

IMPERIAL

Title: Phylogenetic and Functional Characterization of Azole-Resistant *Aspergillus fumigatus* Clinical Isolates in Individuals with Cystic Fibrosis

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Statement of Originality

I certify that this thesis, and the research to which it refers, are the product of my own work, conducted during the current years of the PhD in Clinical Medicine Research (Infectious Disease) at Imperial College London. Any ideas or quotations from the work of other people, published or otherwise, or from my own previous work are fully acknowledged in accordance with the standard referencing practices of the discipline.

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Abstract

Aspergillus fumigatus is a globally distributed fungal pathogen responsible for pulmonary aspergillosis in immunocompromised individuals, including those with asthma, cystic fibrosis (CF), and immune deficiencies. Its capacity to induce diseases ranging from allergies to life-threatening invasive infections stems from rapid adaptation to host microenvironments. This adaptability, a hallmark of its genetic plasticity, drives the emergence of phenotypically diverse strains under selective pressures. Such genetic variation underpins metabolic and phenotypic heterogeneity, including resistance to first-line azole antifungals, compromising treatment efficacy and worsening patient outcomes.

This PhD study aimed to characterize the interplay between genomic and transcriptomic regulation of *A. fumigatus* metabolism by integrating phenotypic microarray metabolomics with multi-omics analyses. Whole-genome sequencing profiling of 145 clinical isolates (azole-resistant [*ARAf*] and azole-sensitive) revealed two phylogenetically distinct clades: Clade A (n = 43) and Clade B (n = 102), with distinct azole-resistance polymorphisms. Clade-specific genetic divergence reflects independent azole-resistance evolution driven by environmental or clinical pressures, with no significant influence from host endotypes.

Phenotypic variation was assessed in 42 isolates across 379 growth conditions using the BioLog OmniLog® platform. Significant differences in 28 metabolic parameters were observed between azole-susceptible and *ARAf* isolates from CF and non-CF hosts. Notably, CF isolates exhibited reduced mean growth rates under stress, indicative of a fitness cost linked to adaptation to the CF lung niche.

To illustrate adaptive mechanisms, genome-wide association studies (GWAS) and transcriptomic (RNA-seq) analyses were performed under baseline conditions and CF-relevant

stressors (histamine and NaCl). GWAS identified 140 significant SNPs mapping to 88 genes, while RNA-seq revealed over 8,000 differentially expressed genes. Gene Ontology (GO) analysis identified 10 genes linked to metabolism and pathogenicity. Integrative multi-omics studies revealed AFUA_2G05620, a histamine catabolism regulator, as a key mediator of phenotypic plasticity, governing critical adaptive traits for survival in dynamic host environments.

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List of Abbreviations

<i>A. fumigatus</i>	<i>Aspergillus fumigatus</i>
ABPA	Allergic bronchopulmonary aspergillosis
ABC	ATP-binding cassette
ARA_f	Azole resistant <i>A. fumigatus</i>
BASR	Base Quality Score Recalibration
BWA	Burrows-Wheeler Aligner
CPA	Chronic pulmonary aspergillosis
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CLSI	Clinical and Laboratory Standards Institute
CNV	Copy number variation
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESCMID	European Society of Clinical Microbiology and Infectious Disease
ECMM	European Confederation of Medical Mycology
ERS	European Respiratory Society
FPPL	Fungal priority pathogens list
GAG	galactosaminogalactan
GWAS	Genome wide association studies
IA	Invasive aspergillosis
IARA	IA risk assessment
HTS	High-throughput sequencing
HSCT	Hematopoietic stem cell transplantation
HICPAC	Healthcare Infection Control Practices Advisory Committee
INDEL	Insertions or deletions
IPA	Invasive pulmonary aspergillosis
ITC	Itraconazole
MIC	Minimum inhibitory concentration
ML	Maximum likelihood
MLST	Multi-locus sequence typing
NCCLS	National Committee for Clinical Laboratory Standards

NGS	Next-generation sequencing
OU	OmniLog Units
OD	Optical density
RAXML	Randomised Axelerated Maximum Likelihood
RBH	Royal Brompton Hospital
R&D	Research and Development
PBMC	Peripheral blood mononuclear cell
PCA	Primary cutaneous aspergillosis
PCR	Polymerase chain reaction
POS	Posaconazole
SCA	Secondary cutaneous aspergillosis
STR	Short tandem repeat
SNP	Single nucleotide polymorphism
TR	Tandem repeat
WGS	Whole genome sequencing
WHO	World Health Organisation
ZRFA-C	Zinc transporter-encoding genes

1 Introduction

1.1 *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus critical to carbon and nitrogen recycling in terrestrial ecosystems. Its ecological niche encompasses soil, decaying organic matter, and compost systems (Tekaiia & Latgé, 2005a). Additionally, *A. fumigatus* colonizes indoor environments, including residential, educational, and healthcare facilities (Araujo et al., 2010). The fungus disperses via asexual reproduction, producing conidial heads bearing phialides that generate chains of airborne conidia (2–3 µm in diameter). These conidia are sufficiently small to penetrate deep into the human respiratory tract, reaching pulmonary alveoli (Figure 1) (Latgé & Chamilos, 2020; J. Zhang et al., 2021a).

Historically, *A. fumigatus* was considered strictly asexual, with genetic diversity attributed to a parasexual cycle involving hyphal anastomosis (Latgé & Chamilos, 2020). However, genomic analyses have challenged this view. Paoletti et al. (2005) identified a near-equal global distribution of *MAT1-1* (α-domain) and *MAT1-2* (HMG domain) mating-type idiomorphs, suggesting cryptic sexual potential. Supporting this, the *A. fumigatus* genome encodes over 200 genes associated with sexual reproduction (Nierman et al., 2005a; Paoletti et al., 2005; Pringle et al., 2005). Later, a mating system consisting of MAT1-2 family HMG gene and a MAT1-1 (alpha domain) idiomorph were discovered in *A. fumigatus* isolates, indicating heterothallism in this species (Pöggeler, 2002). Functional characterization of these loci confirmed heterothallism, with sexual reproduction inducible under laboratory conditions via crosses between compatible *MAT1-1* and *MAT1-2* isolates (O’Gorman et al., 2009). These findings reclassify *A. fumigatus* as a heterothallic, self-sterile species.

1.2 Pathogenesis of *A. fumigatus*

Aspergillus fumigatus is a ubiquitous fungal pathogen whose conidia constitute a significant proportion of airborne particulate matter (O’Gorman, 2011). Globally, inhalation of these spores results in over 3 million chronic infections and 300,000 invasive aspergillosis (IA) cases annually (Bongomin et al., 2017; G. D. Brown et al., 2012a). Pathogenesis initiates with the inhalation of conidia, a process influenced by host immune status and environmental factors. Humans routinely inhale 200–100,000 fungal spores daily (J Konopka, A Casadevall, J Taylor, 2019), with clinical outcomes determined by the host’s ability to mount effective immune responses. In immunocompetent individuals, innate defences typically clear conidia; however, immunocompromised hosts—including cystic fibrosis (CF) individuals, transplant recipients, and individuals with HIV—fail to contain fungal growth, enabling conidial germination into invasive hyphae (Latgé & Chamilos, 2020; Tekaia & Latgé, 2005a). Subsequent hyphal invasion of pulmonary vasculature facilitates hematogenous dissemination, leading to multisystem organ failure (Filler & Sheppard, 2006).

In 2022, the World Health Organization (WHO) designated *A. fumigatus* as a critical priority pathogen due to its high mortality rates (50–95% in IA), rising antifungal resistance, and unmet therapeutic needs (WHO, 2022). Mortality is particularly elevated in hematological malignancy and hematopoietic stem cell transplant (HSCT) populations (dos Santos et al., 2020a; van de Peppel et al., 2018). The WHO Fungal Priority Pathogens List (FPPL) underscores the urgent need for targeted research into host-pathogen interactions, diagnostics, and novel antifungals to mitigate this global burden (<https://www.who.int/publications/i/item/9789240060241>). .

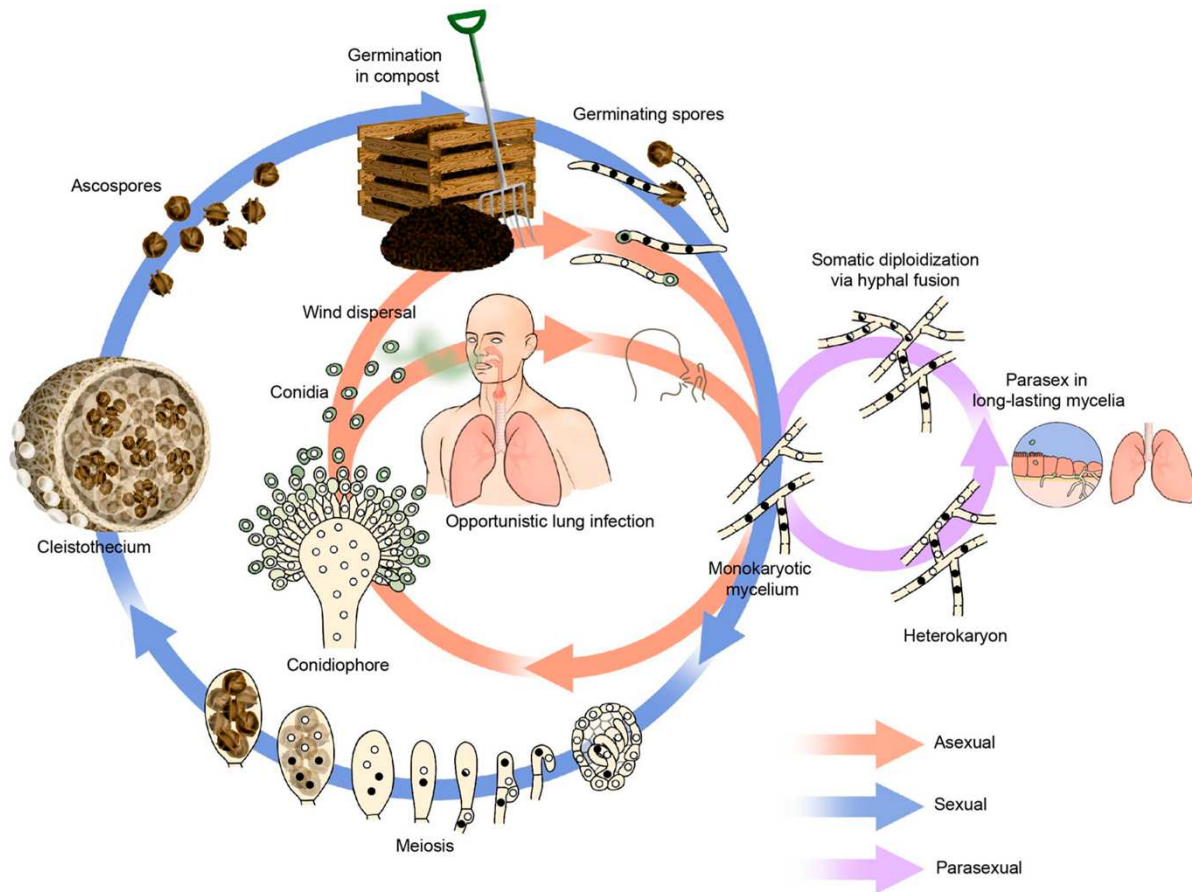


Figure 1. Life cycle of *Aspergillus fumigatus* and its ecological niches. *A. fumigatus* exhibits three distinct reproductive cycles: asexual (orange arrow), parasexual (purple arrow), and sexual (blue arrow). During the sexual reproductive phase, the mycelium forms a specialized structure termed a cleistothecium, which harbours ascospores. These ascospores are subsequently released into the environment, enabling colonization of diverse ecological niches (J. Zhang et al., 2021a).

1.3 Epidemiology of *A. fumigatus* Infections.

The *Aspergillus* genus is the most prevalent cause of fungal infections in humans and can give rise to a variety of severe diseases in both immunocompetent and immunocompromised people. For most patients, disease occurs primarily in the lungs; however, in the most severely predisposed, the infection can spread to virtually any organ. Conidia are easily removed by bronchial ciliary action or are phagocytosed and killed by resident pulmonary macrophages. Consequently, these individuals do not manifest any infectious or allergic symptoms (Latgé & Chamilos, 2020). In 1952, Hinson, Moon and Plummer introduced the term “aspergillosis” to encompass a diverse spectrum of diseases caused by fungi belonging to the genus *Aspergillus*. The disease they considered ranged from mild allergic reactions to life-threatening invasive infections (Morgan et al., 2005a; Shah, 2010; Tahir et al., 2011):

1. Allergic bronchopulmonary aspergillosis (ABPA) is caused by hypersensitivity to *A. fumigatus*. Worldwide, more than four million people are affected by ABPA Neuvégilise (Agarwal et al., 2016a; Breuer et al., 2019; Neuvégilise et al., 1997a; Reece et al., 2019). The mortality rate is low, with most patients responding well to treatment with corticosteroids and antifungal therapy. However, ABPA can affects approximately 1-2% of people with asthma. It has been reported of mortality rates of up to 13% in asthmatics and 7-9% in patients with CF (Agarwal et al., 2016b; Gago et al., 2019).
2. Chronic pulmonary aspergillosis (CPA) is a severe fungal infection usually seen in immunocompetent or mildly immunosuppressed people with lung diseases, such as tuberculosis or COPD. Global epidemiological data indicate that approximately 3 million people suffer from CPA(G. D. Brown et al., 2012a), while 1.2 million are estimated to have CPA as a complication of pulmonary tuberculosis (TB). A further 411,000 have CPA as a complication of allergic bronchopulmonary aspergillosis (ABPA), and 72,000 have CPA as a complication of sarcoidosis contributing to fibrosis and respiratory failure, illustrating

the importance of the problem from a global health perspective (Denning et al., 2011, 2013).

3. Invasive pulmonary aspergillosis (IPA): In the respiratory mucosa, spores may germinate into hyphae, which can then invade the mucosa and cause IPA, which is one of the major causes of morbidity and mortality in immunocompromised patients treated for haematological malignancies or undergoing haematopoietic stem cell transplantation or solid organ transplantation (Chamilos et al., 2006a; Kashefi et al., 2021; Zanganeh et al., 2018; Zarrinfar et al., 2015). The mortality rate associated with invasive pulmonary aspergillosis (IPA) is significant. One study found a 90-day mortality rate of 46% among haematology patients with invasive pulmonary aspergillosis (IPA) receiving voriconazole prophylaxis, compared to 59.3% for those who did not receive prophylaxis (Budin et al., 2021a). The high mortality rate of invasive pulmonary aspergillosis (IPA) was significantly attributed to delayed diagnosis and treatment.
4. *Aspergillus* rhinosinusitis: a form of allergic fungal sinusitis (AFS), characterised by chronic rhinosinusitis and pathological findings similar to those observed in allergic bronchopulmonary aspergillosis (ABPA). This condition can be classified as either invasive or non-invasive according to the histopathological findings of the invaded tissue (Cern Gan & Javer, n.d.; Dagenais & Keller, 2009a). The non-invasive classification of fungal sinusitis is characterised by the absence of hyphae in the mucosal and other tissues of the paranasal sinuses. This category encompasses fungal colonisation, fungal ball, and allergic fungal rhinosinusitis, as described previously (Soler & Schlosser, 2012). While invasive fungal rhinosinusitis is characterised by the presence of fungal hyphae in the paranasal sinus mucosa, submucosa, bone, or blood vessels, it is further classified into acute invasive (fulminant) and chronic invasive subtypes. The latter comprise chronic granulomatous and chronic invasive fungal sinusitis, which persist for durations of more

than 12 weeks (Chakrabarti et al., 2004). Chronic invasive fungal sinusitis is most commonly seen in western populations (Busaba et al., 2002) and Japan (Nomura et al., 2013), whereas granulomatous invasive fungal rhinosinusitis is most commonly observed in Africa (Sudan), Southeast Asia (India, Pakistan and Saudi Arabia) (Alotaibi et al., 2020; Alrajhi et al., 2001; Toyama, 2016) Korea (Kim et al., 2012) and some southwestern US states (Stringer & Ryan, 2000; T. Wang et al., 2020). Fungal sinusitis accounts for approximately 6% to 9% of all rhinosinusitis cases (T. Wang et al., 2020).

5. Primary cutaneous aspergillosis: This refers to skin and soft tissue infections caused by *Aspergillus spp.* and represents a relatively infrequent presentation of aspergillosis. The classification of cutaneous aspergillosis is based on the initial location of involvement and the underlying etiological mechanisms that result in primary or secondary cutaneous aspergillosis (Y. S. Chong & Ro, 2002). The occurrence of primary cutaneous aspergillosis (PCA) is associated with a range of factors, such as burns, catheter inlets and plaster adhesion regions, that damage the skin's integrity. Furthermore, PCA may develop in areas of maceration or repeated skin trauma, in patients who have undergone traumatic experiences, or in surgical wounds due to direct inoculation.
6. Secondary cutaneous aspergillosis (SCA) is a condition that develops through direct spread from adjacent to focal points, such as the lungs or paranasal sinus, or through hematogenous spread. The condition known as SCA primarily impacts individuals with compromised immune systems, particularly those who have received stem cell transplants as treatments for haematological malignancies. The incidence rate is higher for SCA than for PCA (Y. S. Chong & Ro, 2002; Walsh, 1998)
7. Influenza-associated pulmonary aspergillosis (IAPA) is a significant complication observed primarily in critically ill patients with influenza pneumonia, with a notably high incidence reported in European studies, ranging from 12% to 28%. A recent retrospective

cohort study in Alberta, Canada, found a lower incidence of 7.2%, raising questions about geographic variability in IAPA rates, potentially influenced by genetic factors, environmental conditions, and healthcare practices (Huang et al., 2019; Ku et al., 2017; Schauwvlieghe et al., 2018). The presence of galactomannan (GM) as a diagnostic marker has shown promise, with higher sensitivity in bronchoalveolar lavage fluid compared to serum (Boch et al., 2018; Meersseman et al., 2008). Notably, the mortality rate for patients with IAPA is significantly higher, with 51% mortality in a cohort study, suggesting an independent association between IAPA and increased mortality (Rijnders et al., 2020). Future research should focus on establishing a consensus definition of IAPA and identifying additional risk factors to improve diagnosis and treatment strategies (De Pauw et al., 2008; Van De Veerdonk et al., 2018).

1.3.1 Global Distribution and Incidence

Aspergillus fumigatus is a leading cause of invasive fungal infections (IFIs), with invasive aspergillosis (IA) representing a significant global health burden. Geographical disparities in IA incidence are evident: a multicenter study in the Netherlands reported 2–30 IA cases annually per center (average 8 cases/year) (van der Linden et al., 2015). In Germany, the incidence among acute leukemia patients reached 5.84 cases per 100 patients, while intensive care unit (ICU) admissions showed an incidence of 15 cases per 1,000 admissions (Koehler et al., 2021). The COVID-19 pandemic exacerbated IA epidemiology, with COVID-19-associated pulmonary aspergillosis (CAPA) occurring in 1.0%–39.1% of critically ill COVID-19 patients (Arastehfar et al., 2021; White et al., 2021). In the UK, CAPA incidence reached 5% in ICU cohorts, underscoring the need for targeted surveillance (White et al., 2021).

1.3.2 Risk Factors and Patient Populations

Individuals who are immunocompromised are at the highest risk for invasive aspergillosis (IA). This includes patients with hematological malignancies, prolonged neutropenia, solid-organ transplants, or severe lung diseases (Dagenais & Keller, 2009a; Morgan et al., 2005b). The use of corticosteroids and chemotherapy further increases susceptibility to IA. For example, patients undergoing treatment for acute leukaemia are reported to experience IA rates of 5.84 cases per 100 patients (Chamilos et al., 2006b; Kosmidis & Denning, 2015).

In contrast, patients with solid tumours often develop IA as a complication of chronic obstructive pulmonary disease (COPD) or following prior radiotherapy (Kosmidis & Denning, 2015). Additionally, populations with chronic respiratory diseases, such as cystic fibrosis (CF) and those recovering from tuberculosis, are also disproportionately affected by IA. Notably, chronic pulmonary aspergillosis (CPA) complicates approximately 1.2 million cases of tuberculosis worldwide (Denning & Perlin, 2011a).

1.3.3 Mortality Rates

Invasive aspergillosis (IA) poses a significant threat to immunocompromised patients, with overall mortality rates alarmingly high, ranging from 30% to 50%. The 30-day mortality rates for these patients vary widely, reported between 13.6% and 47.8% (Chamilos et al., 2006; Zanganeh et al., 2018). Certain patient groups experience even higher mortality rates. For instance, patients with coronavirus-associated pulmonary aspergillosis (CAPA) have mortality rates that can reach as high as 52.2%. Moreover, those infected with voriconazole-resistant strains of IA face particularly dire outcomes, with 12-week mortality rates exceeding 50% (Bartoletti et al., 2020; Lestrade et al., 2019). Key factors contributing to these poor outcomes include delayed diagnosis and the emergence of antifungal resistance (Budin et al., 2021b).

1.3.4 Antifungal Resistance

Azoles are a cornerstone of antifungal therapy, primarily functioning to inhibit ergosterol biosynthesis by targeting lanosterol 14 α -demethylase (CYP51A), an enzyme essential for maintaining fungal cell membrane integrity. Key azole drugs—such as voriconazole, itraconazole, and posaconazole—are considered first-line agents for treating aspergillosis due to their broad efficacy and favourable oral bioavailability. However, the widespread use of these medications in clinical prophylaxis, agriculture (e.g., crop fungicides), and horticulture has led to the emergence of resistance in *A. fumigatus*.

Resistance primarily develops through mutations in the CYP51A gene, including notable alterations like the TR₃₄/L98H and TR₄₆/Y121F/T289A polymorphisms, which diminish the binding affinity of azoles. Additionally, resistance can occur through the overexpression of efflux pumps (Snelders et al., 2008; Hagiwara et al., 2016). There are significant geographic disparities in azole resistance rates, ranging from 9.6% in the Netherlands to as high as 76% in Brazil and India. These variations reflect regional differences in the use of agricultural azoles and clinical prescribing practices (Chowdhary et al., 2021; van der Linden et al., 2015).

Cross-resistance poses further management challenges, as 22.2% of voriconazole-resistant isolates are also resistant to itraconazole. This situation necessitates reliance on alternative treatments, such as amphotericin B, which can be toxic (Lestrade et al., 2019). Mortality rates for azole-resistant invasive aspergillosis (IA) exceed 50%, prompting the World Health Organization (WHO) to designate *A. fumigatus* as a critical priority pathogen in 2022 (WHO, 2022).

To combat this resistance crisis, novel therapies targeting non-azole pathways are currently in clinical development. These include olorofim, a dihydroorotate dehydrogenase inhibitor, and

fosmanogepix, which blocks GPI-anchored protein biosynthesis (Thompson et al., 2022). Persistent challenges in addressing azole resistance include delays in diagnosis due to reliance on culture-based susceptibility testing, the presence of resistant strains in environmental reservoirs like soil, and the limited effectiveness of salvage therapies. These factors highlight the urgent need for antifungal stewardship and the implementation of rapid molecular diagnostics to mitigate the spread of resistance.

1.3.5 WHO Prioritization and Future Directions

In 2022, the WHO listed *A. fumigatus* as a critical priority pathogen due to its associated high mortality rates, growing antifungal resistance, and significant diagnostic challenges (WHO, 2022). Addressing the burden posed by this pathogen necessitates a multi-faceted approach.

First, enhancing surveillance of resistance patterns is essential to monitor and understand the evolving landscape of antifungal resistance in *A. fumigatus*. This data can guide clinical practices and inform public health strategies. Second, improving diagnostic techniques is crucial. Innovations such as galactomannan assays and polymerase chain reaction (PCR) testing can facilitate earlier and more accurate identification of infections, enabling timely therapeutic interventions.

Finally, there is a pressing need for therapeutic innovations that specifically target the mechanisms *A. fumigatus* employs to adapt to stress. Research efforts should focus on developing new antifungal agents and treatment strategies that can effectively counteract resistance and improve patient outcomes (Latgé & Chamilos, 2020).

By implementing these strategies, we can better combat the challenges posed by *A. fumigatus* and enhance overall patient care in the face of this formidable pathogen.

1.4 The Cystic Fibrosis Microenvironment: Implications for Pathogen Growth and Immune Response

Cystic fibrosis (CF) is a genetic disorder stemming from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, leading to the production of a dysfunctional CFTR protein. This impairment profoundly alters the pulmonary microenvironment, significantly affecting mucus clearance, iron homeostasis, pH regulation, and immune responses. The dysfunctional CFTR protein disrupts ion transport across epithelial surfaces, resulting in dehydrated airway surface liquid (ASL) and the accumulation of thick, viscous mucus that obstructs airflow and hinders effective mucociliary clearance (Boucher, 2007; Mall et al., 2015). The resultant stagnation of mucus creates an environment conducive to the growth of opportunistic pathogens, particularly *A. fumigatus*, which thrives in these altered conditions.

In CF patients, elevated ammonia levels, primarily produced by bacterial metabolism in stagnant mucus, contribute to significant fluctuations in pH that further influence fungal behaviour. The presence of bacteria such as *Pseudomonas aeruginosa* generates ammonia through the breakdown of urea and amino acids, leading to localized alkaline conditions that enhance the secretion of *A. fumigatus* proteases and facilitate tissue invasion (Valsecchi et al., 2020). Concurrently, chronic inflammation and epithelial damage release free iron into the airway lumen, which *A. fumigatus* exploits via specialized iron acquisition mechanisms, including the production of siderophores like fusarinine (Schrettl et al., 2007). This disruption of iron homeostasis, combined with the acidic conditions of the ASL resulting from impaired bicarbonate transport due to CFTR dysfunction, not only enhances iron solubility but also creates a niche where *A. fumigatus* can proliferate unimpeded.

Moreover, the dysregulated pH of the ASL further exacerbates the situation, as reduced bicarbonate transport leads to an acidic environment that compromises the antimicrobial

activity of host defence molecules, such as defensins and lysozymes (Pezzulo et al., 2012; Berkhout et al., 2014). This acidic milieu, coupled with the alkalinity introduced by bacterial metabolism, creates a duality that favours *A. fumigatus* virulence factors, allowing the pathogen to evade host defences and increase the risk of colonization and infection.

The role of CFTR modulators in managing CF offers valuable insights into the implications of *A. fumigatus* infections in this population. CFTR modulators, such as ivacaftor and lumacaftor, target the defective CFTR protein to restore ion transport across epithelial cells, thereby improving mucus clearance and reducing pathogen burden. These modulators function by enhancing the opening of the CFTR chloride channel or promoting the trafficking of the protein to the cell surface, which may alleviate some of the microenvironmental abnormalities associated with CF, including impaired pH regulation and disrupted iron homeostasis (Keating et al., 2018). The restoration of normal ASL hydration and pH could enhance the efficacy of host defence mechanisms against *A. fumigatus*, suggesting a promising therapeutic avenue for reducing the incidence of fungal infections.

Furthermore, iron acquisition mechanisms and pH regulation are critical to understanding the pathophysiology of *A. fumigatus* infections in CF patients. Chronic inflammation and oxidative stress within the CF lung led to dysregulated iron homeostasis, characterized by increased free iron levels in the airway (Moreau-Marquis et al., 2008). *A. fumigatus* employs sophisticated strategies for iron acquisition, with siderophores playing a pivotal role in binding free iron and facilitating its uptake, thereby promoting fungal growth (Schrettl et al., 2007). Additionally, the pH of the ASL influences iron solubility, with acidic conditions enhancing iron availability and promoting *A. fumigatus* virulence (Huang et al., 2016).

The immune response in CF is notably dysregulated, with alterations in both innate and adaptive immunity contributing to increased susceptibility to *A. fumigatus* infections.

