staining 10^5 conidia pre-stained with fluorescent brightener 28 were grown either in RPMI 1640 supplemented with 1.7% MOPS and 0.2% glucose or flow through of 45mg/ml BSA till the swollen stage. Then the spores were gently harvest using a cell scraper, washed once with 1X PBS and then fixed with 4% paraformaldehyde (PFA) for 15 minutes in room temperature. After 3 washes with 1X PBS the spores were permeabilished using 0.1% Triton-X for 10 minutes. Then washed with 1X PBS and blocked for unspecific staining using 5% goat serum in 1hour. After the blocking step the conidia were incubated with rabit anti-Ricin and mouse anti-Cot-H IgG, which were both home-made antibodies which were kindly given by a collaborator, for two hours in Rt (antibody stock is 2mg/ml for ricin and 1mg/ml for Cot-H and used in 1/10 dilution). Then, the spores were washed with Tris-buffer-saline (TBS) containing 0.05% Tween20. Afterwards the spores were counterstained with appropriate secondary antibody in 1/500 dilution in room temperature. Finally washed 3 times with 1 X PBS and placed in a coverslip for examination under Leica SP8 inverted confocal microscopy and quantified for mean fluorescent intensity (MFI).

For FITC-albumin experiments

10^5 ,pre-stained with fluorescent brightener 28, *Rhizopus* conidia were incubated in RPMI-MOPS media supplemented with 35mg/ml BSA plus 10 mg/ml FITC labelled albumin (Sigma-Aldrich, Cat#A9771) for six hours in order the conidia to be in the swelled state. Then, the conidia were kindly harvested and placed on coverslips and

examined under Leica SP8 inverted confocal microscopy. 3-D structured were generated with Leica software.

Human studies

Approval for the collection of clinical information and blood sample from patients with diabetis, hematologic malignancies, cirrhosis and from control individuals was obtained from the Ethics Committee of the University Hospital of Heraklion, Crete, Greece (5159/2014). The patient provided written informed consent in accordance with the Declaration of Helsinki.

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