Neutrophils, integral to the innate immune response, exhibit impaired function in CF, characterized by inadequate phagocytosis of pathogens and enhanced apoptosis, which diminishes their capacity to clear *A. fumigatus* from the lungs (Harris et al., 2015). CFTR dysfunction further compromises phagolysosomal acidification in macrophages, which is essential for the effective killing of internalized fungal spores (di Pietro et al., 2021). The chronic inflammatory milieu, marked by elevated pro-inflammatory cytokines such as IL-1 β and IL-8, exacerbates tissue damage and promotes fungal persistence. Dysregulated immune responses also favour a Th2-dominant environment in conditions like allergic bronchopulmonary aspergillosis (ABPA), leading to inadequate regulation of iron-binding proteins like lactoferrin (Elborn, 2016; Baxter et al., 2013), thereby further disrupting iron balance.

The interplay between dysfunctional CFTR, altered pH, dysregulated iron homeostasis, and immune dysfunction creates a vicious cycle that perpetuates *A. fumigatus* colonization. Addressing these interconnected pathways—through CFTR modulators, iron chelation strategies, and pH-balancing therapies—could disrupt fungal persistence and improve clinical outcomes in CF patients. By targeting the underlying factors contributing to susceptibility to *A. fumigatus*, healthcare providers can enhance the management of infections and improve the quality of life for individuals living with CF. Understanding the complex interactions among ammonia levels, iron homeostasis, pH variations, CFTR modulation, and immune dysfunction is essential for developing effective strategies to mitigate the impact of this pathogen on CF patients, ultimately paving the way for innovative therapeutic approaches that address the multifaceted nature of fungal infections in this vulnerable population.

1.5 Clinical treatment of aspergillosis

The past decade has seen a significant increase in antifungal drug research and the clinical advancement of various compounds and approaches aimed at combating IA (Patterson, 2005).

The following FDA-approved compounds have *in vitro*, *in vivo*, and clinical activity against *Aspergillus* species and are licenced for the treatment of invasive aspergillosis (IA):

1. **Triazoles:** These are the largest class of antifungal drugs. They inhibit the synthesis of ergosterol, a key component of fungal cell membranes. Examples of azoles used to treat *A.fumigatus* infections include voriconazole, itraconazole and posaconazole. Voriconazole is considered the first-line agent for IA, while itraconazole and posaconazole are alternative options (Campoy & Adrio, 2017). Azoles attack fungal pathogens by destabilising the fungal membrane. *cyp51A* encodes the production of 14-lanosterol demethylase (CYP51), an enzyme that catalyses the transformation of lanosterol to ergosterol within the fungal cell membrane. The active site of the enzyme contains an iron protoporphyrin unit. Azoles inhibit the ergosterol biosynthetic pathway by binding to the iron, inhibiting ergosterol synthesis, resulting in the accumulation of 14-methyl sterols. These adversely alter the stability, permeability, and the functionality of the membrane and its associated enzymes (Cowen et al., 2015).
2. **Echinocandins:** This antifungal drug class inhibits the synthesis of 1,3-glucan synthase, another key component of fungal cell walls (Denning, 2002). Four echinocandin antifungal agents are currently approved for use in the US: caspofungin (approved in 2001), micafungin (approved in 2005) anidulafungin (approved in 2006) and Rezafungin (approved in 2023)
3. **Polyenes:** Polyenes are macrolides, amphipathic organic compounds, that are clinically important antifungal agents. The best-known polyene is amphotericin B (AMB), initially isolated from *Streptomyces* (Odds, Brown, and Gow, 2003). The most common mechanism of acquired AmB resistance is attributed to alterations in the sterol

composition of the fungal cell membrane. resulting in depolarization of the membrane leading to increased K^+ and Na^+ permeability, and ultimately cell death. Resistance to polyenes remains rare in comparison to resistance observed in other antifungal drugs but has been observed. Several mutations in the genes of the ergosterol biosynthesis pathway (*ERG* genes) have been implicated as the mechanism of AmB resistance (Ganesan et al., 2022; Geber et al., 1995).intrinsic resistant a Tereus

Since resistance to drugs used to treat aspergillosis has been reported, combination therapy with two or more antifungal drugs may be used in resistant *A. fumigatus* infections or in immunocompromised patients. Combination therapy with voriconazole and an echinocandin can be used in patients with IA who are refractory to monotherapy (Jeans et al., 2012).

While these alterations have frequently been found in environmental isolates, they have not been detected in isolates from patients who underwent long-term triazole treatment, in whom resistance to the drugs may have been expected to have developed. The majority of patients (50%–75%) with TR₃₄/L98H isolates had not been given triazole drugs, which further suggests resistance acquired in the environment (Rocchi et al., 2021a; Sharma et al., 2015; Snelders et al., 2009a). Up to 90% of *A. fumigatus* isolates recovered from patients with azole-resistant invasive aspergillosis exhibit resistance mutations that are associated with resistance selection in the environment specifically (Lestrade et al., 2020). It has been seen that exposure of fungi to azoles in agriculture field develops cross resistance to medical triazoles (Verweij et al., 2009; Chowdhary et al., 2013a; Barber et al., 2020). In the agriculture, fungicides are primarily used for the intent of preventing post-harvest deteriorating of agricultural products and controlling the growth of fungus to which plants are exposed. Before the 1970s, various chemicals, including dithiocarbonate and benzimidazoles, were the most frequently used antimycotics (Russell, 2005). However, in later years around 1980s, azoles gained importance due to their site-specific inhibitory effect, which made them significantly more useful. Nevertheless, the

use of azole-based fungicides has increased, which places a significant selective pressure on pathogenic fungi to persist in the environment. The similar mode of action of azoles used in agriculture and medicine may have resulted in cross-resistance and an increased presence of resistant fungal strains in the environment and in clinics (Berger et al., 2017a).

Overall, prevention of the development and spread of ARAf must consider environmental factors in the management of fungal infections. This may involve reducing the use of azole antifungal agents in agriculture or maintaining use of one drug class exclusively for clinical use. In addition to environmental factors, other factors, such as patient demographics, comorbidities, and previous antifungal exposure, can also contribute to the development of azole resistance in *A. fumigatus*. A multidisciplinary approach that considers all these factors is necessary to effectively manage and prevent ARAf infections.

1.5 Worldwide emergence of Azole-resistant *A. fumigatus*

Azole drugs play an essential role in both the prevention and treatment of infections caused by *Aspergillus* species. The emergence of azole-resistant *A. fumigatus* (ARAf) has become a growing concern in recent years (Denning & Perlin, 2011b). The first reports of ARAf described two individuals who had undergone treatment with itraconazole: one patient died in the early 1990s in California (Denning et al., 1997a), while the second patient made a full recovery during 10 months of therapy (Denning et al., 1997b).

The acquisition of resistance to azole treatment in patients with aspergillosis undergoing long-term azole therapy occurs through two distinct routes: the patient-acquired route, which involves the natural emergence of resistant isolates *in vivo*, and the environment-acquired route, which entails the development of resistant isolates in the environment.

The first resistance mechanism believed to be of environmental origin is linked to a specific resistance mechanism characterized by the presence of a variable number of tandem repeats (TRs) in the promoter region of *cyp51A*, which may or may not be accompanied by point

mutations in the coding gene (Snelders et al., 2008). The most common pan-azole-resistant mutation, TR₃₄/L98H—a polymorphism in *cyp51A* consisting of a 34-bp tandem repeat in the promoter region and a leucine-to-histidine substitution at codon 98—is a dominant resistance mechanism thought to be acquired from the environment (Camps, Rijs, et al., 2012a; Laís Pontes; Caio Augusto Gualtieri Beraquet; Teppei Arai, Guilherme Leite Pigolli; Luzia Lyra; Akira Watanabe, Maria Luiza Moretti, 2019; Toyotome et al., 2017; Wu et al., 2015). Mechanisms of azole resistance are often associated with mutations in genes involved in the *A. fumigatus* ergosterol pathway (Alcazar-Fuoli et al., 2008; Da Silva Ferreira et al., 2005), particularly in *cyp51A*, which encodes the cytochrome P450 14- α -lanosterol demethylase, the main target of azole antifungals (Celia-Sanchez et al., 2022; Parker et al., 2014a; J. Zhang, Li, et al., 2019)

The TR₃₄/L98H polymorphism has become a growing concern in clinical settings due to its association with treatment failures and increased mortality rates in invasive aspergillosis (IA), reported to range from 0.6% to 88% (Dudakova et al., 2017). The prevalence of TR₃₄/L98H in *A. fumigatus* isolates has risen steadily and spread worldwide (Celia-Sanchez et al., 2022; Mellado et al., 2001; Parker et al., 2014a; J. Zhang, Li, et al., 2019), posing a significant challenge for effective antifungal therapy, along with the distribution of *ARAf* in relation to the TR₃₄/L98H polymorphism (Snelders et al., 2008). For instance, itraconazole resistance affects around 10% of *A. fumigatus* strains from the Netherlands, while in the UK, it rose from 0%–5% in 2002–2004 to 17%–20% in 2007–2009 (Bowyer et al., 2011; Howard et al., 2009a).

In recent years, numerous studies have reported *ARAf* cases due to TR₃₄/L98H in Argentina, China, Europe (Portugal, Spain, UK), Japan, Taiwan and the US (Lockhart et al., 2011; Rath et al., 2012; Van Der Linden et al., 2013; Astvad et al., 2014; Ramos et al., 2015; Vermeulen et al., 2015; Chen et al., 2016; Jensen et al., 2016; Wiederhold et al., 2016; I. Montesinos, M.

A. Argudín, M. Hites, F. Ahajjam, C. Dagyan, M. Bakkali, I. Etienne, F. Jacobs, C. Knoop and Lagrou, 2017; Laverne et al., 2017). The origin of this polymorphism remains uncertain. However, genotyping of epidemiologically and geographically unrelated strains indicates that TR₃₄/L98H isolates exhibit lower genetic diversity than wild-type isolates, suggesting a single evolutionary event (Camps, Rijs, et al., 2012b; L. B Denardi, W. J. G. Melchers, J. Zoll, J. Buil, F. Hagen, J. F. G. Meis, S. H. Alves, 2018).

Several azole-based fungicides used in agriculture to protect crops from phytopathogenic molds share chemical similarities with medical triazoles, and cross-resistance between them has been demonstrated (Linden et al., 2012a; J. Zhang et al., 2021b). Many fungicides are effective against wild-type strains but ineffective against isolates with high minimum inhibitory concentrations (MICs) caused by TR₃₄/L98H. These fungicides were introduced in the Netherlands between 1980 and 1996, preceding the discovery of the first clinical TR₃₄/L98H strain (collected in 1998) (Linden et al., 2012b; Schoustra et al., 2019a; van der Linden et al., 2011; Van Paassen et al., 2016). As a result, azole resistance is recognized as a global public health concern (Mortensen et al., 2010; van der Linden et al., 2016; Verweij, Lestrade, et al., 2016).

In addition to TR₃₄/L98H, another fungicide-induced mutation, TR₄₆/Y121F/T289A, was discovered in Nijmegen in 2010. This mutation includes two substitutions (Y121F and T289A) and a 46-bp tandem repeat in the *cyp51A* promoter (TR₄₆/Y121F/T289A). TR₄₆/Y121F/T289A has been identified in environmental and clinical samples across four European countries, as well as in Asia, Africa, and France (first detected in 2009) (Hagiwara, 2018; Laverne et al., 2015; Mushi et al., 2016). A rapid increase in TR₄₆/Y121F/T289A was observed in the Netherlands between December 2009 and January 2011, with 21 clinical isolates identified from 15 patients in six hospitals (van Ingen et al., 2015). Multiple reports describe fatal invasive aspergillosis caused by TR₄₆/Y121F/T289A isolates in patients with acute myeloid

leukaemia (AML) or those undergoing hematopoietic stem cell transplantation, with elevated mortality rates linked to azole-resistant strains (Rybak et al., 2019). *In vivo*, resistance development has primarily been associated with nonsynonymous mutations in *cyp51A* (e.g., G54, G138, M220, and G448 substitutions) or non-*cyp51A*-mediated mechanisms unrelated to tandem repeats. Role of *cyp51A* and *cyp51B* in azole resistance

Azole drugs target the CYP51 enzyme, which catalyzes the demethylation of 14- α -methylated sterols, a critical step in ergosterol biosynthesis. Triazole drugs specifically target CYP51. The two isoforms of cytochrome P450 51, *cyp51A* and *cyp51B* (Hokken et al., 2019; Rodriguez-Tudela, Alcazar-Fuoli, et al., 2008), share 59–63% sequence identity (Hokken et al., 2019; Parker et al., 2014b). A gene essentiality study in *A. fumigatus* concluded that *cyp51A* encodes the key enzyme required for mycelial growth, while the biological role of *cyp51B* remains less understood (Hu et al., 2007). Furthermore, deletion of *cyp51A* reverses resistance to fluconazole, whereas deletion of *cyp51B* does not (P. Chen et al., 2020). Additionally, *cyp51A* has only a weak affinity for triazoles, whereas *cyp51B* exhibits a stronger affinity (Parker et al., 2014b). Both *cyp51A* and *cyp51B* are essential for cell survival; when one is deleted, the other can persist without significantly affecting cell morphology or viability (Hu et al., 2007). Moreover, the p.I301T mutation in *cyp51A* and higher expression levels of *cyp51A* compared to *cyp51B* upon fluconazole exposure are believed to contribute to the intrinsic resistance of *Aspergillus* species to this drug (Blosser & Cramer, 2012).

1.5.1 Environmental factors mediate the development of azole resistance of *A. fumigatus*

The development of cross-resistance to medical and agricultural azoles can be attributed to their comparable structures and activities. (Berger et al., 2017b; Cao et al., 2020; Snelders et al., 2012; Verweij, Chowdhary, et al., 2016a). Azole antifungal agents can persist in the environment, and exposure to these agents can contribute to the selection of resistant *A. fumigatus* strains.

Over 30 azole fungicides, such as propiconazole, bromuconazole, epoxiconazole, difenconazole and tebuconazole, have been examined for their *in vitro* efficacy against *A. fumigatus*. In this particular context, the emergence of triazole resistance is believed to have occurred through a route involving the selection pressure exerted by demethylation inhibitors (DMIs) utilised as fungicides (Chowdhary et al., 2013b; Berger et al., 2017; Sewell et al., 2019). Substantial evidence supports the possibility of an environmentally induced mechanism, as exemplified by the detection of TR azole-resistant strains (TR₃₄/L98H, TR₃₄/L98H/S297T/F495I, TR₄₆/Y121F/T289A, and TR₅₃) across the globe, which show smaller genetic distances amongst themselves compared to other azole-susceptible and non-TR-resistant isolates (Alvarez-Moreno et al., 2017). This observation implies that these strains may have originated from a reduced pool of clonally related strains (Camps, Rijs, et al., 2012c; Garcia-Rubio, Monzon, et al., 2018; Snelders et al., 2008; Van Der Linden et al., 2013). Studies that had investigated the frequency of azole resistance in *Aspergillus* culture collections report finding the TR₃₄/L98H to be the most common mechanism. TR₃₄/L98H and TR₄₆/Y121F/T289A were the cause of 82% to 89% of azole-resistant cases in the Netherlands (van Ingen et al., 2015), while it was present in 64% of cases in Belgium (Montesinos et al., 2013; Vermeulen et al., 2012) and 87% of cases in Turkey (Özmerdiven et al., 2015).

Previous work (Snelders et al., 2009b) has provided evidence suggesting that resistant isolates in the environment may be transmitted to human hosts. The study found that *A. fumigatus* isolates containing the TR₃₄/L98H allele were present in both clinical settings and flower beds outside the hospital, as well as in commercial seeds, leaves, and compost. On the other hand, TR₄₆/Y121F/T289A were first observed in isolates from 2008, with two originating from different cities in Pennsylvania, one from Arizona, and one from another reference laboratory. Similar to previous reports, the MICs for voriconazole and isavuconazole were very high (>16 µg/ml) against the two isolates harbouring TR₄₆/Y121F/T289A mutations, while those of

itraconazole and posaconazole were moderately elevated (Y. Chen et al., 2015; Chowdhary et al., 2014; Isla, Leonardelli, Tiraboschi, Refojo, Hevia, Vivot & Córdoba, 2018; Hagiwara et al., 2016).

The genetic analysis of the environmental isolates revealed a high degree of similarity to the isolates detected in patients, which has since been repeated in other studies (Snelders et al., 2009a; Loeffert et al., 2019; Rhodes et al., 2022a). Other work (Cao et al., 2021) has shown a higher prevalence of *ARAf* in areas where azole fungicides are used extensively in agriculture. For example, a study in the Netherlands reported that *ARAf* was more common in regions with high levels of azole fungicide use in agriculture than in regions with low levels of use (Schoustra et al., 2019b).

1.6 Azole resistance molecular mechanisms

Resistance acquired following prolonged therapy has been recognised since the late 1980s (Russell, 2005). Azole resistance in *A. fumigatus* can occur through several mechanisms, including target site modification, increased efflux pumps, decreased target enzyme affinity, and metabolic bypass pathways (Denning et al., 1997a; Mohammadi et al., 2018; Rivero-Menendez et al., 2019; Siopi et al., 2020; Talbot et al., 2018; Tsitsopoulou et al., 2018). The following are some of the key mechanisms of azole resistance:

1. Target site modification: Azole antifungal agents target the 14 α -demethylase enzyme (*cyp51A*) in the ergosterol biosynthesis pathway, which is essential for fungal growth. The knowledge of the ergosterol biosynthesis route in *A. fumigatus* may prove useful in the design of new antifungal drugs and it could help in the study of antifungal drug resistance mechanisms (Alcazar-Fuoli and Mellado, 2012, Bader et al., 2013; Parker et al., 2014a; Brillowska-Dabrowska et al., 2015; Liu et al., 2015; Jingxiang Zhang et al., 2019; Celia-Sanchez et al., 2022). Mutations in the promoter region of *cyp51A* can also lead to overexpression of the gene, which can result in increased production of the

target enzyme and, consequently, increased resistance (Gonzalez-Jimenez et al., 2020; Moore et al., 2022; Tuohy et al., 2010).

2. Efflux pumps: Azole resistance can also occur through the overexpression of efflux pumps, which are membrane transporters that pump azole antifungal agents out of the cell, thereby reducing intracellular concentrations of the drug. Efflux pumps are categorised into two main classes, the ATP-binding cassette (ABC) that require ATP for activity, encoded by 49 genes, and the major facilitator superfamily (MFS) encoded by 278 genes, which can lead to reduced susceptibility or complete resistance to azole antifungal agents (Cannon et al., 2009; Loiko & Wagener, 2017; Natesan et al., 2013; Pérez-Cantero et al., 2020).
3. Azole resistance can also occur through the activation of metabolic bypass pathways that bypass the target enzyme in the ergosterol biosynthesis pathway. These pathways can lead to the production of alternative sterols that replace ergosterol in the fungal cell membrane, reducing the effectiveness of azole antifungal agents (Shishodia, Tiwari, and Shankar, 2019; Pérez-Cantero et al., 2020).
4. Combination of mechanisms: Azole resistance can also occur through a combination of mechanisms, such as mutations in *cyp51A* and overexpression of efflux pumps. This can lead to high-level resistance to azole antifungal agents (Tuohy et al., 2010; Shishodia, Tiwari, and Shankar, 2019; Gonzalez-Jimenez et al., 2020; Pérez-Cantero et al., 2020; Moore et al., 2022).

In recent years, an increasing focus has been placed on acquired resistance observed in environmental isolates of *A. fumigatus*, potentially attributable to exposure to agricultural azoles. These isolates have been linked to infections in patients who have not been previously treated with azole antifungal medications (Burks et al., 2021; Snelders et al., 2008, 2009c; Verweij et al., 2020). Global surveillance studies indicate that more than half

of the clinical isolates resistant to azoles exhibited shared resistance mechanisms. These mechanisms were characterized by point mutations in *cyp51A* and variable tandem repeat insertions in the promoter region, specifically TR₃₄/L98H or TR46/Y121F/T289A, which confer resistance to multiple azoles and have been frequently detected in environmental samples (Burks et al., 2021; Lockhart et al., 2011; van der Linden et al., 2015; H. C. Wang et al., 2018).

1.7 Identification and diagnosis of resistant *A. fumigatus*

Drug resistance in *A. fumigatus* can be identified and diagnosed using several methods, including microbiological and molecular techniques. The following are some commonly used methods for identifying and diagnosing resistance:

1. Antifungal susceptibility testing is the gold standard for identifying resistance in *A. fumigatus*. In 1968, the Clinical and Laboratory Standards Institute (CLSI) (previously known as the National Committee for Clinical Laboratory Standards [NCCLS]) was created with the objective of achieving uniformity in quality control (QC) and susceptibility testing standardisation for all pathogens. Like the CLSI, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was formed to standardise technical aspects of *in vitro* antimicrobial susceptibility testing and to develop breakpoints. In 1997, the EUCAST antifungal susceptibility testing subcommittee formed, and its first published standard of susceptibility testing for both yeasts and moulds was released in 2008. The goal of AFST is to reliably produce MIC values that may be used to guide patient therapy, inform epidemiological studies, and track rates of antifungal drug resistance (Rodriguez-Tudela, Arendrup, et al., 2008; Wayne, 2002).
2. Molecular methods. *Molecular methods such as real-time polymerase chain reaction (PCR) molecular beacon probes, sequencing, high-resolution melt curve analysis, and Next-generation sequencing (NGS) can detect mutations associated with resistance in*

Aspergillus spp (Heldt et al., 2018; Prattes et al., 2018; Jenks et al., 2019; Rawlings et al., 2019). These methods can be used to identify mutations in genes associated with azole resistance, such as *cyp51A*. Two commercially available multiplex real-time PCR assays are available: AsperGenius® (PathoNostics BV, Maastricht, The Netherlands) and MycoGENIE (Ademtech, Pessac, France). The latter assay detects only *A. fumigatus* DNA and specific TR₃₄ and L98H mutations in *cyp51A*. Its sensitivity and specificity were 92.9% and 90.1%, respectively, using respiratory samples ($n = 88$) and 100% and 84.6%, respectively, with serum samples (Kit, 2017).

3. Clinical response to therapy (therapeutic failure): Clinical response to antifungal therapy can also be used to identify resistance. Patients who do not respond to therapy or who experience recurrent infections may be tested for resistance using microbiological or molecular methods.

An important point to note is that the interpretation of antifungal susceptibility testing can be complex; therefore, the results should be interpreted in the context of the patient's clinical history and the specific antifungal agent being assessed. In addition, the clinical significance of specific mutations associated with resistance may vary depending on the geographic location and the patient population.

1.8 Multi-omics to identify infection and resistance routes

The phenotypic characteristics of *Aspergillus* strains, such as virulence and drug resistance, are encoded in its genome as one of the routes. Whole genome sequencing (WGS) is a powerful tool that can identify infection and resistance routes in *A. fumigatus*. Since 2005 (Nierman et al., 2005a), reference genome sequences for *A. fumigatus* have been available, and they have unquestionably supported significant advances in the field, such as detecting low-frequency mutations, identifying key deletions and insertions, and discovering other genetic changes among strains and virulence factors.

In contrast to the genomes of many other fungal species, which are plastic, genomes of the Aspergillus genus are highly stable, with clinically relevant phenotypic traits diverging considerably between isolates (Gibbons & Rokas, 2013). Our ability to undertake full genomic sequencing in a timely and cost-effective manner is creating avenues for clinical applications, such as species identification and the diagnosis of resistance potential (Arastehfar et al., 2019).

Other sequencing studies have demonstrated that relationships between genomes and phenotypic plasticity can be mapped by comparing genomes and phenotypes across multiple isolates (Wood et al., 2021; Yang et al., 2021). This information can develop new antifungal agents or modify existing drugs to overcome resistance (Abdolrasouli et al., 2015a; Bastos et al., 2020). In addition to identifying infection and resistance routes, WGS can study the population genetics of *A. fumigatus*, including the global distribution of strains and the evolution of drug resistance (Ashu et al., 2017; He et al., 2024a; Rocchi et al., 2021b). These findings can then develop strategies for managing fungal infections and preventing resistance spread (Garcia-rubio et al., 2021; J. Rhodes et al., 2022a).

Omics studies, including WGS and downstream analysis of large fungal strain collections, are likely to identify additional virulence factors and drug targets. Several studies integrating genomic sequencing and subsequent screening have already identified gene products that may play crucial roles in *Aspergillus* pathogenicity (Paulussen et al., 2017a; Schikora-Tamarit & Gabaldón, 2022). The analysis of consecutively obtained isolates from individuals with aspergillosis can uncover mutations acquired during infection (Hagiwara et al., 2014). Additionally, WGS can identify mutations causing azole resistance, particularly in isolates lacking *cyp51* mutations (Sharma et al., 2019a).

Lastly, high-throughput sequencing (HTS) has demonstrated potential for detecting pathogenic species directly through targeted barcode sequencing or whole genome shotgun sequencing, without requiring isolation. HTS can provide thousands to millions of sequence reads and up

to several hundred billion base pairs (bp) of data per sample. Read length, error rate, number of reads, and sequenced bases vary substantially (Lee et al., 2016).

1.9 Genetic determinants of phenotypic diversity in *A. fumigatus*

Phenotypic diversity refers to the variation in observable characteristics of a fungus, such as its morphology, growth rate, and pathogenicity. In *A. fumigatus*, phenotypic diversity is influenced by a range of genetic determinants, including single nucleotide polymorphisms (SNPs), copy number variations (CNVs), and epigenetic modifications.

SNPs are variations in a single nucleotide in the DNA sequence, which can cause amino acid changes, thereby influencing the activity and structure of proteins and leading to changes in the phenotype of the fungus. The SNPs identified in *A. fumigatus* are associated with a range of phenotypic traits, including virulence, antifungal resistance, and growth rate (Abdolrasouli et al., 2015a; A. E. Barber et al., 2021; dos Santos et al., 2020a; Keizer et al., 2021). SNPs in *cyp51A*, particularly amino acid substitutions such as G54, G138, M220, G448, L98H, Y121F, and T289A, have been observed to decrease the binding affinity between azoles and their corresponding targets. Long-term administration of azole drugs is frequently linked to these mutations (Garcia-Rubio, Alcazar-Fuoli, et al., 2018; Pérez-Cantero et al., 2020). Other studies have shown that SNPs in *LAEA* (loss of *aflR*-expression A) are associated with increased virulence in *A. fumigatus*. *LAEA* encodes a transcriptional regulator that controls secondary metabolite biosynthesis. Consequently, SNPs in *LAEA* can alter the expression of secondary metabolites that contribute to virulence and other phenotypic traits (Jin et al., 2005; Keller, 2018; J. M. Palmer & Keller, 2010a; Perrin et al., 2007).

Most genomic studies on A. fumigatus have primarily focused on the analysis of SNPs or large-scale structural variants, with minimal or no emphasis on copy number variation due to the organism's genome stability. Copy Number Variation (CNV) is a form of genetic variation characterised by segments of DNA that exhibit variable copy numbers (CN) relative to a

reference genome (Feuk et al., 2006). CNV has the potential to impact phenotype by directly modifying gene function through gene interruption or gene fusion or by altering gene expression through changes in gene dosage, regulatory element dosage, and position effect (Sjödín & Jakobsson, 2012). The CNVs identified in *A. fumigatus* are associated with a range of phenotypic traits and adaptations (Steenwyk & Rokas, 2018). A recent population genomics study of gene clusters encoding secondary metabolites in *A. fumigatus* revealed significant gene copy number variations (CNVs) that likely contribute to phenotypic variation (Duran et al., 2010a; Ikeda et al., 2013a; Lind et al., 2017; Mhatre V. Ho, Ji-Ann Lee & Dien et al., 2008a; Nierman et al., 2005b; J. M. Palmer & Keller, 2010b; Skinner et al., 2010).

In addition to these genetic determinants, phenotypic diversity in *A. fumigatus* is also influenced by environmental factors, such as temperature, pH, and nutrient availability. These environmental factors can interact with genetic determinants to influence a range of phenotypic traits, including growth rate, virulence, and antifungal resistance (Latgé & Chamilos, 2020; Paulussen et al., 2017b; Pérez-Cantero et al., 2020)

1.10 *A. fumigatus* metabolism: growth conditions, environmental stress resistance and metabolism adaptation

The metabolic plasticity of *A. fumigatus* is essential to its ability to thrive in diverse ecological niches and cause disease in humans (Latgé & Chamilos, 2020; Paulussen et al., 2017b; Tekaia & Latgé, 2005b). *A. fumigatus* metabolism is characterised by its ability to utilise a wide range of carbon and nitrogen sources, including glucose, fructose, galactose, and amino acids (Ries et al., 2021). The fungus can also metabolise complex polysaccharides, such as cellulose and chitin, which are abundant in the environment (Gaderer et al., 2016; Saroj & Korrapati, 2023). These observations indicate that the distinct metabolic features facilitating *A. fumigatus* growth in human tissues reflect its adaptability to adverse conditions in its natural environment. A notable attribute is its marked ability to withstand environmental stressors—such as thermal

stress, alkaline pH, hypoxia, iron depletion, and nitrogen starvation—encountered during infection of a susceptible host.

A. fumigatus exhibits exceptional thermotolerance (up to 70°C), attributed to its adaptation to high temperatures in composting substrates (Bhabhra & Askew, 2005). Furthermore, it is optimally adapted for growth at 37°C, the human body's normal temperature, which is critical for its pathogenicity. Hypoxia, a common condition in compost piles and during invasive pulmonary aspergillosis (IPA) in mice, is also crucial for virulence (Grahl et al., 2011; W. Wang et al., 2007).

Metabolism in *A. fumigatus* is tightly regulated by transcriptional and post-transcriptional mechanisms, enabling responses to nutrient availability, stress, and environmental cues (Ene et al., 2014; Latgé & Chamilos, 2020). These mechanisms include producing secondary metabolites like gliotoxin and fumagillin, which exhibit antifungal and immunosuppressive activities. These metabolites protect the fungus from oxidative/nitrosative stress in the wild and host immune responses during infection (Keller, 2019).

Metabolism in *A. fumigatus* is tightly regulated by a range of transcriptional and post-transcriptional mechanisms. These regulatory mechanisms allow the fungus to respond to changes in nutrient availability, stress, and other environmental cues (Raffa et al., 2019). The transcription factor HAPX regulates iron homeostasis and oxidative stress responses, activating vacuolar iron storage under iron-excess conditions to enhance resistance (Abad et al., 2010; Brandon et al., 2015).

The transcription factor SrbA regulates the expression of genes involved in respiration and metabolism (Willger et al., 2008). SrbA belongs to a family of transcriptional regulators required for hypoxia adaptation in pathogenic fungi, including *A. fumigatus*. This metabolic flexibility, combined with stress resistance and environmental adaptability, makes *A. fumigatus*

a challenging pathogen to treat. However, understanding its metabolic and stress-response mechanisms can guide the development of antifungal therapies targeting these pathways.

1.10.1 Primary metabolism in *A. fumigatus* during infection

Primary metabolism refers to the essential biochemical processes that occur within all living cells and drive growth, reproduction, and maintenance of cellular structures and functions. In fungi, primary metabolism involves pathways and processes critical for growth and survival in their natural environment. During invasive pathogenic growth, *A. fumigatus* encounters dynamic microenvironments within the mammalian lung that change depending on the infection phase. As reviewed by (Braus et al., 2004), the transcription of specific genes and subsequent translation of mRNA into proteins are necessary for metabolic and developmental adaptations enabling fungal survival in diverse in vivo environments.

Amino acids are essential for protein synthesis, and their steady supply is imperative for fungal growth. *A. fumigatus* acquires amino acids through environmental transport mechanisms and by metabolizing precursors from primary carbon/nitrogen metabolism. For example, it degrades host proteins deemed unnecessary during infection to meet nutrient demands. The integration of these processes—transport, precursor metabolism, and protein degradation—depends on precise regulatory control.

Amino acid degradation also plays a significant role in *A. fumigatus* pathogenesis. (Krappmann et al., 2004) demonstrated that the transcription factor CPCA is essential for fungal survival under amino acid deprivation and full pathogenicity. These findings indicate that *A. fumigatus* relies on a cross-pathway control system (General Control [GC] of amino acid biosynthesis) to respond to amino acid scarcity in host microenvironments. This implies the fungus cannot fully acquire essential nutrients from the host during infection.

This was confirmed by studying a gene replacement mutant lacking methylcitrate synthase, a key enzyme in the methylcitrate cycle. Methylcitrate synthase degrades propionyl-CoA, a by-

product of valine, methionine, and isoleucine metabolism (Brock & Buckel, 2004; Maerker et al., 2005). (Ibrahim-Granet et al., 2008) further showed that *A. fumigatus* pathogenicity is significantly influenced by methylcitrate synthase activity, supporting the hypothesis that the fungus metabolizes host proteins during infection.

1.10.2 The host presents a distinct microenvironment for *A. fumigatus* infections

The human host provides nutrients and factors that promote *A. fumigatus* growth. The nutrient-rich respiratory tract contains glucose, amino acids, and other resources the fungus uses to grow and produce secondary metabolites like gliotoxin and fumagillin, which help resist oxidative/nitrative stress and host immune responses (Amich & Krappmann, 2012; J. C. Rhodes, 2006). The respiratory tract is the primary infection site following spore inhalation. In healthy individuals, lung airway surface liquid (ASL) and nasal secretions contain glucose concentrations ~0.5 mM (10× lower than serum) (Baker et al., 2006). *A. fumigatus* accesses nitrogen sources like nitrate, ammonium, and amino acids from host tissue degradation. Ammonium, a preferred nitrogen substrate, is sourced from dietary amino acids or endogenous production by gut and lung microbiota (R. P. Dickson & Huffnagle, 2015; A. J. Richardson et al., 2013; Roberfroid et al., 1995). Respiratory diseases (e.g., cystic fibrosis) increase lung ammonium levels (K. L. Palmer et al., 2007).

A. fumigatus has three zinc transporter genes (*zrfA–C*), regulated by environmental pH and zinc concentration (Amich et al., 2009, 2010a). *zrfA* and *zrfB* are critical for growth in acidic, zinc-limiting conditions (similar to lung tissues), while *zrfC* and *aspf2* are co-regulated by ZafA and PacC under alkaline, zinc-limiting conditions. ZafA upregulates these genes under zinc scarcity, while PacC represses them in acidic environments (Amich et al., 2009, 2010a). Two of these transporters, encoded by *zrfA* and *zrfB*, are transcribed at high levels and are required for fungal growth under acidic zinc-limiting conditions, but are not required for growth in

neutral or alkaline zinc-limiting media (Amich et al., 2009), which are the conditions found in lung tissues. Under alkaline and extreme zinc-limiting conditions, *the transcriptional regulators ZafA and PacC regulate the transcription of zrfC and aspf2*, regardless of the pH of the environment, whereas *PacC* represses the expression of these genes under acidic growth conditions (Amich et al., 2010a).

In addition to providing nutrients, The host environment modulates *A. fumigatus* gene/protein activity (Osharov, 2007). A study conducted by (Nierman et al., 2005a) compared gene expression at 30°C, 37°C, and 48°C, finding no pathogenicity-associated genes upregulated at 37°C versus 48°C, suggesting host temperature alone does not activate virulence genes. By contrast Do, Yamaguchi and Miyano (Do et al., 2009) hypothesised hypothesize thermal tolerance stems from heat shock protein (HSP)-regulated metabolic genes. Proteomic changes at varying temperatures remain understudied, but Kroll et al. (2014) identified 64 differentially expressed proteins (e.g., HSP30/42 and HSP90) in *A. fumigatus* during heat shock (30–48°C). (Kroll et al., 2014; Kwon-Chung & Sugui, 2013; Latgé & Chamilos, 2020).

In Host Immune Modulation, *A. fumigatus* produces virulence factors like cell wall components, which provide structural integrity and mediate host interactions. The cell wall (90% polysaccharides/proteins) includes linear (1–3)-glucans (20–35%), branched (1–6)-glucans (4%), (1–3/1–4)-glucans (10%), chitin, and galactomannans (20–25%) (Amarsaikhan et al., 2014; Gresnigt et al., 2014; Ruiz-Herrera & Ortiz-Castellanos, 2019a).

The polysaccharides include linear (1–3)-glucans (20–35%), of which 4% are branched with (1–6) linkages; linear (1–3/1–4)-glucans (10%); additional (1–3)-glucans (35–46%); and chitins and galactomannans (20–25%). The maintenance of fungal cell wall integrity and functionality—along with structural modifications enabling adaptation to the host environment—may contribute to pathogenesis. Though genes involved in synthesizing most *A. fumigatus* cell wall components have been identified (Millet et al., 2018; Ruiz-Herrera &

Ortiz-Castellanos, 2019b) , studies show that mutant strains lacking key biosynthetic enzymes retain virulence comparable to wild-type strains. However, these mutants exhibit delayed growth in pulmonary tissue (Amich et al., 2009, 2010a).

1.10.3 Targeting transcriptional regulators in *A.fumigatus*

argeting transcriptional regulators represents a promising approach for managing *A. fumigatus* infections. These regulators control gene expression at the transcriptional level, enabling fungal adaptation to the host environment during infection (Bultman et al., 2017a). Identifying and disrupting *A. fumigatus* factors critical for host adaptation can attenuate virulence. For example, deleting *zafA* (a zinc-responsive transcription factor) eliminates virulence in murine models of invasive pulmonary aspergillosis (IPA) (Abad et al., 2010; Amich et al., 2009; Moreno et al., 2007a). Similarly, the calcineurin-dependent transcription factor CRZA is essential for IPA virulence in neutropenic mice (Bultman et al., 2017b; Cramer et al., 2008; Gupta et al., 2022). While targeting transcription factors holds potential, it may not always provide a comprehensive understanding of *in vivo* metabolic status. Instances exist where disrupting master regulators of cellular processes fails to reduce virulence. For example, deleting the transcriptional regulator YapA significantly decreased oxidative stress resistance but did not affect virulence (Lessing et al., 2007). Similarly, removing the gene for the transcriptional regulator PRTT impaired extracellular proteolysis and protein substrate utilization, yet virulence remained unchanged (Sharon et al., 2009). Such outcomes suggest that either the targeted cellular functions do not contribute to pathogenesis in the studied disease model or compensatory mechanisms offset their loss (Bergmann et al., 2009). Overall, targeting transcriptional regulators remains a promising strategy for managing *A. fumigatus* infections. This approach enables detailed study of regulator functions and the development of therapies targeting their activity. However, further research is required to fully characterize the transcriptional regulators involved in the fungus's adaptation to the host environment during

infection. In addition, several transcriptional regulatory pathways in *A. fumigatus* have been characterized. These pathways control genes critical for host adaptation, virulence, and pathogenicity. The HAPX pathway, for instance, regulates transcription in response to iron availability (Liu et al., 2024). HAPX, a transcription factor essential for iron homeostasis under low-iron conditions, controls genes involved in iron acquisition and siderophore biosynthesis (Furukawa et al., 2020). It also represses iron-utilizing genes to conserve iron for essential cellular functions (Furukawa et al., 2020; Liu et al., 2024; Schrettl et al., 2010).

The HAPX pathway is vital for *A. fumigatus* to adapt to the iron-limited host environment. By tightly regulating iron homeostasis, the fungus successfully acquires and utilizes iron for growth and pathogenicity. Transcriptional regulation studies have elucidated mechanisms behind *A. fumigatus* virulence, offering insights into how gene expression controls infection (Liu et al., 2024; López-berges et al., 2021). While progress has been made in understanding transcriptional regulation in *A. fumigatus*, there are still gaps in knowledge regarding specific regulatory mechanisms by which transcriptional regulation occurs in response to host environments, global regulatory networks, and transcriptional dynamics during host adaptation in *A. fumigatus* during infection progression are not completely clear. Studying the temporal regulation of gene expression and how it correlates with different stages of infection could provide valuable insights.

1.10.4 Targeting biosynthetic pathways and essential genes as primary targets

The growth and virulence of *A. fumigatus* within a susceptible host are supported by its metabolic versatility (Ballard et al., 2018). *In vivo* transcriptional profiling studies have provided a comprehensive understanding of the metabolic state of *A. fumigatus* during germination in the lungs of neutropenic mice. The findings suggest that the nutritional status of *A. fumigatus* is characterised by significant nitrogen starvation (Ene et al., 2014; Krappmann

& Braus, 2005). In line with this, various gene products implicated in nitrogen sensing and utilisation can affect the virulence of *A. fumigatus* (Krappmann & Braus, 2005).

Regarding carbon, a thorough examination of a mutant strain deficient in methylcitrate cycling has indicated that proteins could potentially function as the primary source of this macroelement. This discovery aligns with the previously reported complete virulence of a mutant compromised in fatty acid metabolism (Dagenais & Keller, 2009b; Ries et al., 2019a). Through additional mutant analysis, various biosynthetic pathways have been identified that are capable of supporting fungal growth within the nutritionally deficient environment of murine lung tissue.

A well-recognised example is the biosynthesis of uracil, which is represented by *pyrG*. This gene encodes orotidine-5'-phosphate decarboxylase, facilitating the final step in *de novo* UMP biosynthesis. Strains devoid of *pyrG* exhibit auxotrophy for uracil/uridine and demonstrate a significant reduction in virulence in a non-neutropenic murine IA model (D'Enfert, 1996; Sun et al., 2013; Y. Zhang et al., 2020). In addition, the siderophore biosynthesis pathway is crucial, as confirmed by deletion of *sidA*, responsible for encoding L-ornithine-N5-monooxygenase. This enzyme facilitates the first and crucial step in hydroxamate-type siderophore biosynthesis (Hissen et al., 2005; Li et al., 2016; Khan, Singh, and Srivastava, 2018).

The fundamental importance of protein functions in fungal growth implies that the absence of their activity would result in the loss of virulence. Romero (Romero et al., 2003) validated a sophisticated method for identifying essential genes through the implementation of a conditional promoter replacement (CPR) strategy, enhanced by utilising nitrogen-regulated promoters in *A. fumigatus* (Binder et al., 2020; Hu et al., 2007). This approach blocks the expression of the gene of interest when grown in ammonium, whereas the presence of nitrate as a nitrogen source enables appropriate expression under the regulation of *pNiiA*. The efficacy of this system was demonstrated through the phenotypic similarity between repressed *pNiiA*-

CPR strains and their corresponding disruption mutants. Direct virulence testing of these conditional strains in animal models further validates this approach. Hence, CPR methodology constitutes a legitimate and streamlined technique for large-scale identification of crucial genes.

Overall, the biosynthetic routes and essential genes in *A. fumigatus* represent promising targets for developing new antifungal therapies. Inhibiting the ergosterol biosynthesis pathway, secondary metabolite biosynthesis, or essential genes can reduce fungal growth and virulence. Further research is needed to identify and characterise additional targets in *A. fumigatus* and to develop therapies targeting these critical pathways.

1.11 High throughput phenotypic microarray for microbial culturomics: The OmniLog

Functional traits in organisms manifest as phenotypic characteristics, jointly determined by the genome and environment (Houle et al., 2010). A common method for evaluating phenotypes involves assessing replication patterns under specific environmental conditions by analyzing growth curve phases (Casler et al., 2015; Denham et al., 2020; Grinberg et al., 2020; Sikorski & Nevo, 2007). The lag phase duration reflects adaptation efficiency to environmental conditions, while the log phase (cell replication) and stationary phase (growth cessation) indicate how growth is achieved (Brisbin, et al., 1987).

The OmniLog Phenotype MicroArrays (PMs), alongside DNA microarrays and proteomic technologies, represent a key advancement in genomic-era research and drug development. PMs hold promise for studying phenotypes in microorganisms like *A. fumigatus*. The OmniLog system is a semi-high-throughput platform that monitors microbial growth and metabolism across numerous nutrient sources (e.g., carbon, nitrogen, phosphorus) and chemical stressors (Biolog, Inc.) (<https://www.biolog.com/>) (Borglin et al., 2012).

The OmniLog system consists of a 96-well microplate, each containing diverse chemical agents or nutrients (Bochner et al., 2001a; Dunkley et al., 2019). Developed by Biolog (Hayward, CA), the system integrates an incubator and a charge-coupled device (CCD) camera, enabling simultaneous analysis of up to 50 plates. These plates are organized into 25 trays (numbered 1–25), each subdivided into two columns ('a' and 'b'), and incubated at temperatures ranging from 22°C to 45°C ($\pm 2^\circ\text{C}$ tolerance).

The OmniLog digital camera quantifies color intensity in OmniLog Units (OU), a proprietary measure of light transmission. An OU value of 0 corresponds to complete light transmission, while 500 OU indicates no transmission. The OU scale is directly proportional to optical density (OD), calculated as **OU = OD $\times$ 500**.

The microorganism is inoculated into the wells, and cell growth (and thus metabolism) is monitored over time using either a redox dye-based colorimetric assay (e.g., tetrazolium violet) to measure respiration (via NADH activity) or densitometer measurements (Blanco et al., 2018a). The colorimetric assay quantifies metabolic rates by detecting a purple color change in the well, reflecting substrate utilization. This method generates metabolic fingerprints that identify microbes with industrially relevant traits (Franco-Duarte et al., 2019; Greetham, 2014). While the OmniLog PM has been widely used to study bacterial metabolism and identify antibacterial drug targets (Dong et al., 2010; Maugeri et al., 2019), its application to fungi—particularly filamentous fungi like *A. fumigatus*—remains limited. Substrate utilization patterns have proven useful for optimizing secondary metabolite production in fungal cultures, revealing correlations between substrate use, growth, antibacterial activity, and secondary metabolite presence. This technique also distinguishes intraspecies variations (Singh, 2009a). The FF MicroPlate™ (Biolog), leveraging Phenotype Array Technology, enables rapid identification and characterization of filamentous fungi (Blanco et al., 2018b; Singh, 2009a). The Biolog® Phenotype MicroArrays™ (PM) system integrates advanced techniques like

whole-genome sequencing (WGS) (Bochner et al., 2001). PM plates test up to 2000 physiological challenges (e.g., single carbon source metabolism, osmolyte tolerance, inhibitor responses), generating kinetic data that capture longitudinal growth dynamics. These kinetic curves encode biological insights through shape attributes, similar to traditional growth curves. Historically, PM data analysis focused on qualitative assessments (e.g., positive/negative responses), neglecting kinetic details. Despite early calls for advanced analytical strategies (Warringer & Blomberg, 2003) However, the company has launched their own OmniLog PM software (Inc, 2009a), in addition to the native software, as the basic part of an add-on package OPM implemented in the statistical software package R (Vaas et al., 2013); this provides a framework for parameter estimation using model fitting and model-free spline fitting independently, as well as CI-based statistical evaluation of the curves. This application employs the intermediate output, which consists of estimations for parameters that describe the curve, including the length of the lag phase (referred to as λ), the growth rate (indicating the increase in respiration and referred to as μ , akin to “slope”) and the maximum cell growth (reflective of respiration and referred to as A, corresponding to the maximum value recorded, as an additional descriptive attribute of cell parameters).

Overall, the OmniLog PM represents a promising tool for studying the metabolism of fungi and for identifying new targets for drug discovery. The high-throughput nature of the system allows rapid screening of large numbers of compounds and identification of critical pathways and essential genes required for the survival and growth of microorganisms.

1.12 Genetic and Phenotypic Variation in *Aspergillus fumigatus*

The study of genetic and phenotypic variation in *A. fumigatus* isolates is critical for comprehensively understanding the pathogen's adaptability and its implications for treatment outcomes. *A. fumigatus*, as a leading opportunistic pathogen, poses significant challenges in

clinical settings, particularly for immunocompromised individuals, including those with cystic fibrosis. The intricate relationship between genetic mutations and phenotypic expressions elucidates how this fungus not only survives but thrives in diverse environments, further complicating therapeutic interventions.

Genetic variation in *A. fumigatus* is a foundation of its adaptability, with features such as single nucleotide polymorphisms (SNPs), copy number variations (CNVs), and epigenetic modifications playing pivotal roles in its virulence and antifungal resistance (Abdolrasouli et al., 2015a; dos Santos et al., 2020). Furthermore, the presence of CNVs within genes associated with stress response mechanisms can lead to the amplification of genes associated with stress response mechanisms. For example, the upregulation of efflux pump genes, including ATP-binding cassette (ABC) transporters, can enhance the fungus's ability to expel antifungal agents, thereby conferring a survival advantage during treatment. This genetic flexibility highlights the dynamic nature of *A. fumigatus*, which can rapidly adapt to changing conditions, complicating treatment regimens and leading to persistent infections that are difficult to manage.

The selection of a cohort comprising sufficient number of clinical samples of *A. fumigatus* is strategically significant for understanding the pathogen's diversity. Isolates were chosen based on their representation of the phenotypic and genetic variability observed in this specific patient population, which is known to harbour a diverse array of fungal strains due to the unique pulmonary environment created by chronic infection and treatment regimens.

CF patients often experience fluctuating levels of immune response and exposure to various antifungal agents, leading to an evolution of *A. fumigatus* strains that may exhibit distinct metabolic characteristics and resistance profiles. By selecting isolates from this cohort, the study aims to capture the full spectrum of adaptability within the *A. fumigatus* population

thereby supporting the hypothesis that metabolic and phenotypic variability significantly influences treatment outcomes. The unique pressures faced by CF patients—such as recurrent exposure to antifungal therapies, fluctuations in immune status, and the presence of co-infections—create a dynamic environment that fosters the emergence of genetically diverse and phenotypically distinct *A. fumigatus* strains. By capturing this variability, the study aims to elucidate the potential relationships between specific genetic mutations, phenotypic adaptations, and clinical outcomes, enhancing our understanding of the role of *A. fumigatus* in chronic pulmonary infections.

Additionally, in evaluating the adequacy of the sample size of 145 clinical isolates, it is essential to consider the statistical power, and the inclusion of controls is vital for validating the findings of the study. Including azole-susceptible strains as a control group will provide a comparative baseline against which the resistant isolates can be evaluated. This approach will help to elucidate the mechanisms underlying antifungal resistance and the metabolic adaptations that *A. fumigatus* employs in response to therapeutic pressures. By systematically including susceptible strains, the study can establish a clearer connection between observed phenotypic characteristics and the genetic variations associated with resistance, ultimately contributing to a more comprehensive understanding of the pathogen's adaptability.

1.13.4 Phenotypic Diversity in Response to Environmental Stressors

Phenotypic variation is equally significant, encompassing observable traits such as growth rates, metabolic profiles, and resistance patterns, which are crucial for predicting how various *A. fumigatus* isolates will respond to antifungal therapies (Pérez-Cantero et al., 2020; Latgé & Chamilos, 2020). A striking example of this phenotypic flexibility is the fungal response to nutrient scarcity or antifungal exposure. Isolates exposed to sub-lethal concentrations of antifungals can undergo phenotypic changes, such as increased expression of biofilm-forming

genes. This transition to a biofilm phenotype not only enhances resistance to antifungal agents but also provides a protective niche against host immune responses.

Furthermore, phenotypic heterogeneity can result in different growth rates and metabolic adaptations that allow *A. fumigatus* to survive in diverse ecological niches. For example, isolates that can efficiently utilize alternative carbon sources may survive better in nutrient-limited environments, enhancing their virulence and persistence during antifungal treatment. Understanding these adaptive mechanisms is essential for formulating targeted therapeutic strategies that can mitigate the impact of *A. fumigatus* in susceptible populations.

In conclusion, the intricate interplay between genetic and phenotypic variations in *A. fumigatus* reveals a highly adaptable pathogen capable of evading antifungal treatments and establishing chronic infections. Understanding these variations is not merely an academic exercise but a critical component of developing effective therapeutic strategies against this formidable organism. The rationale for selecting a diverse cohort of isolates from patients with and without CF, along with a thorough assessment of sample size and control measures, ensures that the study's findings are robust and relevant. As research continues to unfold the complexities of *A. fumigatus*, it becomes clear that a multifaceted approach, addressing both genetic and phenotypic dimensions, is essential to combat this pathogen effectively and improve patient outcomes.

Hypothesis and Objectives:

The objective of this thesis is to elucidate the metabolic and phenotypic diversity observed in clinical *Aspergillus fumigatus* isolates is underpinned by genotypic and transcriptomic variation, which drives adaptive flexibility during host infection. A keen understanding of the interplay between genetic diversity and metabolic plasticity is imperative for elucidating how

A. fumigatus successfully occupies varied ecological niches, thereby enhancing its persistence within human hosts and facilitating resistance to azole antifungals.

Objectives:

1. To characterize the phylogenomic patterns and population structure of azole-susceptible and azole-resistant *A. fumigatus* clinical isolates. , thus providing a comprehensive framework for understanding their genetic diversity.
2. To determine whether metabolic and phenotypic variability correlates with genomic and transcriptomic diversity in *A.fumigatus* isolates from patients with respiratory diseases such as CF
3. To identify genomic and transcriptomic variations associated with phenotypic differences and to identify largely overlapping gene sets, contributing to the understanding of the functional implications of genetic variation.

2 Materials and Methods

2.1 *Aspergillus fumigatus* isolates

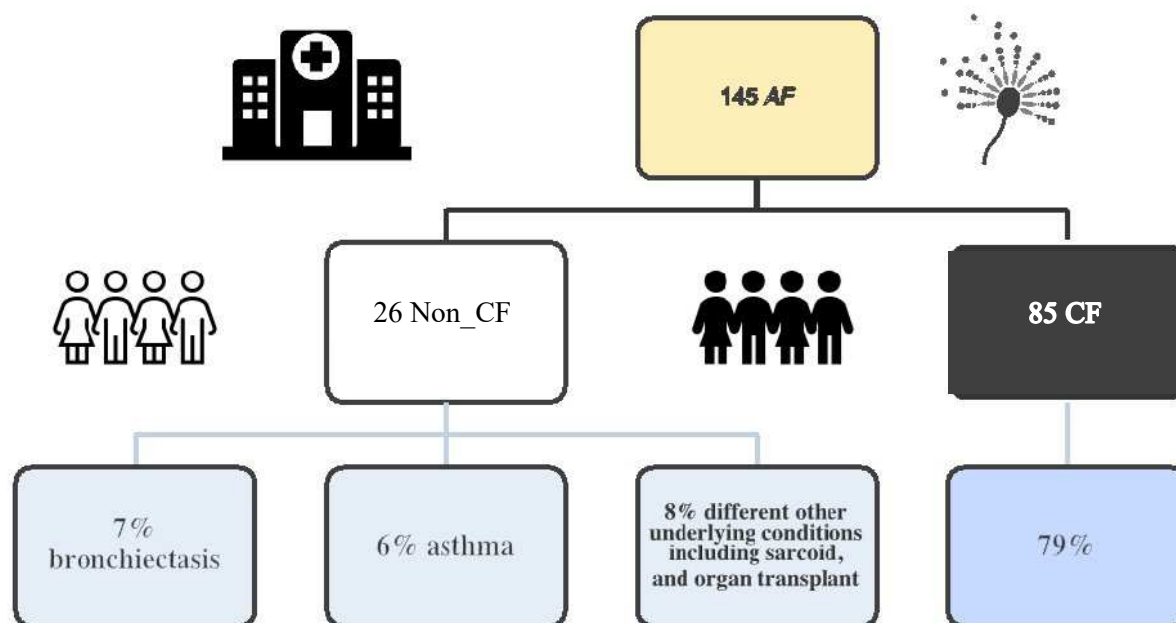


Figure 2: Schematic representation of the patient distribution in a cohort of 145 *A. fumigatus* isolates. The breakdown of patients includes 85 with Cystic Fibrosis (CF) and 32 with Non-CF conditions. The categories are as follows: (1) Non-CF patients consist of 7% with Bronchiectasis, 6% with Asthma, and 8% with various other underlying conditions (including sarcoidosis and organ transplant); (2) CF patients account for the majority at 79% of the total cohort. The flowchart visually communicates the distribution and percentage of each condition among the patient population.

Table 1 presents the analysis of 145 *A. fumigatus* isolates collected from patients at the Royal Brompton Hospital (RBH) in London. These patients exhibited either respiratory colonization with *A. fumigatus* or various forms of aspergillosis. The underlying conditions of these patients were as follows, listed in order of descending prevalence: cystic fibrosis (CF) (79%), bronchiectasis (7%), asthma (6%), and other conditions (8%), which included sarcoidosis and

organ transplants (Figure 2). It is important to note that longitudinal patient information for 50 isolates, specifically from C106 to C173, was unavailable; consequently, each of these isolates was treated as a single isolate corresponding to a single patient. All isolated samples were processed and quantified to extract high-quality, high-molecular-weight DNA using the MasterPure Yeast DNA Purification Kit (Epicentre) with bead beating, as described previously (Rhodes 2022). Genomic DNA libraries were constructed using the Illumina TruSeq Nano Kit at the NERC Biomolecular Analysis Facility, University of Edinburgh, UK. Whole-genome sequence libraries were then sequenced on an Illumina HiSeq 2500 sequencer, generating 150 bp paired-end reads in high-output mode. Isolation and sequencing were performed by Darius Armstrong-James, Andrew Scourfield, and Alireza Abdolrasouli, and data analysis was conducted by Renad Aljohani. Data were stored in the High-Performance Computing facility at the Research Computing Service, Imperial College London. All raw reads have been submitted to the European Nucleotide Archive under accession number PRJEB63143. An additional 24 isolates, sequenced as part of Abdolrasouli et al. (2015a), were submitted under accession number PRJEB8623 (Abdolrasouli et al., 2015a).

Table 1: *A. fumigatus* clinical isolates from RBH including isolate IDs, year of isolation, genotype, underlying diagnosis of patient, isolates' mating type, antifungal susceptibility, and location on the phylogenetic tree.

Id	Phenotyping ID	Longitudinal patients	Cyp51A Genotype	Underlying Diagnosis	Matting type	Antifungal susceptibility	Clade
C306	IGF11953	RBHp 1	TR ₃₄ /L98H	Coronary artery disease	MAT1-1	Resistant	A
C307	IGF11954	RBHp 2	TR ₃₄ /L98H	Asthma	MAT1-1	Resistant	A
C308	IGF11955	RBHp 3	TR ₃₄ /L98H	Bronchiectasis	MAT1-1	Resistant	A
C309	IGF11956	RBHp 4	TR ₃₄ /L98H	CF	MAT1-2	Resistant	A
C310	IGF11957	RBHp 5	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C311	IGF11958	RBHp 5	Wildtype	CF	MAT1-2	Resistant	B
C312	IGF11959	RBHp 6	Wildtype	CF	MAT1-1	Susceptible	B
C313	IGF11960	RBHp 6	Wildtype	CF	MAT1-1	Resistant	B
C314	IGF11961	RBHp 7	Wildtype	CF	MAT1-1	Resistant	B
C315	IGF11962	RBHp 8	Wildtype	Bronchiectasis	MAT1-1	Resistant	B
C316	IGF11963	RBHp 9	Wildtype	Sarcoid	MAT1-1	Resistant	B
C317	IGF11964	RBHp 10	M220K	Non_CF	MAT1-1	Resistant	B
C318	IGF11965	RBHp 10	TR ₃₄ /L98H	Non_CF	MAT1-1	Resistant	A
C319	IGF11966	RBHp 11	TR ₃₄ /L98H	Bronchiectasis	MAT1-1	Resistant	A

C320	IGF11967	RBHp 12	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C321	IGF11968	RBHp 12	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C322	IGF11969	RBHp 12	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C323	IGF11970	RBHp 13	TR ₃₄ /L98H	Asthma	MAT1-2	Resistant	A
C324	IGF11971	RBHp 14	Wildtype	CF	MAT1-2	Susceptible	B
C325	IGF11972	RBHp 14	Wildtype	CF	MAT1-2	Resistant	B
C326	IGF11973	RBHp 14	Wildtype	CF	MAT1-2	Resistant	B
C327	IGF11974	RBHp 14	Wildtype	CF	MAT1-1	Resistant	B
C328	IGF11975	RBHp 15	Wildtype	CF	MAT1-1	Resistant	B
C329	IGF11976	RBHp 16	Wildtype	CF	MAT1-1	Resistant	B
C330	IGF11977	RBHp 17	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C331	IGF11978	RBHp 18	Wildtype	CF	MAT1-2	Resistant	B
C332	IGF11979	RBHp 18	Wildtype	CF	MAT1-2	Resistant	B
C333	IGF11980	RBHp 19	Wildtype	CF	MAT1-2	Resistant	B
C334	IGF11981	RBHp 20	Wildtype	CF	MAT1-1	Resistant	B
C335	IGF11982	RBHp 21	Wildtype	Sarcoid	MAT1-2	Resistant	B
C336	IGF11983	RBHp 22	Wildtype	CF	MAT1-1	Resistant	B
C337	IGF11984	RBHp 22	Wildtype	CF	MAT1-1	Resistant	B
C338	IGF11985	RBHp 22	Wildtype	CF	MAT1-2	Resistant	B
C339	IGF11986	RBHp 23	TR ₁₃₀	CF	MAT1-1	Susceptible	B
C340	IGF11987	RBHp 23	TR ₁₃₀	CF	MAT1-1	Resistant	B
C341	IGF11988	RBHp 23	TR ₁₃₀	CF	MAT1-2	Resistant	B
C342	IGF11989	RBHp 23	TR ₁₃₀	CF	MAT1-2	Resistant	B
C343	IGF11990	RBHp 23	Wildtype	CF	MAT1-1	Susceptible	B

	IGF11991	RBHp 23	TR ₁₃₀	CF	MAT1-1	Resistant	B
C344	IGF11992	RBHp 4	Wildtype	CF	MAT1-1	Resistant	B
C345	IGF11993	RBHp 4	Wildtype	CF	MAT1-1	Susceptible	A
C346	IGF11994	RBHp 24	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C347	IGF11995	RBHp 25	Wildtype	CF	MAT1-1	Resistant	B
C348	IGF11996	RBHp 26	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C349	IGF11997	RBHp 27	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C350	IGF11998	RBHp 27	Wildtype	CF	MAT1-2	Susceptible	B
C351	IGF11999	RBHp 27	L98H/M220K	CF	MAT1-2	Resistant	B
C352	IGF12000	RBHp 27	L98H/M220K	CF	MAT1-2	Resistant	B
C353	IGF12001	RBHp 28	L98H/M220K	CF	MAT1-1	Resistant	B
C354	IGF12002	RBHp 28	Wildtype	CF	MAT1-2	Susceptible	B
C355	IGF12003	RBHp 29	Wildtype	CF	MAT1-2	Susceptible	B
C356	IGF12004	RBHp 29	Wildtype	CF	MAT1-2	Susceptible	A
C357	IGF12005	RBHp 10	Wildtype	CF	MAT1-1	Susceptible	B
C358	IGF12006	RBHp 11	TR ₃₄ /L98H	Bronchiectasis	MAT1-1	Resistant	A
C360	IGF12007	RBHp 30	Wildtype	Asthma	MAT1-2	Susceptible	B
C361	IGF12008	RBHp 30	Wildtype	Bronchiectasis	MAT1-1	Susceptible	B
C362	IGF12009	RBHp 18	Wildtype	CF	MAT1-2	Susceptible	B
C363	IGF12010	RBHp 31	Wildtype	CF	MAT1-1	Susceptible	B

C364	IGF12011	RBHp 31	Wildtype	CF	MAT1-2	Susceptible	B
C365	IGF12012	RBHp 32	Wildtype	Asthma	MAT1-1	Susceptible	A
C366	IGF12013	RBHp 15	Wildtype	Bronchiectasis	MAT1-2	Susceptible	B
C367	IGF12014	RBHp 33	Wildtype	Sarcoid	MAT1-1	Susceptible	A
C368	IGF12015	RBHp 34	Wildtype	CF	MAT1-2	Susceptible	B
C369	IGF12016	RBHp 35	Wildtype	CF	MAT1-2	Susceptible	B
C370	IGF12017	RBHp 36	Wildtype	Sarcoid	MAT1-2	Susceptible	B
C371	IGF12018	RBHp 37	Wildtype	CF	MAT1-2	Susceptible	B
C372	IGF12019	RBHp 14	G54W	Non_CF	MAT1-2	Susceptible	B
C373	IGF12020	RBHp 38	Wildtype	CF	MAT1-2	Susceptible	B
C374	IGF12021	RBHp 39	Wildtype	Non_CF	MAT1-1	Susceptible	A
C375	IGF12022	RBHp 40	Wildtype	CF	MAT1-2	Susceptible	B
C376	IGF12023	RBHp 41	Wildtype	Asthma	MAT1-2	Susceptible	B
C377	IGF12024	RBHp 5	TR ₃₄ /L98H	CF	MAT1-2	Resistant	A
C378	IGF12025	RBHp 42	Wildtype	CF	MAT1-2	Susceptible	B
C379	IGF12026	RBHp 43	Wildtype	CF	MAT1-2	Susceptible	A
C380	IGF12027	RBHp 18	Wildtype	CF	MAT1-1	Susceptible	B
C381	IGF12028	RBHp 44	Wildtype	CF	MAT1-2	Susceptible	B
C382	IGF12029	RBHp 45	Wildtype	CF	MAT1-2	Susceptible	B
C383	IGF12030	RBHp 27	Wildtype	Bronchiectasis	MAT1-1	Susceptible	B
C384	IGF12031	RBHp 46	Wildtype	CF	MAT1-2	Susceptible	B
C385	IGF12033	RBHp 47	Wildtype	CF	MAT1-1	Susceptible	B
C386	IGF12034	RBHp 48	Wildtype	CF	MAT1-1	Susceptible	A

C387	IGF12035	RBHp 49	Wildtype	Bronchiectasis	MAT1-2	Susceptible	A
C388	IGF12036	RBHp 50	Wildtype	Asthma	MAT1-2	Susceptible	B
C389	IGF12037	RBHp 51	Wildtype	CF	MAT1-2	Susceptible	B
C390	IGF12038	RBHp 52	Wildtype	CF	MAT1-2	Susceptible	B
C391	IGF12039	RBHp 53	Wildtype	CF	MAT1-2	Susceptible	A
C392	IGF12040	RBHp 54	Wildtype	CF	MAT1-1	Susceptible	A
C393	IGF12041	RBHp 55	Wildtype	CF	MAT1-1	Susceptible	B
C395	IGF12042	RBHp 56	Wildtype	Bronchiectasis	MAT1-2	Susceptible	B
C396	IGF12043	RBHp 57	Wildtype	Bronchiectasis	MAT1-1	Susceptible	A
C397	IGF12044	RBHp 58	Wildtype	CF	MAT1-2	Resistant	A
C398	IGF12045	RBHp 59	Wildtype	CF	MAT1-2	Susceptible	B
C399	IGF12046	RBHp 60	Wildtype	CF	MAT1-2	Susceptible	B
C400	IGF12047	RBHp 61	Wildtype	Pulmonary metastasis	MAT1-2	Susceptible	B
C106	C124	RBHp 62	G54W	Interstitial lung disease	MAT1-1	Resistant	B
C107	C125	RBHp 63	TR ₃₄ /L98H	CF	MAT1-2	Resistant	A
C108	C126	RBHp 64	G54W	Aortic valve replacement	MAT1-1	Resistant	B
C109	C127	RBHp 65	Wildtype	CF	MAT1-2	Resistant	B
C110	C128	RBHp 66	TR ₁₃₀	CF	MAT1-2	Resistant	B
C111	C129	RBHp 67	Wildtype	CF	MAT1-2	Resistant	B
C112	C130	RBHp 68	G54W	CF	MAT1-2	Resistant	B

C113	C131	RBHp 69	G54W	CF	MAT1-2	Resistant	B
C114	C132	RBHp 70	G54W	CF	MAT1-2	Resistant	B
C115	C133	RBHp 71	TR ₃₄ /L98H	L.VENTRICULAR ASSIST	MAT1-2	Resistant	A
C116	C134	RBHp 72	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C117	C135	RBHp 73	G54W	CF	MAT1-2	Resistant	B
C118	C136	RBHp 74	TR ₁₃₀	CF	MAT1-2	Resistant	B
C119	C137	RBHp 75	TR ₁₃₀	CF	MAT1-2	Resistant	B
C120	C138	RBHp 76	P216L	Non_CF	MAT1-1	Resistant	B
C121	C139	RBHp 77	Wildtype	CF	MAT1-2	Resistant	B
C122	C140	RBHp 78	G54W	Bronchiectasis	MAT1-1	Resistant	B
C123	C141	RBHp 79	TR ₃₄ /L98H	CF	MAT1-1	Resistant	A
C124	C142	RBHp 80	G54W	Bronchiectasis	MAT1-1	Resistant	A
C125	C143	RBHp 81	TR ₃₄ /L98H	CF	MAT1-2	Resistant	A
C126	C144	RBHp 82	TR ₃₄ /L98H	CF	MAT1-2	Resistant	A
C127	C145	RBHp 83	P216L	CF	MAT1-2	Susceptible	B
C146	C164	RBHp 84	Wildtype	CF	MAT1-2	Resistant	B
C147	C165	RBHp 85	Wildtype	CF	MAT1-1	Resistant	B
C148	C166	RBHp 86	Wildtype	CF	MAT1-2	Susceptible	B
C149	C167	RBHp 87	Wildtype	CF	MAT1-1	Susceptible	B
C150	C168	RBHp 88	Wildtype	CF	MAT1-1	Resistant	B
C151	C169	RBHp 89	Wildtype	CF	MAT1-2	Susceptible	B

C152	C170	RBHp 90	Wildtype	CF	MAT1-2	Susceptible	B
C153	C171	RBHp 91	TR ₃₄ /L98H	CF	MAT1-2	Resistant	A
C154	C172	RBHp 92	Wildtype	CF	MAT1-2	Susceptible	B
C155	C173	RBHp 93	Wildtype	CF	MAT1-1	Susceptible	B
C156	C174	RBHp 94	Wildtype	CF	MAT1-1	Susceptible	B
C157	C175	RBHp 95	Wildtype	CF	MAT1-2	Susceptible	A
C158	C176	RBHp 96	Wildtype	CF	MAT1-1	Susceptible	B
C159	C177	RBHp 97	Wildtype	CF	MAT1-1	Susceptible	A
C160	C178	RBHp 98	Wildtype	CF	MAT1-2	Susceptible	B
C161	C179	RBHp 99	Wildtype	CF	MAT1-1	Susceptible	B
C162	C180	RBHp 100	Wildtype	CF	MAT1-2	Susceptible	B
C163	C181	RBHp 101	Wildtype	CF	MAT1-1	Susceptible	B
C164	C182	RBHp 102	Wildtype	CF	MAT1-2	Susceptible	A
C165	C183	RBHp 103	Wildtype	CF	MAT1-2	Susceptible	A
C166	C184	RBHp 104	Wildtype	CF	MAT1-2	Susceptible	B
C167	C185	RBHp 105	Wildtype	CF	MAT1-1	Susceptible	B
C168	C186	RBHp 106	Wildtype	CF	MAT1-1	Susceptible	A
C169	C187	RBHp 107	Wildtype	CF	MAT1-2	Susceptible	B
C170	C188	RBHp 108	Wildtype	CF	MAT1-1	Susceptible	A
C171	C189	RBHp 109	G54R	CF	MAT1-2	Resistant	B
C172	C190	RBHp 110	Wildtype	CF	MAT1-1	Susceptible	B
C173	C191	RBHp 111	Wildtype	CF	MAT1-2	Susceptible	B

2.1 Bioinformatics workflow for WGS :

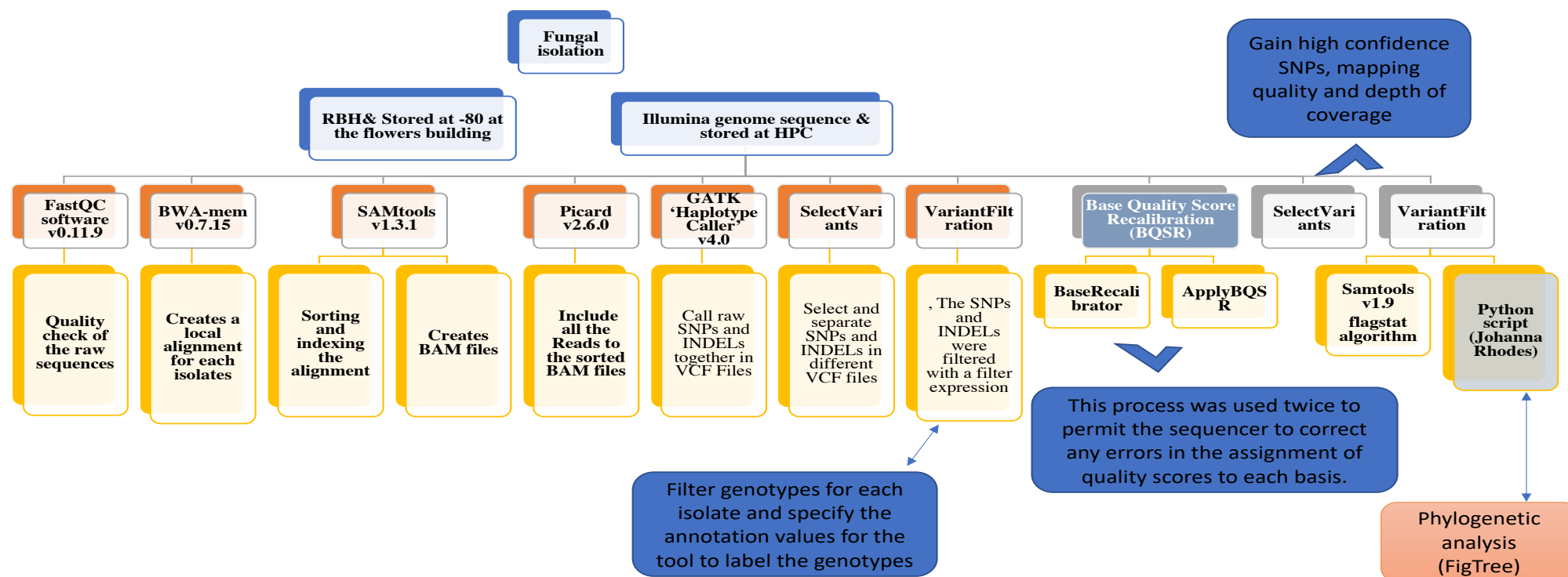


Figure 3: Schematic representations of the workflow perform the analysis **for bioinformatics analysis** developed (by **Johanna Rhodes**) to analyse a total of 145 *A. fumigatus* isolates, using Af293 as a reference genome. The bioinformatics workflow for WGS falls into the following steps: (1) raw read quality control; (2) data pre-processing; (3) alignment; (4) variant calling; (5) genome assembly; (6) genome annotation; (7) phylogenetic analysis. Assessment of the mapping quality and coverage statistics revealed a mean coverage across the series of 13X, with an average single nucleotide polymorphisms (SNPs) count of 47,920 per isolate. On average, 96.8% of the reads were successfully mapped to the reference genome. A maximum likelihood phylogenetic tree was constructed using Randomised Axelerated Maximum Likelihood (RAXML) and visualised using FigTree and Microreact software(Rhodes *et al.*, 2022b).

2.1.1 Mapping to the Af293 *A. fumigatus* reference genome

The complete whole genome sequence of the reference genome Af293 is 29.4 MB, which consists of 9,926 predicted genes on eight chromosomes plus a mitochondrial genome (Nierman et al., 2005a). Af293 was the first *A. fumigatus* isolate to be sequenced and is used as a reference genome in many analyses (Abdolrasouli et al., 2015b; He et al., 2024b; Nierman et al., 2005b; J. Rhodes et al., 2022b; Sharma et al., 2019b) . It also has high quality gene annotations, required for downstream analyses, hence Af293 was chosen as the reference for this purpose. The bioinformatics workflow is illustrated in Figure 3.

Illumina reads were quality checked using FastQC version 0.11.9 (Babraham Institute) (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and FastQ Screen v0.13.0 (https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/) ('FastQC', 2015).

Three separate pieces of software were used to align, and map reads from each sample to Af293 in order to call high-confidence variants needed for downstream analyses. Read mapping was carried out using the short-read aligner (BWA) 'mem' v0.7.15 (Li and Durbin, 2009) and outputs isolates' reads to a reference genome alignment as a SAM (Sequence Alignment Map format) file. SAMtools v1.3.1 was used to improve the alignment by marking duplicates and flag low-quality mapping. The Genome Analysis Tool Kit (GATK, v4.0) was used to improve the alignment through base score recalibration before calling variants, excluding repetitive regions identified using RepeatMasker v4.0.6 (<https://www.repeatmasker.org/RepeatMasker/>). Both SNPs and indels were called and filtered using the following expression: "QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0 || SOR > 4.0". This resulted in a VCF containing high-confidence SNPs.

The assessment of alignment quality was conducted through implementation of Samtools v1.9 flagstat. This algorithm provided a comprehensive summary of the number of successfully

mapped reads, properly paired reads mapped to the sequence, improperly paired reads, and singleton reads where one read was unmapped while its mate was mapped. The depth of coverage for each isolate throughout the entire genome was computed using picard “CollectWgsMetrics”. It also recorded the percentage of the genome of each isolate that was covered by at least of one read. The Python script filterGatkGenotypes.py was employed to further refine the VCF, with the aim of retaining solely the SNPs of high quality (Table 13A and 5).

2.2 Phylogenetic analysis:

Following assessment of the data gathered through whole-genome sequencing of the 145 isolates (Table 1) the VCFs were combined through the use of 'vcf-merge' from VCFtools v0.1.166, resulting in a single merged VCF for all 145 *A. fumigatus* isolates. Subsequently, the VCF was transformed into a ped file using PLINK v1.90 (Purcell et al., 2007) and converted to a to Phylip file wherein each isolate was represented by a single row. The construction of the Maximum Likelihood (ML) phylogenetic tree was accomplished through the use of the Randomised Axelerated Maximum Likelihood (RAXML) v8.2.10 with the BINCAT substitution module (Stamatakis, 2014). The ML phylogeny tree was visualised using both FigTree v1.4.4 and Microreact software (Argimón et al., 2016a).

2.3 Phenotype microarray analysis

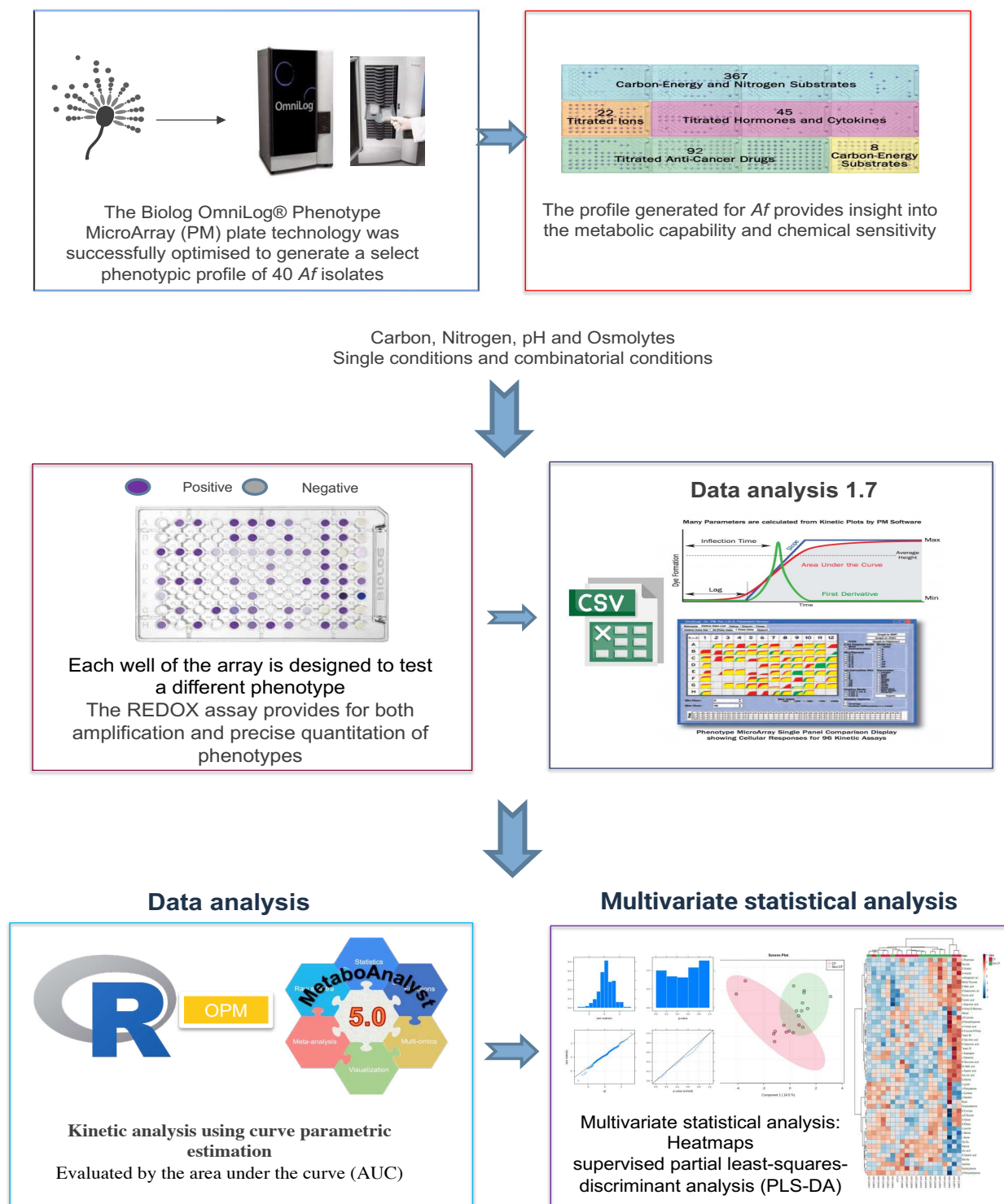


Figure 4 outlines the workflow of the Omnilog system, beginning with fresh cultures from frozen stock streaked onto SAB plates and incubated at 37°C for 4 to 7 days. Spores or conidia are collected with sterile applicators to minimize nutrient transfer, followed by suspension in FF-IF inoculating fluids and inoculation into plates. After incubation in the OmniLog reader at 37°C for 120 hours, the metabolic activity of isolates is monitored. The preparation of stock solutions and inoculating fluids is detailed in Tables 2 and 3, while subsequent data visualization and statistical analysis enhance the reliability of the results.

2.3.1 PM Isolates selection and growth media:

A. fumigatus cultures used in this project were stored frozen at -80°C in 50% glycerol and were revived on a Sabouraud agar (Sigma-Aldrich) for 5–7 days at 37°C .

The PM plates' capacity to assess the growth rate of several isolates simultaneously creates a chance to examine individual isolates under various growth conditions. 42 representative isolates were selected for the initial screening along with Af293, the same isolate used as reference for the mapping of short-reads, and CEA10, an alternative reference isolate. CEA10 was included in this study for the differences observed previously (Bertuzzi et al., 2021) when comparing Af293 to the CEA10 lineage. It was observed that Af293 had a slower growth rate on solid *Aspergillus* minimal media, however, it had a faster growth rate in liquid media and showed increased sensitivity to hypoxic conditions. Additionally, Af293 was found to be less pathogenic than CEA10 in a triamcinolone murine model of infection (Bertuzzi et al., 2021). CEA10 has been also the primary control strain in the laboratory. The selection of isolates was made based on the respiratory/fungal diseases of the patients, the fungal genotype and mating type. All experiments were carried out in triplicate to assess reproducibility and validate the observed metabolic/phenotypic variability.

2.3.2 Antifungal susceptibility testing

VIPcheck plates (VIPcheck, Boven- Leeuwen, The Netherlands) were used to confirm the antifungal drug susceptible profile of the selected 42 isolates. The wells contain either 4 mg/L itraconazole (ITC), 2 mg/L voriconazole (VRC), or 0.5 mg/L posaconazole (POS). The fourth well is the growth control (Arendrup et al., 2017). Briefly, a disposable pipette was used to add one drop of the suspension (10 μl) to each of the four wells. The lid was put on the VIPcheck plate and incubated for 48 h at 37°C . Growth was determined after 48 hr and measured in a binary unite from 1 no growth to 4 formed full colony.

2.3.3 The Phenotype MicroArray (PM) instruments (The OmniLog):

The OmniLog ID System (Biolog, 21124 Cabot Blvd, Hayward, CA) is a fully automated approach for microbial identification and stress fitness cost evaluation. It involves the simultaneous incubation, reading, and analysis of up to 50 microplates using a charged-coupled device camera system. The system software provides an image of kinetic curves visualization at all steps of the testing process for each loaded plate.

The OmniLog digital camera measures the colour intensity and density of each well in OmniLog Units (OU), which is a proprietary scale that quantifies light transmission. An OU of 0 indicates 100% light transmission, while an OU of 500 indicates 0% transmission. OU and optical density (OD) are equivalent, and OU can be calculated as 500 times the OD. Additional microplates can be loaded and processed at any time upon availability. Quantitative responses were recorded automatically every 15 min using a charge-coupled device (CCD) camera. Readouts were stored using the OmniLog file management software version 12.0; Technopath). Data saved as D5E format can be read, analysed, and exported by the native OmniLog® PM Data Analysis 1.7 software (Inc, 2009b).

2.3.4 PM Microplates:

Pre-made PM microplates included in this study; PM1 (Carbon sources), PM3B (Nitrogen sources), PM9 (Osmolytes stress) and PM10 (pH stress) presented in Figure 5 and table 1A. Plates were purchased from Biolog (21124 Cabot Blvd, Hayward, CA) and stored at 4 °C until needed. The full list of the PM microplate compound and their KEGG numbers can be found in Table 1A and Figure 5.

A

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Gluconic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Mellobiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucoaminic Acid	D4 1,3-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 D-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 myo-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-D-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 α-Hydroxy Phenyl Acetic Acid	H3 α-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Palose	H6 L-Xylose	H7 Glucuronamide	H8 Pyruvic Acid	H9 D-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

B

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Bluret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D1 N-Acetyl-L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyrogutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 S-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alofan	G5 Alantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 α-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 α-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

C

PM9 MicroPlate™ Osmolytes

A1 NaCl 1%	A2 NaCl 2%	A3 NaCl 3%	A4 NaCl 4%	A5 NaCl 5%	A6 NaCl 5.5%	A7 NaCl 6%	A8 NaCl 6.5%	A9 NaCl 7%	A10 NaCl 8%	A11 NaCl 9%	A12 NaCl 10%
B1 NaCl 6%	B2 NaCl 6% + Betaine	B3 NaCl 6% + N,N-Dimethyl Glycine	B4 NaCl 6% + Barcosine	B5 NaCl 6% + Barcosine	B6 NaCl 6% + MOPS	B7 NaCl 6% + Glycerol	B8 NaCl 6% + Choline	B9 NaCl 6% + Phosphoryl Choline	B10 NaCl 6% + Creatine	B11 NaCl 6% + L-Carnitine	B12 NaCl 6% + L-Leucine
C1 NaCl 6% + KCl	C2 NaCl 6% + L-Proline	C3 NaCl 6% + N-Acetyl L-Glutamine	C4 NaCl 6% + β-γ-Amino-N-Butyric Acid	C5 NaCl 6% + γ-Amino-N-Butyric Acid	C6 NaCl 6% + Glutathione	C7 NaCl 6% + Glycerol	C8 NaCl 6% + Trehalose	C9 NaCl 6% + Trimethylamine-N-oxide	C10 NaCl 6% + Trimethylamine	C11 NaCl 6% + Octopine	C12 NaCl 6% + Trigonaline
D1 Potassium chloride 1%	D2 Potassium chloride 2%	D3 Potassium chloride 3%	D4 Potassium chloride 4%	D5 Potassium sulfate 5%	D6 Sodium sulfate 3%	D7 Sodium sulfate 5%	D8 Sodium sulfate 5%	D9 Ethylene glycol 5%	D10 Ethylene glycol 10%	D11 Ethylene glycol 15%	D12 Ethylene glycol 20%
E1 Sodium formate 1%	E2 Sodium formate 2%	E3 Sodium formate 3%	E4 Sodium formate 4%	E5 Sodium formate 5%	E6 Sodium formate 6%	E7 Urea 2%	E8 Urea 3%	E9 Urea 4%	E10 Urea 5%	E11 Urea 6%	E12 Urea 7%
F1 Sodium Lactate 1%	F2 Sodium Lactate 2%	F3 Sodium Lactate 3%	F4 Sodium Lactate 4%	F5 Sodium Lactate 5%	F6 Sodium Lactate 6%	F7 Sodium Lactate 7%	F8 Sodium Lactate 8%	F9 Sodium Lactate 9%	F10 Sodium Lactate 10%	F11 Sodium Lactate 12%	F12 Sodium Lactate 15%
G1 Sodium Phosphate pH 7 20mM	G2 Sodium Phosphate pH 7 50mM	G3 Sodium Phosphate pH 7 100mM	G4 Sodium Phosphate pH 7 200mM	G5 Sodium Benzoate pH 5.2 20mM	G6 Sodium Benzoate pH 5.2 50mM	G7 Sodium Benzoate pH 5.2 100mM	G8 Sodium Benzoate pH 5.2 200mM	G9 Ammonium sulfate pH 8 10mM	G10 Ammonium sulfate pH 8 20mM	G11 Ammonium sulfate pH 8 50mM	G12 Ammonium sulfate pH 8 100mM
H1 Sodium Nitrate 10mM	H2 Sodium Nitrate 20mM	H3 Sodium Nitrate 40mM	H4 Sodium Nitrate 60mM	H5 Sodium Nitrate 80mM	H6 Sodium Nitrate 100mM	H7 Sodium Nitrate 150mM	H8 Sodium Nitrate 200mM	H9 Sodium Nitrate 400mM	H10 Sodium Nitrate 600mM	H11 Sodium Nitrate 800mM	H12 Sodium Nitrate 1000mM

D

PM10 MicroPlate™ pH

A1 pH 3.5	A2 pH 4	A3 pH 4.5	A4 pH 5	A5 pH 5.5	A6 pH 6	A7 pH 7	A8 pH 8	A9 pH 8.5	A10 pH 9	A11 pH 9.5	A12 pH 10
B1 pH 4.5	B2 pH 4.5 + L-Alanine	B3 pH 4.5 + L-Arginine	B4 pH 4.5 + L-Asparagine	B5 pH 4.5 + L-Aspartic Acid	B6 pH 4.5 + L-Glutamic Acid	B7 pH 4.5 + L-Glutamine	B8 pH 4.5 + Glycine	B9 pH 4.5 + L-Histidine	B10 pH 4.5 + L-Isoleucine	B11 pH 4.5 + L-Leucine	B12 pH 4.5 + L-Lysine
C1 pH 4.5 + L-Methionine	C2 pH 4.5 + L-Phenylalanine	C3 pH 4.5 + L-Proline	C4 pH 4.5 + L-Serine	C5 pH 4.5 + L-Threonine	C6 pH 4.5 + L-Tryptophan	C7 pH 4.5 + L-Citrulline	C8 pH 4.5 + L-Valine	C9 pH 4.5 + Hydroxy-L-Proline	C10 pH 4.5 + L-Ornithine	C11 pH 4.5 + L-Homoarginine	C12 pH 4.5 + L-Homoserine
D-1 pH 4.5 + Anthranilic Acid	D2 pH 4.5 + L-Norleucine	D3 pH 4.5 + L-Norvaline	D4 pH 4.5 + α-Amino-N-Butyric Acid	D5 pH 4.5 + p-Amino-Benzoic Acid	D6 pH 4.5 + D-Cysteic Acid	D7 pH 4.5 + D-Lysine	D8 pH 4.5 + 5-Hydroxy Lysine	D9 pH 4.5 + 5-Hydroxy Tryptophan	D10 pH 4.5 + D,L-Diamino-Pyruvic Acid	D11 pH 4.5 + Trimethylamine-N-oxide	D12 pH 4.5 + Urea
E1 pH 9.5	E2 pH 9.5 + L-Alanine	E3 pH 9.5 + L-Arginine	E4 pH 9.5 + L-Asparagine	E5 pH 9.5 + L-Aspartic Acid	E6 pH 9.5 + L-Glutamic Acid	E7 pH 9.5 + L-Glutamine	E8 pH 9.5 + Glycine	E9 pH 9.5 + L-Histidine	E10 pH 9.5 + L-Isoleucine	E11 pH 9.5 + L-Leucine	E12 pH 9.5 + L-Lysine
F1 pH 9.5 + L-Methionine	F2 pH 9.5 + L-Phenylalanine	F3 pH 9.5 + L-Proline	F4 pH 9.5 + L-Serine	F5 pH 9.5 + L-Threonine	F6 pH 9.5 + L-Tryptophan	F7 pH 9.5 + L-Tyrosine	F8 pH 9.5 + L-Valine	F9 pH 9.5 + Hydroxy-L-Proline	F10 pH 9.5 + L-Ornithine	F11 pH 9.5 + L-Homoarginine	F12 pH 9.5 + L-Homoserine
G1 pH 9.5 + Anthranilic Acid	G2 pH 9.5 + L-Norleucine	G3 pH 9.5 + L-Norvaline	G4 pH 9.5 + Agmatine	G5 pH 9.5 + Cadaverine	G6 pH 9.5 + Putrescine	G7 pH 9.5 + Histamine	G8 pH 9.5 + Phenylethylamine	G9 pH 9.5 + Tyramine	G10 pH 9.5 + Creatine	G11 pH 9.5 + Trimethylamine-N-oxide	G12 pH 9.5 + Urea
H1 X-Caprylate	H2 X-α-D-Glucoside	H3 X-β-D-Glucoside	H4 X-β-D-Galactoside	H5 X-β-D-Galactoside	H6 X-α-D-Glucuronide	H7 X-β-D-Glucuronide	H8 X-β-D-Glucosaminide	H9 X-β-D-Galactosaminide	H10 X-α-D-Mannoside	H11 X-PD4	H12 X-SD4

Figure 5: fungal metabolic and chemical sensitivity microplates. A) PM1 (carbon sources), B) PM3B (Nitrogen sources), C) PM9 (Osmolytes stress) and D) PM10 (pH stress). List of the substrates in each plate is in table 1A

2.3.5 FF-IF broth:

FF-IF broth used in the experiment was composed of mixed 0.25% Phytigel (P8169 from Sigma Chemical Co., St. Louis, MO) and 0.03% Tween 40 (P1504 from Sigma) in DI water. The broth was stored at room temperature.

2.3.6 PM Inoculum preparation:

Fresh culture from the frozen stock were streaked onto SAB plates and incubated at 37°C for 4 to 7 days depending on the isolates growth rate. Sterile cotton-tipped applicators (Pur-Wraps from Hardwood Products Company, Guilford, ME) were used to collect spores or conidia from agar plates, with the aim to avoid the transfer of nutrients from the agar medium. Conidia suspensions were made according to the manufacturer's protocol (Table 2 and 3); conidia were suspended in FF-IF inoculating fluids in a 20 mm diameter tube at a density equivalent to 75% transmittance using a turbidimeter (Biolog, Inc) along with the supplements required for each different type of plate. Each well was inoculated with 100 µL. Then, the plates were subjected to incubation at a temperature of 37°C within an OmniLog reader (manufactured by Biolog) for a duration of 120 hours (Figure 4).

Table 2: List of Chemicals and Materials needed for the preparation of additive stock solution.

Preparation of stock solutions Ingredient	Concentration (mM)	Grams/100 ml	Concentration Factor
D-glucose	3200	57.664	32x
potassium phosphate monobasic anhydrous (pH6.0)	60	0.8166	12x
sodium sulfate	24	0.341	12x
yeast nitrogen base		8.04	12x

*Dissolve all ingredients in distilled water. Q.S. to 100 ml and sterilize by filtration. Store at 4°C

Table 3: preparation for PM Inoculating Fluids from Stock Solutions

Recipe for PM Inoculating Fluids from Stock Solutions PM Stock Solution	PM1 (ml)	PM3 (ml)	PM9+ PM10 (ml)
FF-IF	20.00	12.5	15
D-glucose (32x)	-	0.4	1.125
PM additive (12x)	-	potassium phosphate monobasic anhydrous (pH6.0) solution = 1.25	yeast base =3 nitrogen solution
cells	0.050	0.03	0.075
sterile water	3.95	0.75	16.8
Total	24.00	14.93	36

2.3.7 Visualization of PM raw data:

The estimated parameters length of the area under the curve (AUC) using the basic part of the *OPM* package v1.3.77 (Vaas et al., 2013) in R. PM curves were visualised as heat maps using the function `ph heatmap()` (Figure 2A and 3A) (Inc, 2009b; Jacobsen et al., n.d.). All curves were visually inspected to identify the most potential artefacts, the range of potential curve dot shape, and any other issues that could impact the reproducibility of readings. By using the function `xyplot()` from the *lattice* v 0.22.6 and *ggplot2* v3.5.1 packages (<http://lmdvr.r-forge.r-project.org/figures/figures.html>) (Xie, 2016). An open-source MetaboAnalyst v6.0 was used for these specifications and showed that curve kinetics offer a more powerful data visualization than heatmap (<https://www.metaboanalyst.ca>). Modifications were made to the raw data files to include the names of the substrates, rather than just the coordinates of the wells. The name of the substrates was requested from the Biolog and listed in Table 1A for only the plates that were used in this project

2.3.8 Metabolomics Data Analysis and Normalisation:

The pre-processed raw data and post-processed normalized data are provided in the Appendixes (Tables 2A-5A in <https://github.com/renad122/aljohani-re.git>). AUC values were calculated using the *OPM* package v1.3.77 (Vaas et al., 2013), and MetaboAnalyst v6.0, an open-source R-based program designed specifically for metabolomics analysis (Processing, 2011).

The outcomes of phenotype microarray measurements can be vulnerable to biological and experimental noise, biases, and systematic errors. Therefore, analysing PM data could be advantageous by focusing on removing and normalizing unwanted data characteristics (Fritsche-Guenther *et al.*, 2020). Data was normalized to the control well for each sample and was used as based line, followed by log transformation with a base of 10. Standardization scaling of metabolite and isolates were performed by subtracting the mean and dividing by the standard deviation. Biological triplicates were performed for 42 isolates including the control isolates Af293 and CEA10. Comparison of the replicated sets of PM data was indicated using the median relative standard deviation (RSD) as a metric for analytical reproducibility for carbon and nitrogen sources, with values below 10%, which is an acceptable threshold for metabolomics. For osmotic and pH stressors, the median RSD was less than 30%, indicating good analytical reproducibility (Fritsche-Guenther et al., 2020).

2.3.9 PM Statistical analysis and visualization:

The normalized and processed AUC raw PM data can be utilized as input for various types of supervised and unsupervised statistical data analysis, using the resulting matrix of values corresponding to sample-by-growth condition. Multivariate statistical analysis was conducted using RStudio v4.2.2. Initially, a comparative untargeted metabolomics approach was employed, wherein the 42 selected isolates were divided into two cohorts: isolates from patients with CF ($n = 20$) and those with a different respiratory disorder (referred to collectively as

non-CF) ($n = 20$), as well as isolates exhibiting resistance to azole antifungal drugs ($n = 20$) (isolates were resistant to either itraconazole or posaconazole or both), which their antifungal profile was confirmed as describe above, and those that were susceptible ($n = 20$). Tests were conducted using an adjusted p -value ≤ 0.05 , which is considered statistically significant.

Supervised multivariate classification techniques offer valuable insights into the relationships between PM growth conditions and samples. This type of exploratory analysis can assist in the confirmation of hypotheses, identification of outliers, and provide biological understanding based on the resulting clusters.

Heatmap visualisation was performed using the normalised area under the curve (AUC) of top 25 metabolites from the partial least squares discriminant analysis (PLSDA) module. PLS-DA tests were used to distinguish metabolites that contributed significantly to the differentiation between groups. Then, the variable importance in projection (VIP) plot from PLS-DA ranked the top 15 discriminating metabolites that contributed the most to the separation of the previously divided groups.

The list of identified metabolites was determined through a series of two-tailed Student's t-tests to establish the statistical significance of the association between each metabolite and the groups being assessed. The false discovery rate (FDR) was used to adjust the p-values to account for multiple testing. Metabolites exhibiting significant differential abundance (DA) were identified by applying a q-value threshold of $q \leq 0.05$.

2.3.10 Metabolic Pathway and Enrichment analysis:

Metabolic pathway analysis was carried out using MetaboAnalyst v5.0 (J. Chong & Xia, 2018; Processing, 2011) which combines Metabolite Set Enrichment Analysis (MSEA) and Metabolomic Pathway Analysis (MetPA). The data were analysed with MetaboAnalyst and annotated based on compound names. Accepted metabolites were manually verified using different databases such as KEGG (Kanehisa & Goto, 2000). The pathway

library of *Aspergillus* spp was selected for pathway analysis. Metabolite set enrichment was conducted using the KEGG database, which contains 84 metabolite sets with at least two compounds representing each set.

2.4 Correlation of radial growth rate with the PM growth to validate the OmniLog data:

PM plates have been compared favourably against radial growth rate. Our aim was to determine whether PM assays possess the required sensitivity and accuracy to detect new collateral metabolic and sensitivities to establish extensive sensitivity/resistance networks. To accomplish this, I compared the results obtained from our PM assays with radial growth rate assays. Radial growth rate of the isolates was determined with 2 μ l (one colony/isolate) point inoculates on *Aspergillus* minimal medium (AMM) (Weidner et al., 1998). Agar plates were supplemented with 1 mM, 3 mM and 5 mM Histidine, 3 mM, and 5 mM Histamine, and 1%, 3%, 5.5% NaCl. Fungal growth was measured after 48h in orthogonal directions using a ruler from which the mean value of the two diameters was calculated. Three technical and biological replicates were carried out for each condition.

2.5 Genome-wide association study (GWAS):

The present study employed *treeWAS* (Collins & Didelot, 2018a) to characterise 42 *A. fumigatus* isolates (Table 1) exhibiting various levels of azole resistance and fitness enhancement under two distinct conditions, namely histamine, and NaCl. *TreeWAS* applies the null distribution to allow for strict control over the false positive rate. This provides the chance to enhance power by employing numerous independent tests of association. Thus, *treeWAS* evaluates the statistical correlation between phenotype and genotype across all loci to identify significant associations. The genes showing a significant correlation were reported to validate any differences between the control and the conditions.

In detail, the input data used in *treeWAS* consisted of phenotypic variables for each individual, a phylogenetic tree generated by RAxML v8.2.10, and ClonalFrameML v1.13 (It used to infer recombination in genome) (Didelot & Wilson, 2015) (<https://github.com/xavierdidelot/ClonalFrameML>). A ClonalFrameML step produced five files that were used in *treeWAS*. ML_sequence.fasta, Position_cross_reference.txt, Em.txt, Emsim.txt, Importation_status.txt and labelled_tree.newick. data then were converted using “read.CFML” function to *treeWAS* format. Therefore, measurement of the association between each genetic locus and the phenotype were made with three separate tests (subsequent, terminal and simultaneous), which contained set of significant loci for all three tests. All three tests can be used on binary genetic data, such as SNPs and continuous phenotypic data (Collins & Didelot, 2018b)(Collins and Didelot, 2018b).

1. Score 1, also known as the "Terminal Score", quantifies the overall relationship among the samples across the branches of the phylogenetic tree. It aims to discover overall patterns of correlation without making assumptions based on reconstructions of ancestral states.
2. The "Simultaneous Score," or score 2, quantifies how much the phenotype and genotype are changing simultaneously in different tree branches.
3. The "Subsequent Score" or score 3 quantifies the extent to which the genotype and phenotype coexist within the tree. Individually, each association test detects a group of genetically significant loci that have statistical significance. this score may allow us to identify broad, if imperfect, patterns of association.

“Out\$treeWAS.combined” was used to print out the pooled set of significant loci identified by any association score. At the upper tail of the null distribution, a threshold of significance is drawn at the quantile corresponding to 1 - (a base p-value corrected for multiple testing). Any loci in the real dataset that have association score values lying above this threshold are deemed

to be significantly associated to the phenotype. P-value correction was performed by Bonferonni correction (Collins & Didelot, 2018b).

2.6 RNA extraction

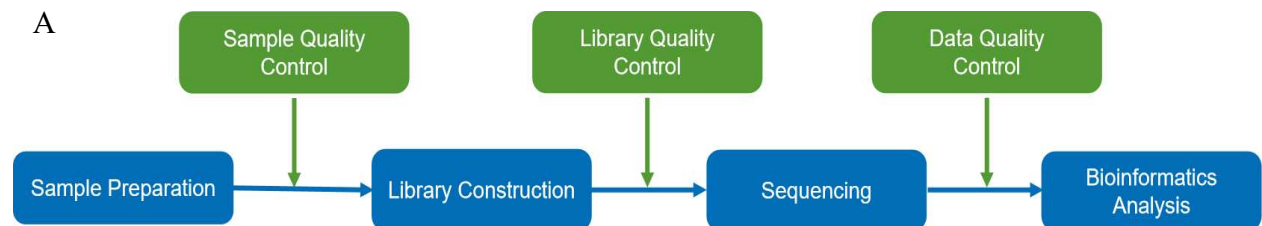
CEA10 and Af293 along with 9 selected isolates were subjected for RNA extraction and sequencing. These isolates were selected based on GWAS, phenotype significance, their location on the phylogenetic tree and their ITR and AMR profiles.

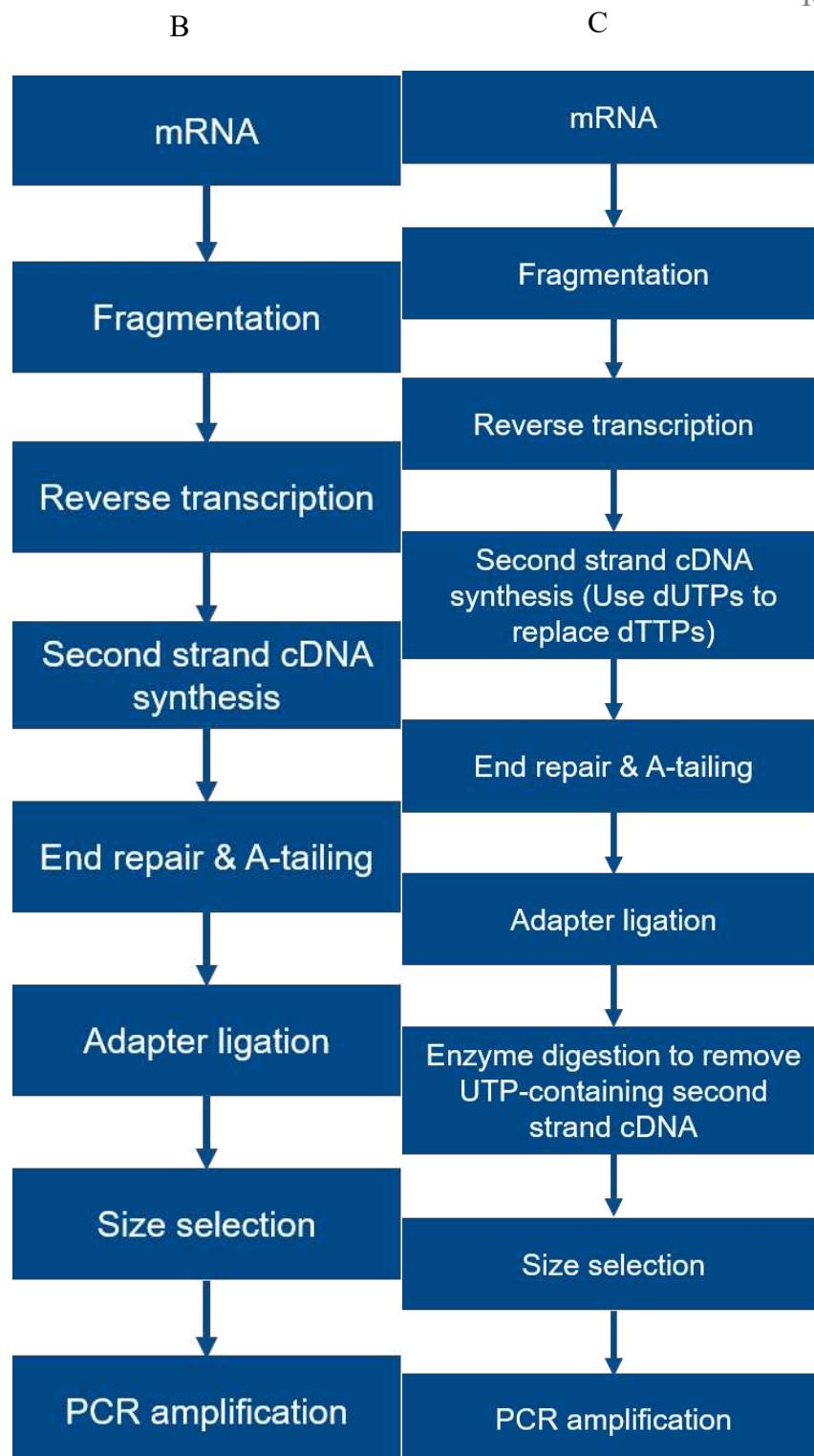
The protocol used in the experiment was as follows: 5 ul of the glycerol stock in -20 °C was inoculated in 15 mL of AMM supplemented with two distinct growth conditions: NaCl and histamine. These conditions were selected based on association observed in GWAS results. Experiments were carried out in three biologicals triplicate. After 18 hours of growth at 37 °C in an orbital shaker, the mycelia were extracted and divided into three 15 mL tubes as follows: control, 800 mM NaCl, and 5 mM histamine. These tubes were then incubated for 2 hours at 37 °C in an orbital shaker. Mycelia were harvested by passing the culture through miracloth and were directly frozen in liquid nitrogen. RNA was isolated from the harvested hyphae using the RNeasy plant mini kit (Qiagen, CA, USA), following the manufacturer's protocols (<https://www.qiagen.com/us/resources/resourcedetail?id=246847e7-0095-43e4-8d1d-41df3f9153dd&lang=en>).

The mycelium was grinded with liquid nitrogen using a mortar and pestle. The powdered samples were resuspended in 450 L of Buffer RLC, containing 10 L of 2-mercaptoethanol per 1 mL of buffer, and vigorously homogenised without disrupting the particle. Afterwards, 500 L of 98% ethanol was added to each sample, and the mixture was homogenised by pipetting. 650 ul of the sample with any precipitate was transferred to the pink RNeasy min spin column and centrifuge for 15 s at ≥ 10000 rpm. The RNeasy column was washed once with RW1 buffer for 15 at ≥ 10000 rpm and twice with RPE buffer. Finally, RNeasy was added to the spin column

before transfer to a new 2 ml tube and centrifuged for 2 minutes. 50ul.of RNeasy-free water was added to elute the RNA (RNeasy ® Plant Mini Kit, 2016).

2.7 RNA sequencing and transcriptomic analysis:





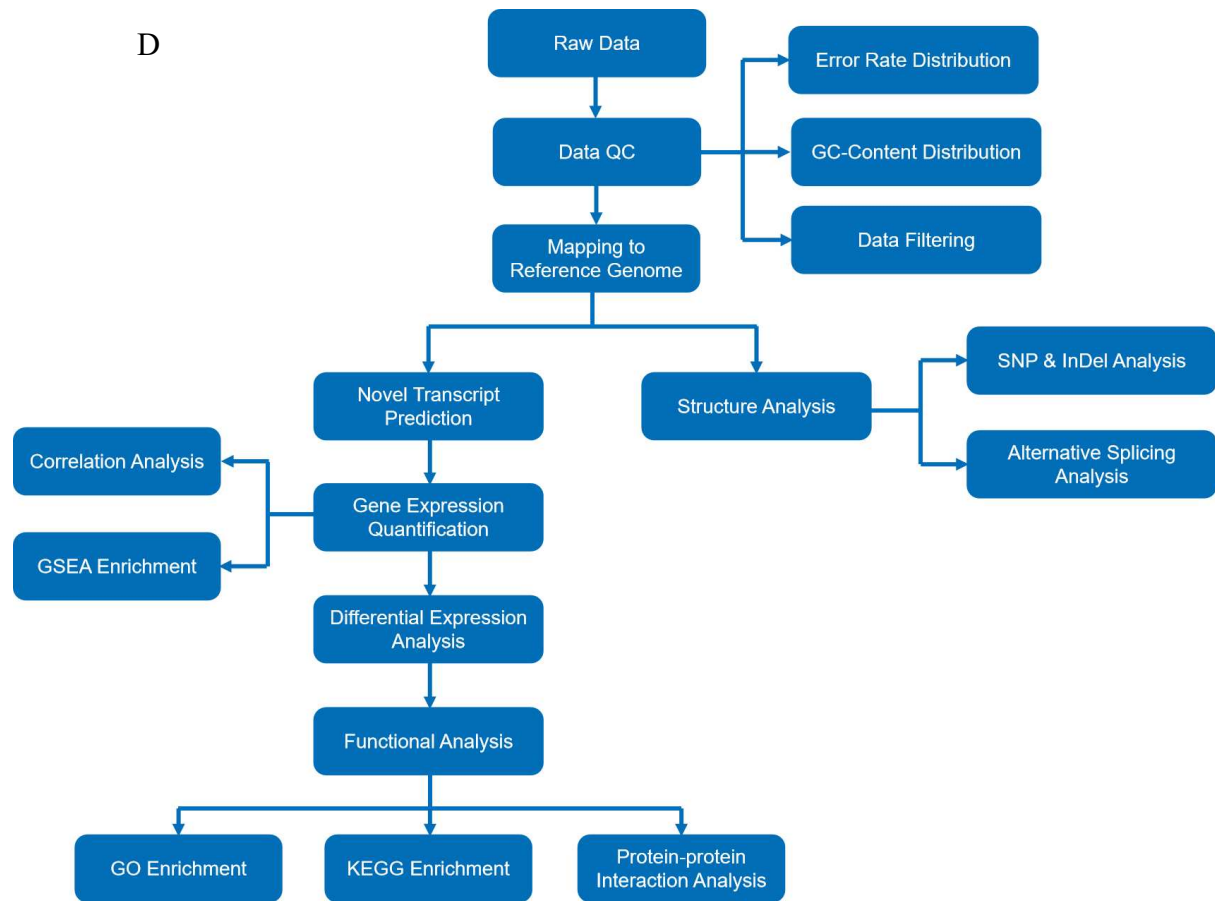


Figure 6: Schematic representations of the workflow performed via Illumina platforms. The messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis using either dUTP for directional library or dTTP for non-directional library. (B) A workflow of non-directional library construction. (C) A workflow of directional library construction. (D) RNA-seq information analysis technology flow.

Purification of Messenger RNA was done by Novogene (Figure6-A). For the non-directional library, it was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification (Figure 6-B). For the directional library, it was ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification, and purification (Figure 6-C). Raw data (paired ends) in fastq format was processed through in-house Perl scripts at Novogene. In this step, clean reads were obtained by removing reads containing adapter sequences, as well as poly-N and low-quality reads. All the downstream analyses were based on the high-quality clean data. The Af293 reference genome (GCF_00002655_ASM265v1) and gene model annotation files were downloaded from NCBI directly (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/655/GCF_000002655.1_ASM265v1/GCF_000002655.1_ASM265v1_genomic.fna.gz). Examination of sequencing error rate for each base can be transformed by the Phred score as in equation 1. "e" represents sequencing error rate (Figure 6-D). Sequencing error rate and base quality varies depending on sequencers, reagent residues, and different sample types (sequencing data error rate distribution figures and data table can be found in <https://github.com/renad122/aljohani-re.git>). GC content distribution detects potential AT/GC separation, which affects subsequent gene expression quantification. In view of random fragmentation and biological law of G/C-A/T content, G and C, A and T should be respectively equal, and the content should be stable throughout the entire sequencing process for non-stranded library (Parkhomchuk et al., 2009) (If the library is strand specific, AT separation or GC separation may occur). A large variation of sequencing error in the first 6-7 bases is allowed considering the use of random primer in library construction, in which it is normal that the first few bases have certain preference in existing high-throughput sequencing technology

Bioinformatics workflow for RNASeq

The index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. Hisat2 was selected as the mapping tool because it can generate a database of splice junctions based on the gene model annotation file and generate a better mapping result than other non-splice mapping tools. Mapped reads were assembled by StringTie v1.3.3 (Pertea et al., 2015) using a reference-based strategy. StringTie employs a novel network flow algorithm and an optional de novo assembly phase to construct and quantify full-length transcripts that represent multiple splice variants for each gene locus. Then Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels. Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by the *edgeR* v 3.22.5 and *DESeq2* v1.44.0 packages in R through one scaling normalized factor. Differential expression analysis of two conditions was performed using *edgeR* v 3.22.5. The *p*-values were adjusted using the Benjamini & Hochberg method. A corrected *p*-value of 0.05 and absolute foldchange of 2 were set as the threshold for significantly differential expression. Differential expression analysis of two conditions/groups (three biological replicates per condition) was performed using the *DESeq2R* package v1.44.0 in R. *DESeq2* provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution (Figure 6).

3 Results

Contributorship statement

I am the primary contributor to this work. Darius Armstrong-James, Johanna Rhodes, and I designed the experiments, with the majority of the experiments conducted by me. Laila Alsharqi conducted the second and third replicates of the OmniLog experiment involving 18 isolates, as well as three replicates of 2 isolates included in Chapter 3.2. Darius Armstrong-James, Andrew Scourfield, and Alireza Abdolrasouli collected and sequenced the clinical samples. I carried out the data analyses, and I also predominantly completed the writing of the manuscript. This results chapter has been written as part of a manuscript that is being prepared for submission.

3.1 Phylogenomic Patterns and Mutations Associated with Clinical Diseases in Azole-Resistant *Aspergillus fumigatus* Clinical Isolates.

3.1.1 Preface

A. fumigatus is frequently prevalent in the respiratory tracts of patients with immunocompromise. Given the increase in resistance to azole drugs globally, understanding how antifungal resistance mechanisms evolve in the host is of critical clinical and biological relevance. Whole genome sequencing was performed for genome-scale comparisons of various isolates using single nucleotide polymorphism (SNP) analysis. In this chapter 3.1, a population genomic analysis of 145 of serial *A. fumigatus* isolates was performed. Isolates were collected from patients cared for by the Royal Brompton and Harefield Hospitals in London (Table 1 and Figure 2). It was hypothesized that phenotypic variation of AR*Af* isolates, was genetically dependent. The aim was to characterize phylogenomic patterns and population genomics in azole resistant and susceptible *A. fumigatus*.

3.1.2 Bioinformatics analysis

A total of 145 isolates were studied, using *Af293* as a reference genome. Assessment of the mapping quality and coverage statistics revealed a mean coverage across the series of 13X, with an average SNPs count of 47920 per isolate (Table 13A). On average, 96.8% of the SNPs were successfully mapped, with an average of 95.5% properly paired and 0.2% singletons determined from the average total number of reads (Table 13A & 5).

The low-confidence SNPs were calculated at an average of 5192 SNPs per isolate. The confidence of these SNPs is assigned using several metrics, including consensus scores, genotype quality, and coverage depth. SNPs with lower consensus scores, indicating fewer supporting reads, or those with insufficient depth of coverage are classified as low confidence. The genomic variations across *A. fumigatus* isolates were studied by constructing a

phylogenetic tree using whole-genome SNPs. The variation in SNP numbers in these isolates ranged from 33 to 71884, with reference isolate *Af293* having only 33 SNPs (Table 4). Notably, this range underscores the differing degrees of confidence associated with the SNP calls, as SNPs classed with higher confidence are more likely to represent true genomic variations.

This approach was used to validate the quality of the sequencing data and the reliability of the bioinformatics pipeline for analysing the remaining isolates (J. Rhodes et al., 2022b). The confidence of SNP calls was systematically assessed through metrics such as consensus scores and genotype quality, ensuring that low-confidence SNPs were minimized (Figure 3). The SNP-based phylogenetic tree showed that Clades A and B had significantly different numbers of SNPs, with a minimum difference of over 1,000 SNPs. These statistics indicated that all of the sequenced isolates yielded high-quality reference-based assemblies containing nearly complete genomic content and gene inventories, which could be used for downstream analysis (Tables 4 and 5). The substantial differences in SNP counts between the clades further affirm the robustness of the sequencing data and its potential for illuminating genomic diversity within *A. fumigatus*.

Table 4 : Summary of unfiltered and filtered SNPs, pass filtered SNPs and low confidence SNPs and the percentage of SNPs that were filtered out in the WGS analysis of 145 *A. fumigatus* clinical isolates.

SEQ ID	LOCAL REF	unfiltered SNPs	filtered SNPs	python PASS Filtered SNPs	Python script filtered out	Low confidence SNPs	% filtered out
IGF11953	71	62120	61382	59846	194	2468	3.97295557
IGF11954	134	65168	63950	62014	332	3486	5.349251166
IGF11955	122	64816	63983	62597	349	2568	3.961984695
IGF11956	172	63582	62251	59451	343	4474	7.036582681
IGF11957	54	60874	59608	56935	725	4664	7.661727503
IGF11958	123	60389	58733	56107	213	4495	7.443408568
IGF11959	28	32192	30786	28123	364	4433	13.77050199
IGF11960	2	52690	51297	47258	210	5642	10.70791422
IGF11961	131	18563	17667	11338	127	7352	39.60566719
IGF11962	159	52441	51307	47300	168	5309	10.12375813
IGF11963	128	53907	52768	50219	173	3861	7.162335133
IGF11964	160	54014	53136	51053	156	3117	5.770726108
IGF11965	161	60542	58899	56078	218	4682	7.733474282
IGF11966	10	63897	62993	61142	285	3040	4.757656854
IGF11967	55	62423	60997	58505	288	4206	6.737901094
IGF11968	162	61559	55663	42991	1031	19599	31.83774915
IGF11969	163	62806	61426	58692	343	4457	7.096455753
IGF11970	29	66235	64771	62499	328	4064	6.135728844
IGF11971	155	51931	50921	46812	152	5271	10.15000674
IGF11972	1	51936	50901	47021	102	5017	9.659966112

IGF11973	166	51608	50712	46878	158	4888	9.471399783
IGF11974	167	49723	48963	45045	1125	5803	11.67065543
IGF11975	164	51573	50738	47147	128	4554	8.83020185
IGF11976	43	43279	40378	18450	381	25210	58.24995956
IGF11977	4	62632	61451	59852	131	2911	4.64778388
IGF11978	144	52351	51437	49740	121	2732	5.218620466
IGF11979	124	52226	51341	49423	256	3059	5.85723586
IGF11980	165	18109	17154	14086	27	4050	22.3645701
IGF11981	3	52499	51504	49488	234	3245	6.181070116
IGF11982	168	60625	59240	56299	270	4596	7.581030928
IGF11983	154	57484	55375	39120	427	18791	32.6890961
IGF11984	125	55190	54217	50251	227	5166	9.360391375
IGF11985	44	55490	54327	49920	304	5874	10.58569112
IGF11986	158	49872	49076	44868	498	5502	11.03224254
IGF11987	82	51338	50160	45772	294	5860	11.41454673
IGF11988	80	50661	49350	45745	217	5133	10.13205424
IGF11989	19	51119	50068	45835	405	5689	11.12893445
IGF11990	32	55806	54677	44445	526	11887	21.300577
IGF11991	169	50664	49508	45815	183	5032	9.93210169
IGF11992	151	72130	69996	40299	941	32772	45.43463191
IGF11993	25	62301	61117	58725	294	3870	6.211778302
IGF11994	129	52337	51622	48918	247	3666	7.004604773
IGF11995	170	64964	63194	60009	357	5312	8.176836402
IGF11996	39	67889	66871	65399	250	2740	4.035999941
IGF11997	171	59135	58000	54417	227	4945	8.362222034
IGF11998	133	53894	52912	50677	184	3401	6.310535496
IGF11999	67	53935	53049	50872	161	3224	5.977565588

IGF12000	140	53891	53004	50983	171	3079	5.713384424
IGF12001	47	52748	51685	48586	1466	5628	10.66959885
IGF12002	94	50348	49230	47197	224	3375	6.703344721
IGF12003	86	48061	47406	45649	162	2574	5.355693806
IGF12004	135	44708	44028	42728	118	2098	4.692672452
IGF12005	69	59321	57954	54931	420	4810	8.108427033
IGF12007	21	50124	47136	41975	1796	9945	19.84079483
IGF12008	109	56441	55347	51216	495	5720	10.13447671
IGF12009	157	54689	53651	49399	258	5548	10.14463603
IGF12010	17	14170	13900	13072	42	1140	8.045165843
IGF12011	63	11807	11377	9683	1184	3308	28.01727789
IGF12012	139	71728	70371	68549	390	3569	4.975741691
IGF12013	64	52981	52250	48704	299	4576	8.637058568
IGF12014	12	64842	63698	61584	431	3689	5.689213781
IGF12015	49	53092	52306	47685	1597	7004	13.19219468
IGF12016	68	39828	38781	33835	4193	10186	25.57497238
IGF12017	9	29331	27835	19459	8198	18070	61.6071733
IGF12018	33	56453	55555	52935	453	3971	7.034170018
IGF12019	155	58749	57965	56361	165	2553	4.345605883
IGF12020	30	52461	51073	46939	155	5677	10.82137207
IGF12021	72	72375	70988	68409	480	4446	6.143005181
IGF12022	100	52964	52179	50731	111	2344	4.42564761
IGF12023	136	59339	58273	48961	409	10787	18.17860092
IGF12024	175	62175	61038	58443	296	4028	6.478488138
IGF12025	76	54525	53199	47627	127	7025	12.88399817
IGF12026	117	54706	53417	51504	125	3327	6.081599825
IGF12027	178	14006	13725	13292	233	947	6.761387977

IGF12028	45	49514	49172	47022	1093	3585	7.240376459
IGF12029	98	48690	48337	46384	198	2504	5.142739782
IGF12030	174	50451	50005	48372	187	2266	4.491486789
IGF12031	52	56423	55529	54200	184	2407	4.265990819
IGF12032	13	46868	45908	42369	223	4722	10.07510455
IGF12033	16	51692	50816	44160	269	7801	15.09131007
IGF12034	57	70977	69968	68027	214	3164	4.457782098
IGF12035	148	58221	57334	55357	200	3064	5.262705896
IGF12036	48	53607	52784	50781	248	3074	5.734325741
IGF12037	88	43947	43322	42148	103	1902	4.327940474
IGF12038	41	62632	61709	59759	613	3486	5.565844935
IGF12039	42	76201	74855	71884	386	4703	6.171835015
IGF12040	89	52578	52260	50657	370	2291	4.357335768
IGF12042	5	50428	49752	45918	194	4704	9.328151027
IGF12043	11	52170	51256	49289	188	3069	5.882691202
IGF12044	177	53020	51851	50035	182	3167	5.973217654
IGF12045	176	62914	62028	60338	288	2864	4.552245923
IGF12046	118	50894	50072	47931	171	3134	6.157896805
IGF12047	105	43883	43377	42121	91	1853	4.222591892
IGF12048	AF293	292	239	33	5	264	90.4109589
C124		59436	51774		1849	9272	15.59997308
C125		62796	58895		1170	6560	10.44652526
C126		59467	51764		1183	9265	15.58006962
C127		54939	51121		1313	5667	10.31507672
C128		50297	45952		1328	5515	10.96486868
C129		55979	50638		1240	6524	11.65437039
C130		53223	50344		2226	4192	7.876294083

C131		53561	50417		1747	4472	8.349358675
C132		53582	50574		1123	4248	7.928035534
C133		62766	58829		1053	6163	9.819010292
C134		58827	54789		1077	5785	9.833919799
C135		53241	50344		1244	4020	7.550571928
C136		50448	46048		937	5453	10.80915002
C137		50413	46077		1362	5413	10.73730982
C138		53992	47281		1521	7955	14.73366425
C139		56237	50769		2174	6405	11.38929886
C140		59863	51935		2062	9290	15.51876785
C141		58972	54802		1560	5691	9.650342535
C142		75520	69515		845	8179	10.83024364
C143		62828	58802		1174	6088	9.689947157
C144		70611	66896		935	5275	7.470507428
C145		42645	40597		1002	2893	6.783913706
C164		54122	50734		1308	4515	8.342263774
C165		51803	47272		1111	5322	10.27353628
C166		56612	54191		849	3459	6.110012012
C167		55057	47925		737	8440	15.32956754
C168		55894	53064		2187	3941	7.050846245
C169		52442	49957		311	3334	6.357499714
C170		52500	49989		646	3248	6.186666667
C171		69816	62014		649	9989	14.30760857
C172		53456	50597		1305	3170	5.930110745
C173		43069	40678		3380	3037	7.051475539
C174		43308	40674		1350	3283	7.580585573
C175		54037	50021		5735	5321	9.846956715

C176		57859	54909		662	6330	10.94038957
C177		65323	62699		1697	3974	6.083615266
C178		62714	46765		1016	21684	34.57601174
C179		54663	52552		952	2773	5.072901231
C180		56191	51335		1194	6553	11.66200993
C181		54728	49488		1316	6256	11.43107733
C182		50855	48161		1388	3646	7.169403205
C183		52982	49979		1623	4197	7.921558265
C184		52898	48265		909	5949	11.24617188
C185		59897	56635		1115	4650	7.763327045
C186		66681	63855		2102	4449	6.672065506
C187		56570	50812		1154	6667	11.78539862
C188		61020	58479		1445	3656	5.991478204
C189		58324	54229		2102	6197	10.62512859
C190		53976	47390		1154	7740	14.33970654
C191		59651	54258		1445	6838	11.46334512

3.1.1 Whole-Genome Sequencing and Phylogenetic Analyses Reveal Significant Genetic Diversity and Two Major Clades Within 145 *A. fumigatus* Isolates.

To better understand the genetic relationships between these *A. fumigatus* isolates, phylogenetic analysis was performed. 111 *A. fumigatus* genomes from CF clinical isolates, and 34 of *A. fumigatus* genomes from non-CF clinical isolates (Table 1) were included. A phylogenetic tree was constructed for all 145 *A. fumigatus* genomes from patients with CF and patients with different fungal disease endotypes using RAxML v8.0.25 and visualised in Microreact v200 (<https://microreact.org/project/oQjWGgYh5i1kpygfQMshHJ>) with bootstrap values of 70% over 1000 iterations and rooted to the reference strain Af293.

The maximum likelihood phylogenetic tree was also visualised using FigTree v1.4.4 (Figure 1A). A bifurcating clade structure was observed, with two clades with well-supported (bootstrap 70-90%) branches (Figure 7). These two clades were labelled as “A” and “B”.

Clade A contained the majority of isolates with *cyp51A* resistance polymorphisms: 36.2% ($n = 21/58$) contained the TR₃₄/L98H polymorphism and they were mostly clustered closely to each other, and all TR₃₄/L98H lay in Clade A, except IGF11990 that was clustered closer to the reference isolate Af293 in Clade B. 25.86% of isolates ($n = 15/58$) were the wildtype *cyp51A* genotype suggests these isolates do not have the known resistance mutations and may still be susceptible to azoles.

while *cyp51A* wild-type isolates were distributed across both Clades, Clade B housed the majority of isolates with wild-type/non-wild-type (WT/non-WT) alleles. Among the tested isolates, 48% ($n = 69$) demonstrated resistance to azole drugs, while 52% ($n = 76$) were classified as azole susceptible. Notably, 30.6% ($n = 34/111$) of the azole-resistant isolates originated from cystic fibrosis (CF) patients and exhibited insertional mutations in *cyp51A*, including TR₃₄/L98H ($n = 26$; 23.4%), G54W and P216L ($n = 5$; 4.5%), and L98H/M220K (n

= 3; 2.7%). Conversely, 69% (n = 77/111) of the azole-resistant isolates maintained wild-type alleles.

Additionally, 43% (n = 14/32) of azole-resistant isolates were sourced from non-CF patients; within this group, 8% contained the drug resistance polymorphism TR₃₄/L98H, 6% harbored G54W, P216L, and M220K mutations, and 56.2% (n = 18/32) displayed wild-type *cyp51A*. The analysis found no clear associations between phylogenetic relationships and pathological conditions, with isolates from CF patients dispersed throughout both clades. This observation implies that genetics of the pathogen influence pathology. .

Furthermore, no correlations were evident between the *cyp51A* genotypes of the isolates and the patients' clinical presentations (Figure 7). A similar phylogenetic tree was derived from sequences collected in the U.K. (Rhodes et al., 2022b), reinforcing the robustness of the findings. Importantly, WT/non-WT *cyp51A* infections represented 70% of the wild-type isolates. Additionally, a larger tandem repeat, the TR130 expansion within the *cyp51A* promoter region, was detected in vivo in 7% of the cases, indicating the potential for this genetic variation to influence phenotypic outcomes in the context of azole resistance.

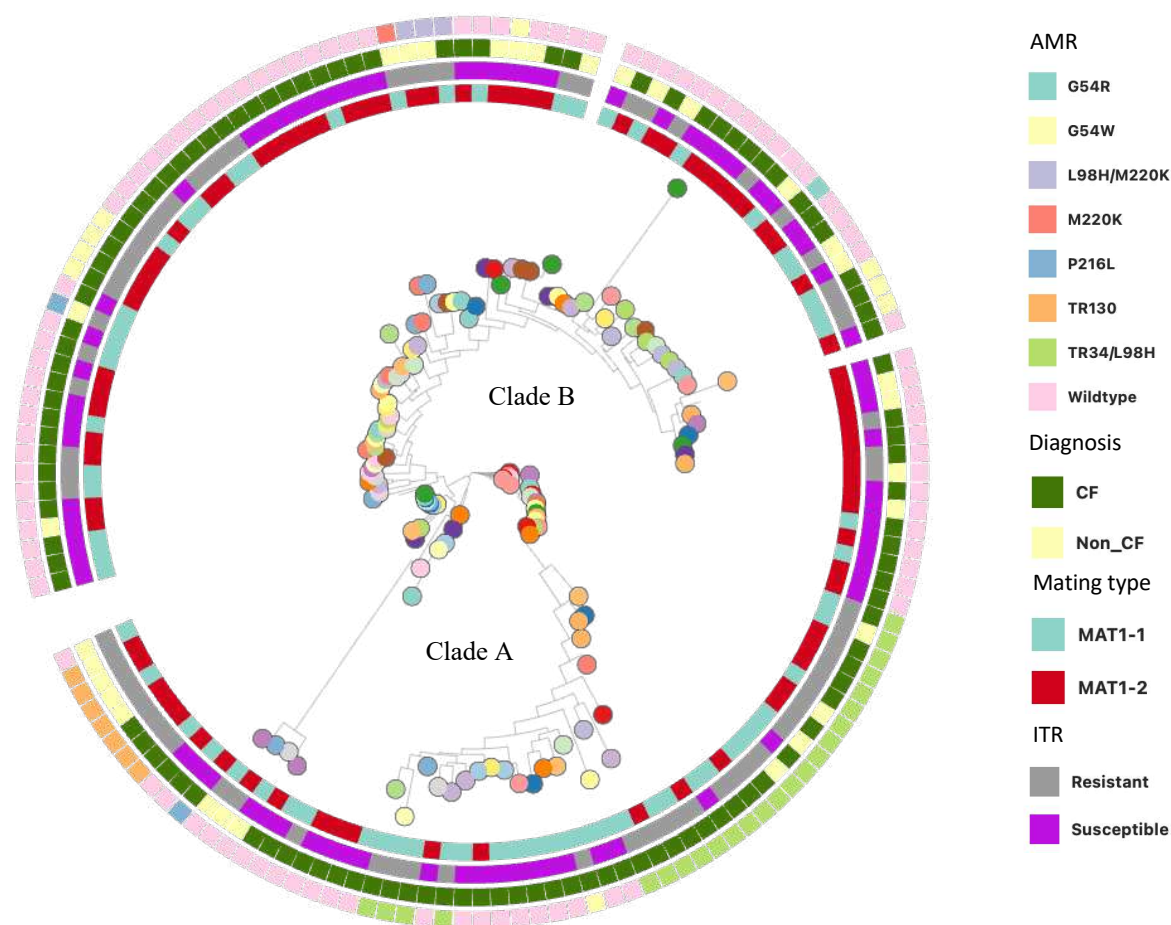
The analysis revealed that multiple isolates from a single patient exhibited high genotypic diversity, indicating an infection characterized by a single strain that subsequently diversified. Notably, isolates IGF11986, IGF11987, IGF11988, IGF11989, IGF11991, C136, and C139 were serially collected from a single cystic fibrosis (CF) patient undergoing azole treatment between 2015 and 2019 (Table 1). These isolates were clustered as a distinct subclade within Clade B, supported by 65% bootstrap value, alongside isolate C128 from a second patient (Figure 7). The findings indicate that the first isolate, obtained in March 2015, was azole-sensitive; however, subsequent isolates demonstrated azole resistance and exhibited more than two repeats of the TR130 sequence in the *cyp51A* promoter region. This is significant, as the

eight isolates with multiple copies of TR130 also developed phenotypic resistance to azole drugs (Figure 7). Interestingly, the isolate IGF11990 from the same patient clustered in a different subclade and positioned closer to the reference isolate Af293, suggesting that at least two distinct strains were present, indicative of a mixed strain infection.

In contrast, isolates IGF11961, IGF19980, IGF12011, IGF12012, and IGF12018, which displayed wild-type genotype profiles, were placed in separate subclades. The long branch lengths for these isolates suggest significant phylogenetic dissimilarity from each other and from the main Clade B. Furthermore, the analysis did not reveal any *cyp51A* polymorphisms among these isolates (Table 1), emphasizing the distinct genetic landscape of this subset and potentially indicating different evolutionary pressures or infection dynamics at play.

Overall, these observations highlight the complexities of within-host diversity and the emergence of resistance in *A. fumigatus* infections, particularly in patients with cystic fibrosis, and underscore the importance of monitoring clonal evolution during antifungal treatment.

Figure 7: Phylogenomic analysis of 145 *A. fumigatus* isolated in London from the Royal Brompton Hospital (constructed in RAxML using genome-wide SNPs) generated using the Microreact tool. Two divergent clades with 100% bootstrap support were identified. Clade A consists mainly of azole-resistant *A. fumigatus* isolates carrying the TR₃₄/L98H allele, whereas Clade B consists of azole-resistant and azole-susceptible *A. fumigatus* isolates carrying other *cyp51A* drug-resistance polymorphisms and wild-type alleles, as indicated by the colour markers in the outer rings. The inner circle refers to the mating type. The *itraconazole* (*ITR*) profile of each isolate is shown on the circle node of the tree. The diagnoses of patients' samples collected from all isolates are provided in Table 1.



3.1.3 Discussion

The increasing global rates of azole resistance in *A. fumigatus* pose significant public health challenges. Evidence suggests a rapid dissemination of resistant strains in a short time frame (Jeanvoine et al., 2020; Moghnieh et al., 2017; M. Richardson & Lass-Flörl, 2008a). *A. fumigatus* thrives in various environmental niches, resulting in substantial genotypic and phenotypic diversity among its isolates. This heterogeneity complicates the efforts of mycological laboratories to elucidate the intricate relationships between diverse genomes and their biological characteristics, including pathogenicity and antifungal resistance. Genomic analysis has proven essential in enhancing our understanding of *A. fumigatus* diversity. Whole-genome sequencing (WGS) has facilitated high-resolution analyses that distinguish closely related strains (Popovich & Snitkin, 2017). In this chapter, WGS was used to perform genome-scale comparisons, specifically focusing on single nucleotide polymorphisms (SNPs) relative to the reference isolate Af293.

The data revealed that *A. fumigatus* isolates can be divided into two distinct clades, A and B, each characterized by varying SNP counts. Notably, most isolates harbouring azole resistance polymorphisms were clustered within Clade A, which has been well-documented in various studies. According to the findings presented by (Etienne et al. 2021), a significant proportion of azole-resistant *A. fumigatus* isolates from the United States, especially those with the TR₃₄ resistance marker, were clustered within this clade. This clustering implies a shared evolutionary origin for resistance mechanisms, suggesting that mutations enabling azole resistance may have emerged once and dispersed through recombination and gene flow among fungal populations (Rhodes et al., 2022b). Furthermore, recent studies have reported a concerning increase in the TR₃₄/L98H substitution rate, which has reportedly doubled since its initial detection in the late 2000s (Mortensen et al., 2011). A notable proportion of azole-

resistant isolates from the U.S. displayed a mixture of alleles from both clades, indicative of ongoing recombination processes. This observation is consistent with findings from , which demonstrated that multi-azole-resistant *A. fumigatus* isolates exhibit elevated mutation rates, accelerating the evolution of antifungal resistance. A variant in the DNA mismatch repair (MMR) gene *msh6* was identified, significantly increasing mutation rates and contributing to additional antifungal resistance, further indicating that the TR₃₄/L98H mutation may be part of a broader genetic framework facilitating resistance evolution under selective pressure from environmental and clinical antifungal agents.

The implications of these findings extend beyond the laboratory, highlighting an urgent need for enhanced surveillance and monitoring of *A. fumigatus* populations. The increasing use of azole fungicides in agriculture likely drives the selection of resistant strains in the environment, potentially infecting patients, particularly those with compromised immune systems (Verweij et al., 2019). This cycle of resistance necessitates integrating environmental and clinical data to inform public health strategies aimed at curbing the spread of azole-resistant *A. fumigatus*.

Isolates with wildtype *cyp51A* and those harbouring point mutations such as G54 and M220 were identified within both Clades A and B; however, they predominantly clustered together within Clade B. This suggests that these point mutations, which are often associated with different levels of antifungal resistance, may not be strongly linked to a specific phylogenetic lineage but can be dispersed across multiple groups. In contrast, the TR₃₄/L98H polymorphism, a well-documented resistance-associated polymorphism, was predominantly grouped within Clade A. This clustering indicates a possible selective advantage or a shared evolutionary origin among isolates carrying this mutation. This phenomenon is supported by previous

research indicating the TR₃₄/L98H polymorphism is a common mechanism of azole resistance and has been observed globally. which has been supported by previous studies (Bader, 2021; Bottery et al., 2024; Gong et al., 2024). Sewell *et al.* (2019) demonstrated that isolates with TR₃₄/L98H or TR₄₆/Y121F/T289A mutations tend to be less genetically diverse than wildtype (WT) isolates, implying a potential clonal expansion or recent emergence of these resistant strains.

A noteworthy finding from our analysis is the presence of a TR₁₃₀ expansion within the *cyp51A* promoter region in eight isolates from cystic fibrosis (CF) patients. This expansion revealed a significant increase in the size of the promoter region, identifying multiple tandem repeat configurations: six isolates exhibited a four-fold repeat, one isolate had a three-fold repeat, and another featured a two-fold repeat of the same 130 bp sequence. This observation aligns with existing literature correlating tandem repeats with azole resistance mechanisms (Shelton et al., 2022). Gene expression studies indicated a significant upregulation of *cyp51A* expression in isolates with TR₁₃₀, correlating with the number of repeats and levels of azole resistance (Romero et al., 2003b; Shelton et al., 2022). This suggests an evolutionary adaptation concerning the number of repeats and corresponding azole susceptibility profiles.

Moreover, the emergence of *cyp51A* polymorphisms and tandem repeats is strongly associated with prolonged azole treatment, leading to increased minimum inhibitory concentrations (MICs) for azole resistance (Camps, Linden, et al., 2012; Howard et al., 2009b; Tashiro et al., 2012). The prevalence of wild-type and non-wild-type *cyp51A* infections highlights the

necessity for continuous monitoring of antifungal resistance in clinical settings, particularly since TR₁₃₀ expansion signifies adaptive evolution in response to the drug pressure experienced by CF patients.

Our genomic analyses corroborate findings from prior studies examining intraspecies diversity within this opportunistic pathogen (Verweij et al., 2009; Chowdhary et al., 2012; Simone M.T. Camps et al., 2012a; Risum et al., 2020b; Rocchi et al., 2021b; Zhou et al., 2023a, 2023b). Specifically, CF patients may be colonized by more than one *A. fumigatus* genotype over time (Verweij et al., 1996; Zhou et al., 2023b). the SNP differences documented in our study ranged from several hundred to tens of thousands of variants, reinforcing the hypothesis that various *A. fumigatus* genotypes can concurrently colonize the CF lung (Neuvéglise et al., 1997b).

These findings confirm a high degree of diversity between isolates from hospitalized CF and non-CF patients, suggesting that CF pulmonary aspergillosis infections are primarily associated with a predominant isolate. This raises the possibility that environmental isolates possess enhanced capabilities for infecting the lungs of individuals with cystic fibrosis or that these patients are consistently exposed to azoles, leading to the acquisition of resistant environmental isolates. Consequently, these environmental isolates may facilitate transmission among cystic fibrosis patients (Pontes et al., 2024). Our data showed that isolates from individuals with CF were dispersed across both clades, indicating no direct association with CF phenotype or disease representation

The significant diversity in *A. fumigatus* populations among patients, along with the variability within isolates from the same patient, emphasizes the need for awareness regarding the presence of *A. fumigatus* in clinical environments (van der Torre et al., 2021; Vanhee et al., 2008). The identification of *A. fumigatus* in aerosol samples within a cystic fibrosis treatment facility further underscores the potential for airborne transmission of this microorganism (Engel et al., 2019). Genomic analyses of the TR₁₃₀ genotype, represented by isolates recovered from two CF individuals with the same genotype, suggests possible person-to-person transmission (Breuer et al., 2019; Neuvéglise et al., 1997a). This evidence highlights the critical need to address cross-contamination risks, particularly in environments where patients undergo extended azole treatment, increasing their vulnerability to the development and exchange of resistant strains.

Limitation

It has been utilised to study the population structure of various fungi and to evaluate potential geographical patterns of recent outbreaks of fungal infections (Etienne et al., 2012; Litvintseva et al., 2014). Although WGS analyses can provide a valuable amount of novel information, there are some **limitations** that should be considered.

Firstly, analyses based on genome-wide comparative data and single nucleotide polymorphism (SNP) calling are heavily dependent on the quality of sequencing, assembly, variant calling criteria, and the careful selection of the reference genome. Poor sequencing quality or inappropriate reference selection can lead to inaccurate interpretations of genetic variations.

Secondly, the interpretation of detected SNPs can vary significantly based on the genetic similarity between the reference genome and the strains being analysed (Kowalski et al., 2016a). This variability can introduce challenges in assessing the evolutionary relationships among different strains, particularly when genetic divergence is substantial. Phylogenies derived from whole genomes serve as powerful tools for reconstructing the evolutionary relationships of all living organisms (Brown et al., 2001). However, the accuracy of these phylogenetic trees is contingent upon the robustness of the underlying data. for reconstructing the evolutionary tree of all living organisms (Brown et al., 2001) .

Additionally, it is important to note that 50 isolates included in our analysis lacked specific patient information. These isolates were treated as though they originated from a single patient with a single isolation event. This assumption may limit the scope of our findings, as it does not account for potential variations or complexities that could arise from multiple infections in different patients.

The rapid increase in the number of sequenced genomes presents a unique opportunity to address longstanding inquiries about the evolutionary history of specific species groupings and enhance our understanding of their evolution. To further this endeavour, current WGS genetic data are being integrated and compared with clinical and environmental isolates obtained from London and the broader U.K., merging them with data from the global dataset Microreact (Argimón et al., 2016b). This approach aims to create a more comprehensive understanding of the genetic diversity and evolutionary dynamics of fungal populations.

3.2 Application of BIOLOG™ Phenotype MicroArrays technology to Investigate the Acquisition of Nutrients and Subsequent Metabolic Activities of Clinical Azole-Resistant *A. fumigatus* Isolates.

3.2.1 Preface

Aspergillus fumigatus is a filamentous fungus known for its role in both environmental processes and human health. It is primarily a saprophytic organism, playing a crucial role in decomposing organic materials and recycling nutrients in the ecosystem, particularly in the carbon and nitrogen cycles. Its metabolic processes are integral to nutrient cycling (G. D. Brown et al., 2012b; Friedman & Schwartz, 2019; Kauffman, 2006; Pathakumari et al., 2020b; M. Richardson & Lass-Flörl, 2008b; Suleyman & Alangaden, 2016; VandenBergh et al., 1999). Its ability to metabolize different carbon and nitrogen sources is vital for its growth and survival, especially in nutrient-limited conditions. This fungus can assimilate nitrogen from various sources, enabling it to thrive in diverse environments. A phenotypic method has been developed for rapid characterisation in fungi such as *Aspergillus fumigatus*. Consequently, Phenotype MicroArray (PM) techniques (Biolog, Hayward, CA, USA) offer high throughput assays to study and analyse the characteristics of microbial cells and metabolic activity. The Biolog metabolic characterisation for fungus is determined by the use of thousands distinct tests conducted simultaneously, resulting in a distinctive reaction pattern referred to as a "fingerprint". The fingerprint response pattern yields extensive data regarding the metabolic and phenotypic characteristics of each isolate examined (Bochner et al., 2001). It becomes possible to observe and record biological responses, such as cellular respiration or growth, over a specific period. This enables the evaluation of metabolic changes occurring in targeted cells and the correlation of functional phenotypes with genotypes. In this chapter, the aim is to assess azole resistance in *A. fumigatus* isolates and susceptible strains in relation to patients with

cystic fibrosis (CF) and fungal endotypes, using BIOLOG™ PM microplates. Additionally, this chapter will highlight the benefits and limitations of this technique.

Metabolic Profiles of Azole-Resistant *A. fumigatus* Clinical Isolates in the Presence of Different Carbon and Nitrogen Sources Reveal Growth Phenotypic Heterogeneity Among *A. fumigatus* Isolates.

In vitro high-throughput microarray phenotypic assays of 379 distinct conditions were conducted to study the response of 42 *A. fumigatus* isolates. Only isolates from patients with known genotype information were included in the analysis. Forty isolates were categorized into two groups based on their patient endotypes and antifungal resistance (AMR), while two reference strains were included for comparative purposes. The results were visualized using a heatmap (Figure 8). The relative average quantities of each metabolite's concentration are compared across three biological replicates for each isolate against the reference strains. By calculating these average quantities, researchers can determine which metabolites are consistently produced at significantly different levels between the two groups, further elucidating the metabolic differences associated with the phenotypic variations observed among the isolates.

The results revealed remarkable growth phenotypic heterogeneity compared to reference *A. fumigatus* strains Af293 and CEA10. Twelve isolates, including IGF11963, IGF11957, IGF11988, IGF12034, IGF11982, IGF11966, IGF12012, IGF11969, IGF11970, IGF11989, IGF12017, and IGF11958, exhibited a similar phenotypic profile to CEA10. Twenty-four isolates displayed a similar phenotype to Af293. Additionally, four isolates showed distinct characteristics not observed in the reference strains. Specifically, IGF11953 and IGF11956, which harbour the TR₃₄/L98H polymorphism, showed varying growth levels in response to the identified sugars and carbohydrates, namely, Glycolic acid, Mucic acid, α -Hydroxyglutaric

acid- γ -Lactone, Methyl Pyruvate, Tween 40, and Adonitol, with absorbance generally below 0.50. This indicates there may be a significant deficiency in the necessary enzymes for substrate metabolism (Figure 8, Table 5).

Table 5 : Summary of *Aspergillus fumigatus* Clinical Isolates presents a comprehensive overview of 42 *Aspergillus fumigatus* clinical isolates, detailing each isolate's underlying diseases, antifungal resistance (AMR) status, genotype, and mating type.

ID	Underlying diseases / <i>Af</i> infection	ITR	Genotype/AMR	Mating type
IGF11959	CF/Asp bronchitis	Susceptible	Wildtype	MAT1-1
IGF11990	CF/Asp bronchitis	Susceptible	Wildtype	MAT1-1
IGF11986	CF/Asp bronchitis	Susceptible	TR ₁₃₀ /2R	MAT1-1
IGF11989	CF/Asp bronchitis	Resistant	TR ₁₃₀ /3R	MAT1-2
IGF11988	CF/Asp bronchitis	Resistant	TR ₁₃₀ /4R	MAT1-2
IGF11991	CF/Asp bronchitis	Resistant	TR ₁₃₀ /4R	MAT1-1
IGF11987	CF/Asp bronchitis	Resistant	TR ₁₃₀ /4R	MAT1-1
C136	CF/Asp bronchitis	Resistant	TR ₁₃₀ /4R	MAT1-2
C128	CF	Resistant	TR ₁₃₀ /4R	MAT1-2
C137	CF/Asp bronchitis	Resistant	TR ₁₃₀ /4R	MAT1-2
IGF11957	CF/Asp Bronchitis	Resistant	TR ₃₄ /L98H	MAT1-1
IGF11953	CF/Invasive Pulmonary Aspergillosis	Resistant	TR ₃₄ /L98H	MAT1-1
IGF12048	Reference	Susceptible	Wildtype	MAT1-2
C32	Reference	Susceptible	Wildtype	MAT1-1
IGF11958	CF/Asp Bronchitis	Resistant	Wildtype	MAT1-2
IGF11968	CF/ABPA	Resistant	TR ₃₄ /L98H	MAT1-1
IGF12037	CF/Asp bronchitis	Susceptible	Wildtype	MAT1-2
IGF12024	CF/Asp bronchitis	Resistant	TR ₃₄ /L98H	MAT1-2
IGF11979	CF/Asp colonisation	Susceptible	Wildtype	MAT1-2
IGF11956	CF/CPA	Resistant	TR ₃₄ /L98H	MAT1-2
IGF11969	CF/ABPA	Resistant	TR ₃₄ /L98H	MAT1-1
IGF11961	CF/Asp colonisation	Resistant	Wildtype	MAT1-1
IGF11955	Bronchiectasis/CPA	resistant	TR ₃₄ /L98H	MAT1-1
IGF11954	Asthma/ABPA	Resistant	TR ₃₄ /L98H	MAT1-1
IGF12023	Asthma/ABPA	Susceptible	Wildtype	MAT1-2
IGF12012	Asthma/ABPA	Susceptible	Wildtype	MAT1-1
IGF12035	Asthma/ABPA	Susceptible	Wildtype	MAT1-2
IGF11966	Bronchiectasis	Resistant	TR ₃₄ /L98H	MAT1-1
IGF11970	Asthma/ABPA	Resistant	TR ₃₄ /L98H	MAT1-2
IGF12007	Asthma/ABPA	Resistant	TR ₃₄ /L98H	MAT1-2

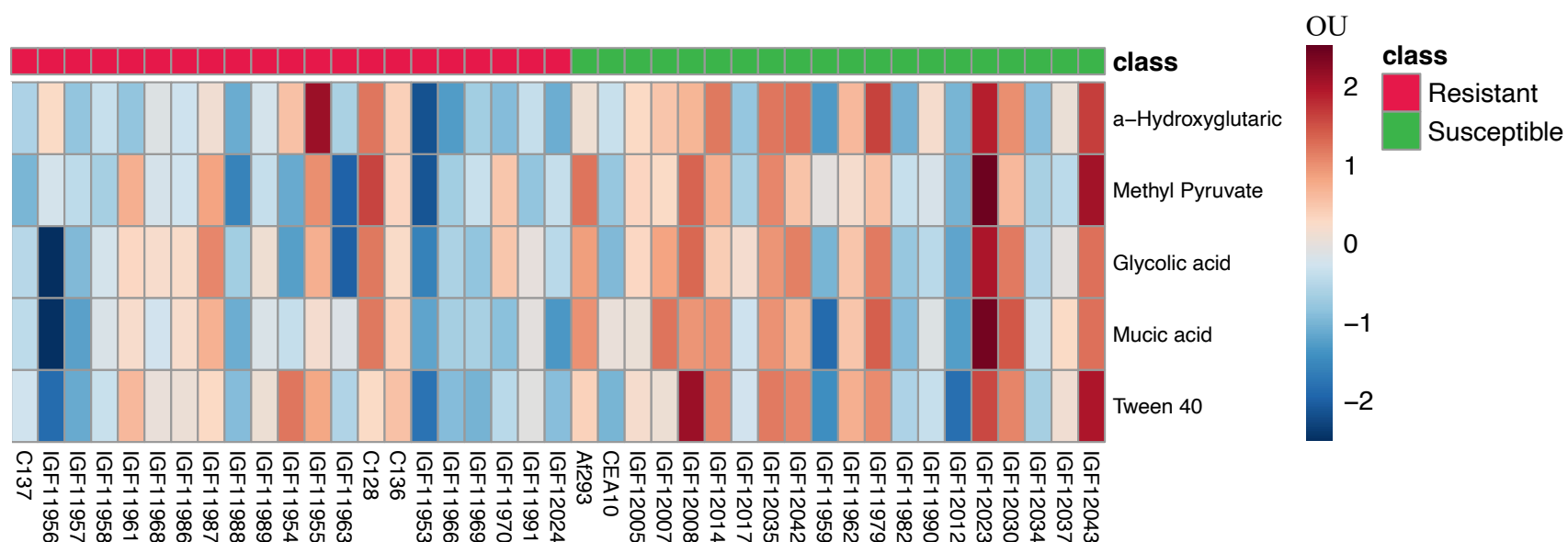
IGF12013	Bronchiectasis/ABPA	Susceptible	Wildtype	MAT1-2
IGF12014	Sarcoid/CPA	Susceptible	Wildtype	MAT1-1
IGF12017	Sarcoid/CPA	Susceptible	Wildtype	MAT1-2
IGF12042	Bronchiectasis/ABPA	Susceptible	Wildtype	MAT1-2
IGF12043	Bronchiectasis/Asp colonisation	Susceptible	Wildtype	MAT1-1
IGF12005	Bronchiectasis/ABPA	resistant	TR ₃₄ /L98H	MAT1-1
IGF11982	Sarcoid/CPA	resistant	Wildtype	MAT1-2
IGF11963	Sarcoid/CPA	resistant	Wildtype	MAT1-1
IGF12008	Bronchiectasis/ABPA	Susceptible	Wildtype	MAT1-1
IGF12034	Bronchiectasis/Asp colonisation	Susceptible	Wildtype	MAT1-2
IGF11962	Bronchiectasis/Asp colonisation	Susceptible	Wildtype	MAT1-1
IGF12030	Bronchiectasis/Asp bronchitis	Susceptible	Wildtype	MAT1-1

While the WT *cyp51A* isolates IGF12043 and IGF120123 showed a significant increase in growth on the previously listed substrates. In table 6, the statistical approach utilized for extracting significant metabolites from the data involved the use of the significance analysis of microarrays (SAM) test, which is specifically designed for identifying differentially expressed metabolites in high-dimensional data. In this analysis, the SAM test calculates a t-statistic for each metabolite, comparing its expression levels between the resistant and susceptible *A. fumigatus* isolates. This method accounts for multiple testing by applying a false discovery rate (FDR) correction, which helps to control for type I errors. The resulting p-values indicate the probability of observing the data under the null hypothesis, with lower p-values suggesting stronger evidence against the null. In this case, metabolites with p-values below a predetermined threshold (< 0.05) are deemed statistically significant, highlighting their potential roles in differentiating between resistant and susceptible isolates. The t-statistics further indicate the direction and magnitude of the differences, with negative values suggesting lower levels in resistant isolates compared to susceptible ones. Af293 and CEA10 exhibited markedly different growth phenotypes on the nitrogen and carbon plates, with Af293 utilizing these sources more effectively than CEA10 (Figure 8, Figure 11, and Table 6). In comparison

to the remaining isolates shown in Figure 8, the isolates IGF12043 and IGF120123 demonstrated significantly higher levels of amino acid utilization on both carbon and nitrogen plates.

Although a link between the TR₃₄/L98H resistance genotype and azole resistance phenotype was observed, no relevant correlation was detected between the phylogeny and the phenotype distribution (Figure 11). However, significant variation in carbon and nitrogen source metabolism was greater in resistant isolates when compared to susceptible isolates (Figure 8). This suggests that the resistance mechanisms in these isolates may be more closely linked to genetic mutations and/or metabolic pathways.

These findings indicate that the resistance genotype may play a more direct role in influencing the metabolic activity of the isolates. Additionally, exploring the underlying mechanisms driving this metabolic variation could provide valuable insights into the development of targeted interventions against resistant isolates. Understanding these complex interactions may be crucial for effective management strategies to combat antimicrobial resistance.



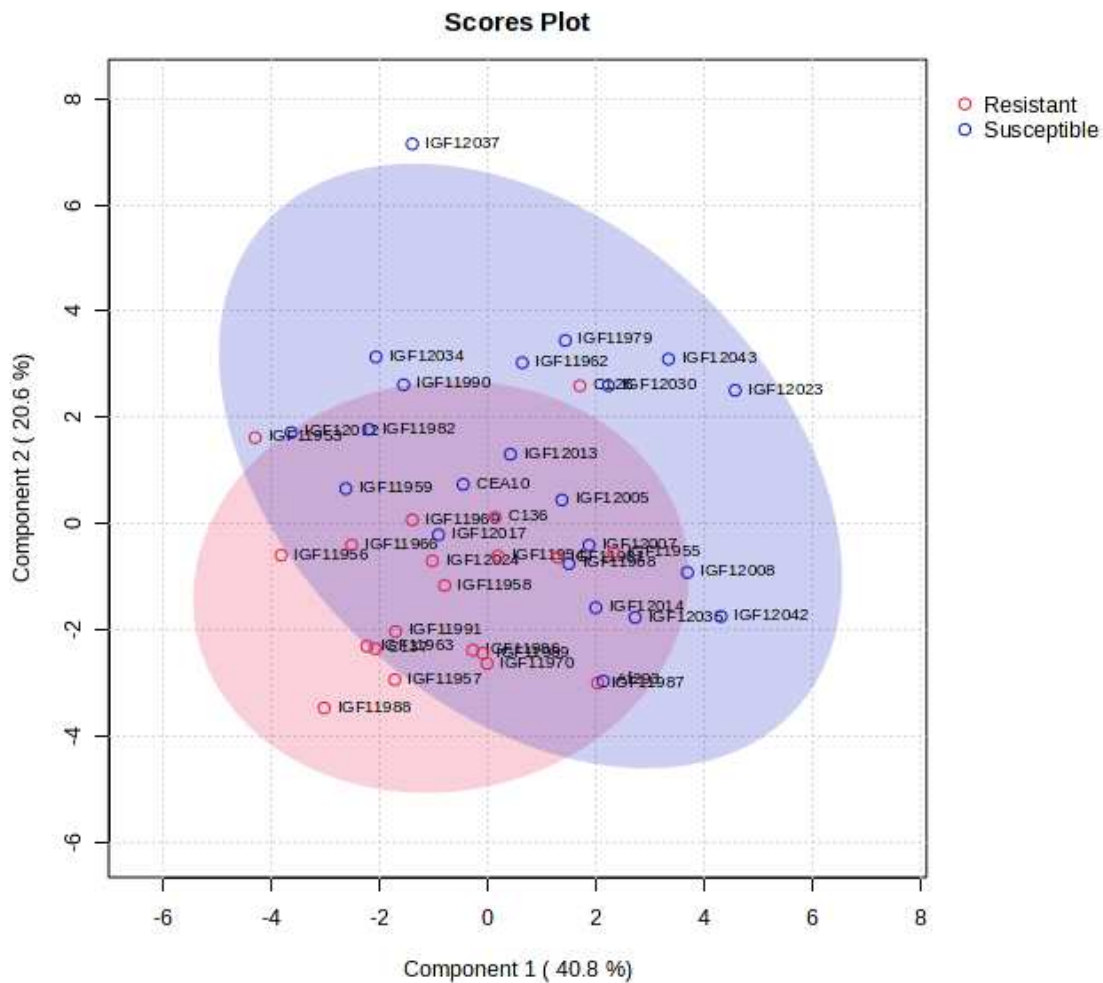


Figure 9: Multivariate data analysis of carbon and nitrogen metabolites in resistant and susceptible *A. fumigatus* isolates. Two-dimensional Partial Least Squares-Discriminant Analysis (PLS-DA) score plots of resistant versus susceptible isolates are presented. Shaded areas represent the 95% confidence regions for each group. Predictive component 1 and component 2 differentiate the susceptible and resistant isolates. The plot displays two principal components: Component 1 (40.8%) and Component 2 (20.6%), which together capture a significant portion of the variance in the data. Isolates are color-coded, with red circles representing azole-resistant strains and blue circles indicating azole-susceptible strains. The overlapping regions suggest metabolic similarities between some resistant and susceptible isolates, while distinct clusters highlight differences in metabolic activity related to antifungal resistance. Notably, the positioning of isolates within the plot provides insights into the underlying metabolic variations associated with their resistance phenotypes.

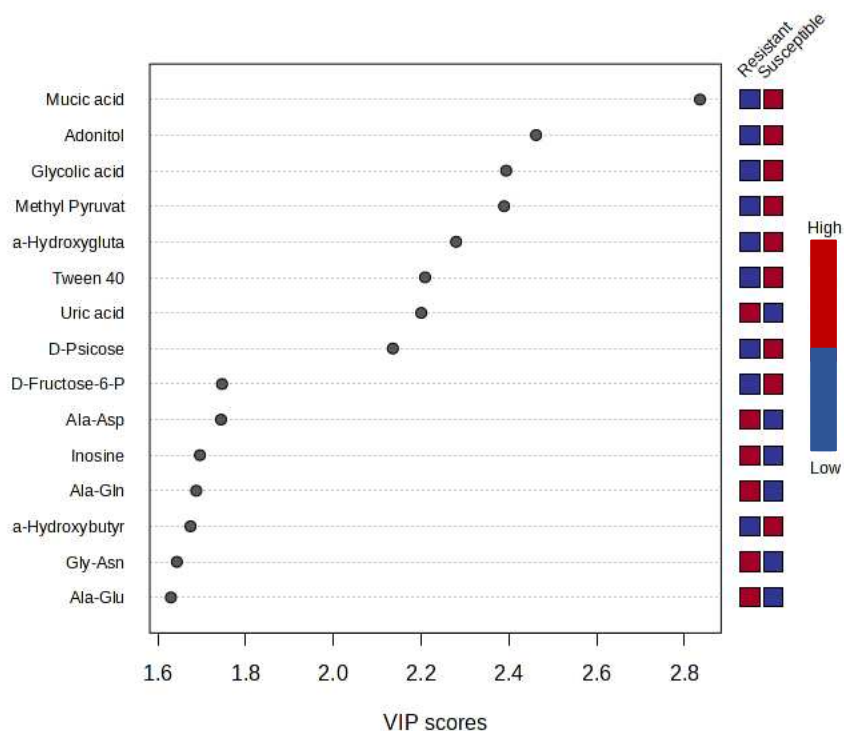


Figure 10: Variable Importance in Projection (VIP) scores for various metabolites identified in resistant and susceptible *A. fumigatus* isolates. Each point on the graph corresponds to a specific metabolite, with the VIP score indicating its contribution to the differentiation between the two groups. The metabolites are ranked along the x-axis, with higher scores reflecting greater importance in distinguishing between resistant (red) and susceptible (blue) isolates. The colour gradient on the right side of the figure illustrates the relative abundance of each metabolite, where darker colours represent higher concentrations. Notable metabolites include mucic acid and adonitol, which exhibit the highest VIP scores, indicating their significant role in metabolic pathways associated with antifungal resistance. This analysis provides valuable insights into the metabolic differences underlying the resistance phenotypes of *A. fumigatus*.

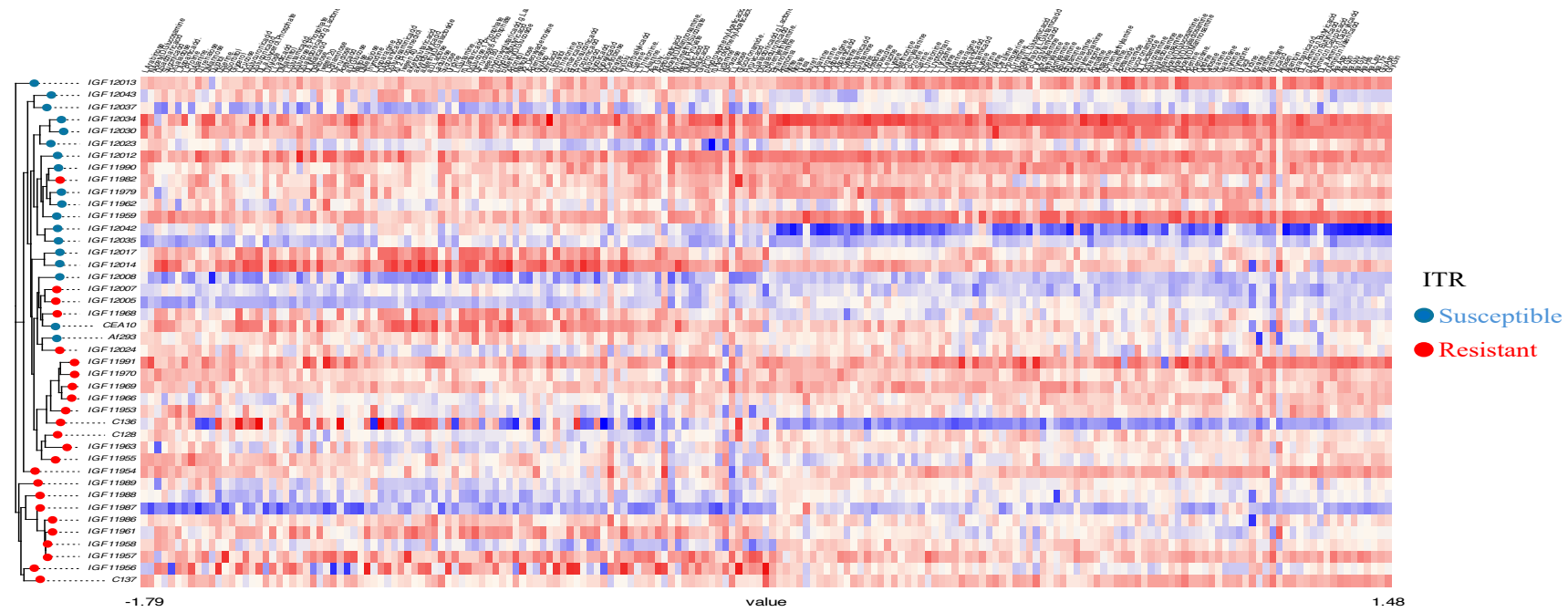


Figure 11: This heatmap displays the expression levels of carbon and nitrogen metabolites in resistant and susceptible *A. fumigatus* isolates, visualized across a 42 of isolates. The colour gradient represents the relative abundance of each metabolite, with red indicating higher values and blue signifying lower values. Each column corresponds to a specific metabolite, while the row represents individual isolates categorized as either resistant (red circles) or susceptible (blue circles) to antifungal treatment. The dendrograms on the left of the heatmap illustrate phylogeny clustering of the 42 isolates, highlighting patterns of similarity and dissimilarity in metabolic profiles. Notably, the OU value is indicated along the x-axis, providing context for the metabolic activity associated with antifungal resistance. This heatmap allows for a comprehensive examination of metabolic variations and their potential implications for understanding resistance mechanisms in *A. fumigatus*.

The metabolite compositions from fungal growth under different conditions were further investigated using the partial least square discriminate function (PLS-DA) to provide important insights into the metabolic profiles of the two groups (Figure 9). The PLS-DA analysis rendered a two-component model as calculated by cross-validation (CV). The PLS-DA analysis scores for susceptible and resistant isolates showed no clusters scores, where the metabolites analysed from resistant isolates positioned on the negative component 1 and the susceptible isolates positioned on the positive side of component 1 (Figure 9). Explanation of 87.4% of the analysed data was accomplished (i.e., $R^2 = 0.97$ and $Q^2 = 0.95$) and the eigenvalue for component 1 was 14.5 as compared to 2.1 for component 2. As shown in the PLS-DA in figure 9, the metabolites analysed from susceptible isolates were partially clustered separately from those of the resistant isolates for Mucic acid, Glycolic acid, α -Hydroxy glutaric acid - γ -lactone, methyl pyruvate and adonitol (Figure 8), suggesting that there are distinct metabolic differences between susceptible and resistant isolates. The metabolites that contributed to the classification of susceptible and resistant isolates were identified using the VIP scores (Figure 10). Metabolites with greater VIP scores make a more important contribution to the separation between the two groups, while those with smaller VIP scores have less influence on the model. Metabolites such as D-melibiose, Gly-Glu and Met-Ala had VIP scores greater than 1.5 for carbon and 0.7 for nitrogen sources, indicating that these metabolites played an essential role in the discrimination of metabolic profiles of susceptible and resistant isolates (Figure 9 and 10). The resistant isolates growing under carbon and nitrogen were characterised by a great reduction in the growth level of metabolites Mucic acid, Glycolic acid (figure 10). This reduction suggests that the resistant isolates may have altered metabolic pathways compared to susceptible isolates. Further investigation is needed to understand the implications of these changes on antibiotic resistance mechanisms.

Table 6: Summary statistics for the differential metabolite profiles between azole-resistant and azole-susceptible *A. fumigatus* isolates using sam test. **t. stat** displays the t-statistic for each metabolite, reflecting the magnitude and direction of differences in expression levels between resistant and susceptible isolates. Negative values indicate lower levels in resistant isolates. **p.value:** shows the p-value associated with each t-statistic, representing the probability of observing the data under the null hypothesis. A lower p-value indicates stronger evidence for a significant difference, with values below 0.05 considered statistically significant.

Metabolites	t. stat	p.value
Glycolic acid	-2.6444	0.011829
Mucic acid	-2.5289	0.015715
a-Hydroxyglutaric acid-g-Lactone	-2.355	0.023792
Methyl Pyruvate	-2.3083	0.026519
Tween 40	-2.247	0.030528
Adonitol	-2.1378	0.039028

3.2.2 Azole resistant *A. fumigatus* isolates display halotolerance to osmolyte/ions stress conditions and pH optimum.

fumigatus isolates differed in their phenotypic traits under various osmotic stresses. Isolates with the azole-resistant phenotype exhibited increased stress tolerance to 100 mM ammonium sulfate at pH 8 ($p = 0.03$), 10 mM sodium nitrite ($p = 0.02$), up to 5% potassium chloride ($p = 0.012$), and up to 6% NaCl ($p = 0.04$; Figure 12 and 14, Table 7). In contrast, these isolates demonstrated significantly attenuated tolerance to urea ($\geq 6\%$), 20 mM sodium phosphate, and 12% sodium lactate (plate PM 9, wells F1 to F12), as well as up to 20% ethylene glycol (plate PM 9, wells D9 to D12) and up to 6% sodium formate (plate PM 9, wells E1 to E6) (Table 7). Interestingly, the wild-type IGF12008 and IGF12014 isolates showed enhanced adaptation in 50 mM sodium benzoate at pH 5.5, while the other three wild-type isolates could only utilize up to 20 mM sodium benzoate. This suggests that greater resistance to sodium benzoate may

account for the widespread environmental prevalence of *A. fumigatus* (Figure 12 and 14, Table 4A).

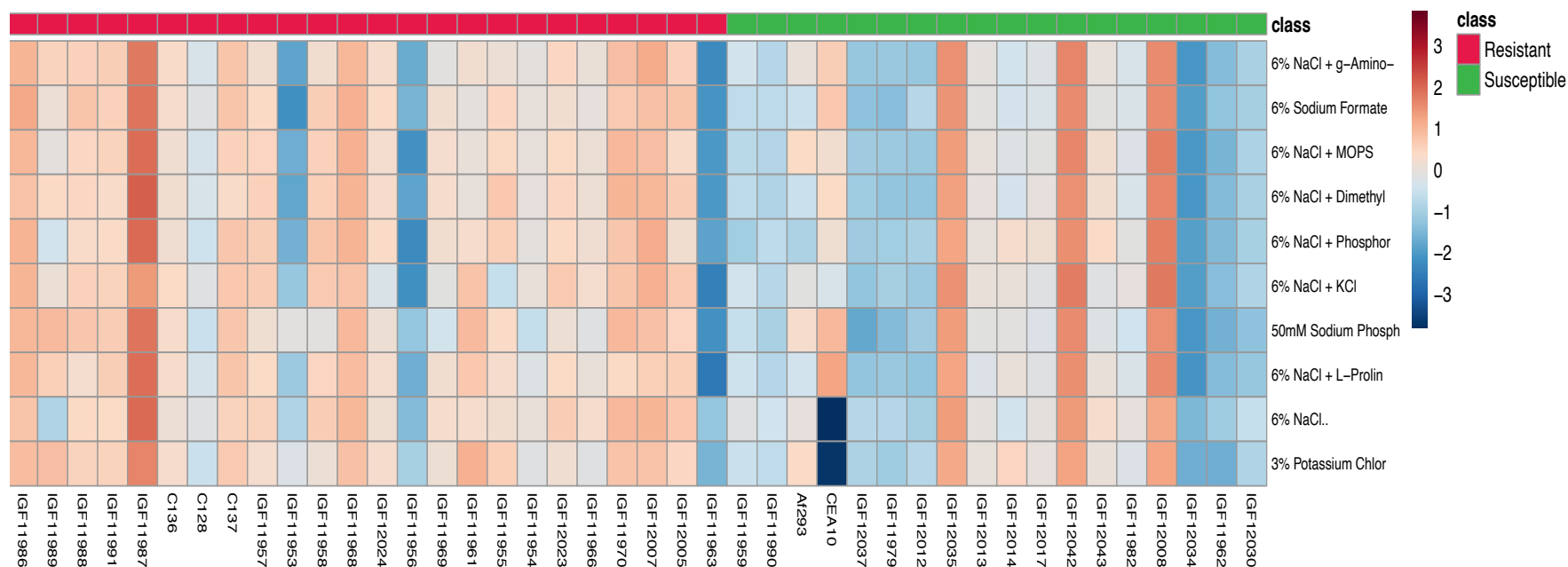


Figure 12: This heatmap visualizes the expression levels of various stressors across different doses administered to resistant and susceptible *A. fumigatus* isolates. The colour intensity represents the relative average quantities of stressors using the Omnilog unite (OU), with higher values indicated in red and lower values in blue. *A. fumigatus* isolates are categorized into azole-resistant (indicated by the red bar) and azole-susceptible (indicated by the green bar) classes. The heatmap highlights significant variations in metabolite utilization across the isolates, revealing notable differences in metabolic activity associated with azole resistance.

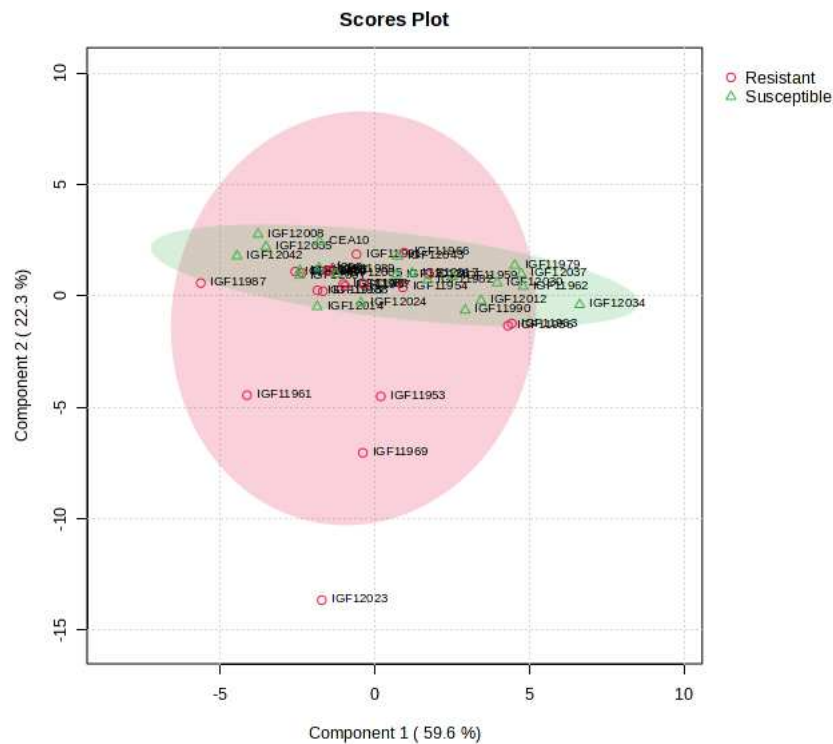


Figure 13: Scores Plot illustrating the distribution of sample groups based Two-dimensional Partial Least Squares-Discriminant Analysis (PLS-DA). The plot displays two distinct groups: Resistant isolates, represented by red circles, and Susceptible isolates, indicated by green triangles. The ellipses surrounding each group highlight the confidence intervals for the respective sample distributions. Component 1 (59.6%) and Component 2 are the axes representing the principal components derived from the dataset, capturing the variance within the data. The separation between the two groups suggests distinct characteristics associated with resistance and susceptibility.

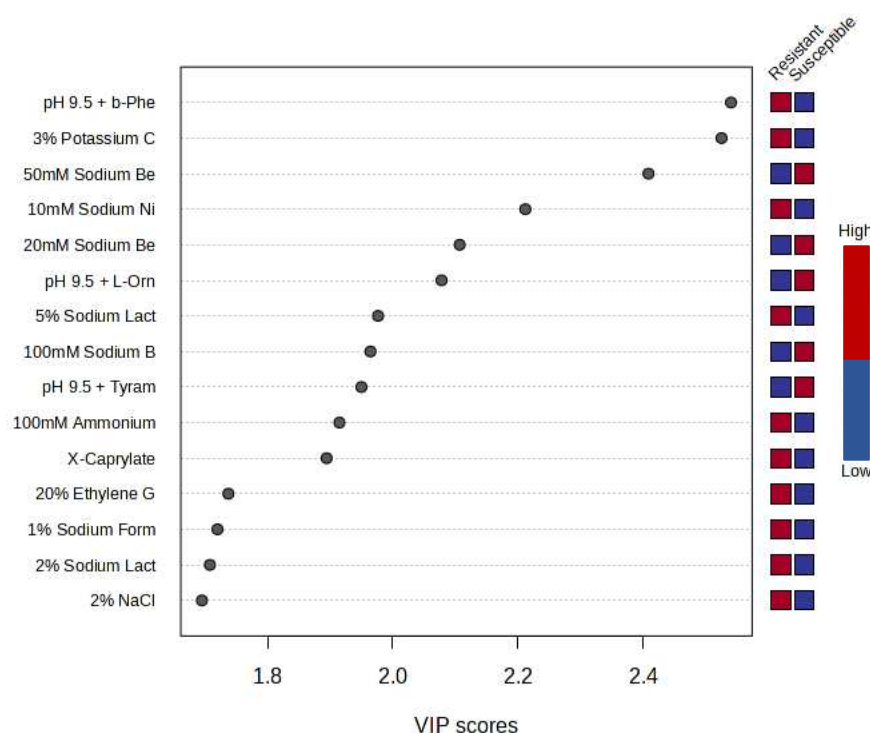


Figure 14: Variable Importance in Projection (VIP) scores plot showcasing the significance of various factors in differentiating between "Resistant" and "Susceptible" sample groups. Each dot represents a variable, plotted against its VIP score, indicating its contribution to the model's predictive capability. The colour gradient on the right side illustrates the correlation strength; darker shades signify higher importance in distinguishing between the two groups. Variables such as pH levels, sodium concentrations, and other chemical treatments are displayed, reflecting their varying influences on the outcomes. This plot aids in identifying key variables that may play critical roles in the observed resistance and susceptibility traits. Data were normalized to the control well, log-transformed, and Pareto scaled (mean-cantered and divided by the square root of the standard deviation of each variable).

Table 7: Summary statistics of the differential metabolite profiles between azole-resistant and azole-susceptible *A. fumigatus* isolates. **t. stat** displays the t-statistic for each metabolite, reflecting the magnitude and direction of differences in expression levels between resistant and susceptible isolates. Negative values indicate lower levels in resistant isolates. **p.value:** shows the p-value associated with each t-statistic, representing the probability of observing the data under the null hypothesis. A lower p-value indicates stronger evidence for a significant difference, with values below 0.05 considered statistically significant.

Metabolites	t.stat	p.value
5% Potassium Chloride	2.4386	0.019278
10mM Sodium Nitrate	2.2658	0.028947
100mM Ammonium Sulfate pH 8	2.2429	0.030513
5% Sodium Lactate	2.2359	0.031007
6% NaCl.	2.0557	0.046383
50mM Sodium Phosphate pH 7	1.9365	0.059888

Assessment of tolerance to pH levels revealed that all isolates were capable of surviving within a pH range of 3.5 to 9.5. However, the azole-resistant isolates demonstrated notable resistance to strong alkalis, specifically 100 mM ammonium sulfate at pH 8- and 50-mM sodium phosphate at pH 7 (refer to Figures 12 and 14, Table 7). Remarkably, these isolates maintained active metabolism utilizing multiple nitrogen sources at both pH 4.5 and 9.5. For instance, at pH 9.5 with L-isoleucine and at pH 4.5 with putrescine and L-isoleucine, they exhibited a heightened capacity for pH regulation.

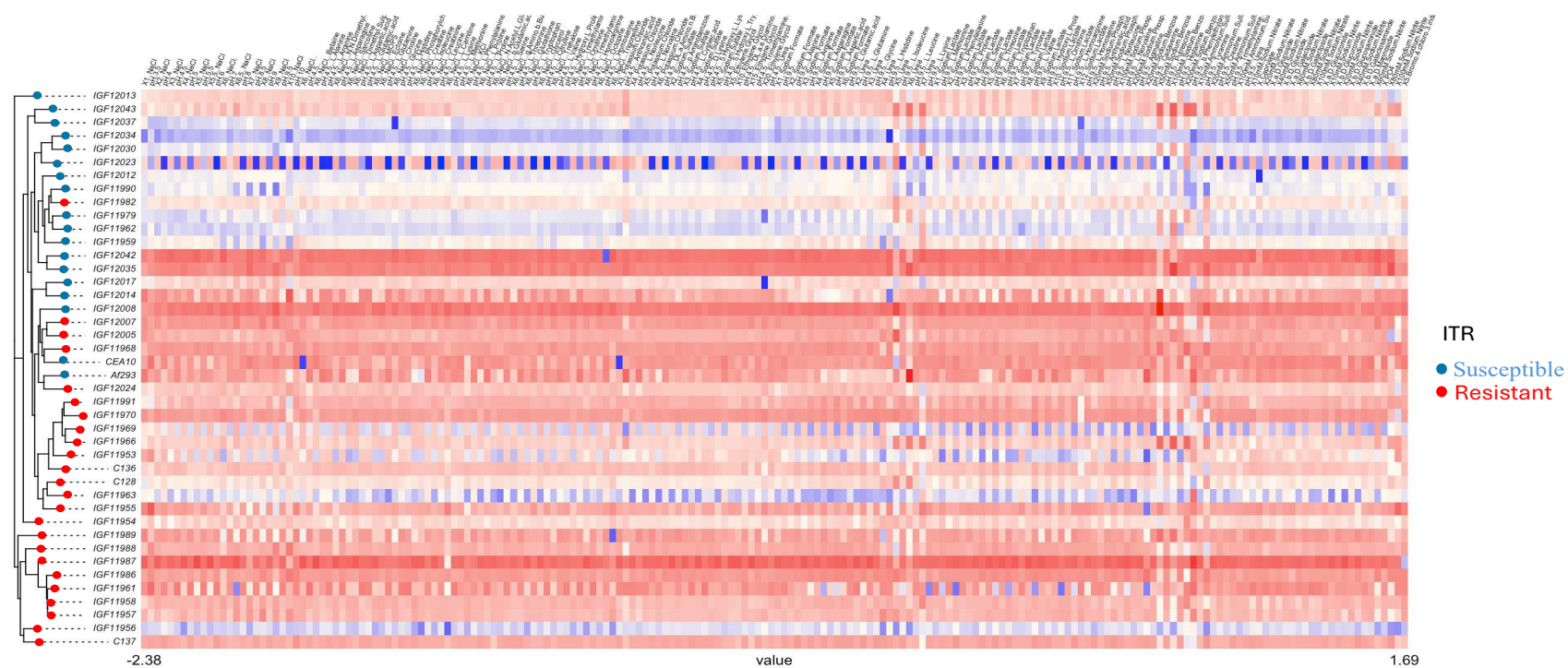
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The metabolic profiles of susceptible and resistant isolates were further analyzed through Partial Least Squares-Discriminant Analysis (PLS-DA) (see Figure 13). This analysis produced a two-component model, validated by cross-validation (CV). The PLS-DA scores illustrated two distinct clusters separated by component 1: metabolites from susceptible isolates were positioned on the positive side, while those from resistant isolates were found on the negative side (Figure 13). The significant difference in eigenvalues indicated that the majority of data variation was associated with the clustering of the resistant isolates. Additionally, the PLS-DA graph showed that metabolites from susceptible isolates largely clustered together, with the exception of IGF11953, IGF11961, IGF11969, and IGF12023, which were associated with resistant isolates. This suggests distinct metabolic profiles between the two groups (see Figures 13 and 14).

To further evaluate the importance of variables in the projection analysis, we identified the top metabolites contributing to the differences in metabolic profiles between susceptible and resistant isolates (Figures 13 and 14). Metabolites with a VIP score of 1.0 or higher were deemed significant contributors. As illustrated in Figures 14 and 15, key metabolites such as 100 mM sodium nitrate, 4% urea, pH 4.5, pH 9.5 with β -phenylethylamine, X-SO₄, and pH 9.5 with putrescine were identified as critical factors in differentiating between the susceptible and resistant isolates.

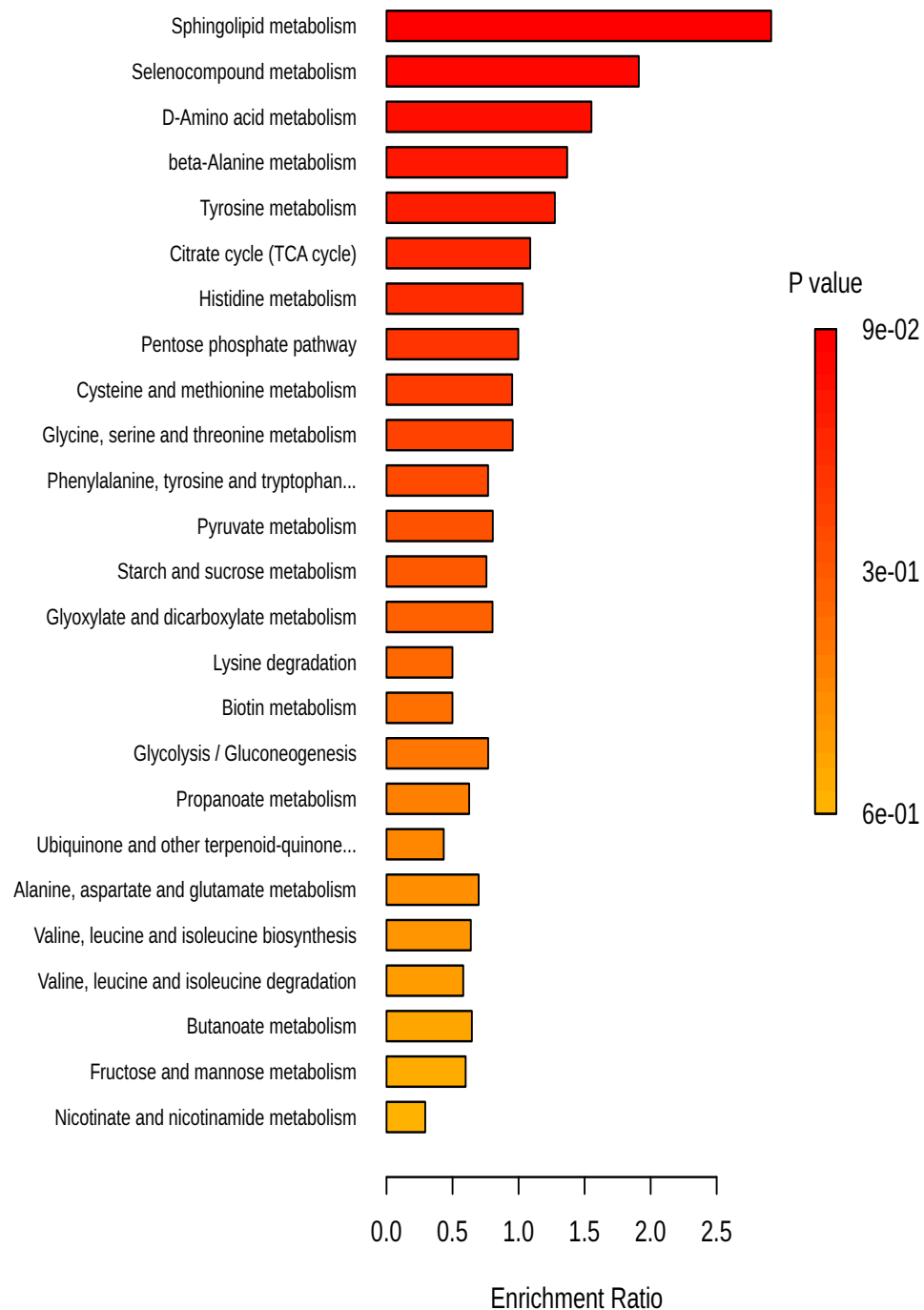
Figure 15: Figure 16: Heatmap displaying the growth responses of various isolates in the presence of osmolytes and pH metabolites. The color gradient indicates varying levels of response, with shades of red representing susceptible isolates and shades of blue indicating resistant ones. A notable correlation is observed between the phylogenetic relationships of the isolates and their respective phenotypic distributions.



3.2.3 Metabolic Enrichment and Metabolic Pathway Analysis Associated with the Azole-Resistant Phenotype.

Metabolite set enrichment and metabolic pathway analyses were performed using MetaboAnalyst v6.0 to determine the most enriched metabolic pathways associated with the azole-resistant phenotype. The pathway impact value was calculated from topology pathway analysis, defining those with scores higher than 0.10 as significant metabolic pathways. The metabolic set enrichment overview revealed that the analyzed metabolites were involved in the perturbation of 14 metabolic pathways related to carbon and nitrogen sources in azole-resistant isolates, including sphingolipid and selenocompound metabolism, glycine, serine, and threonine metabolism, glycerophospholipid metabolism, thiamine metabolism, purine metabolism, and pyrimidine metabolism (Figure 18). The quantitative enrichment analysis (MESA) was used to determine the major perturbed pathways associated with the azole-resistant phenotype in the fungal isolates (Figure 18). Metabolic pathway analysis indicated that 25 pathways were differentially regulated in response to carbon and nitrogen sources (Figure 18), seven of which were significantly enriched for sphingolipid (L-Serine) ($p < 0.05$) and selenocompound metabolism (L-Alanine) ($p < 0.012$). Sphingolipids represent a category of natural lipids characterised by a backbone of sphingoid base, specifically sphingosine. Sphingosine acylated with fatty acids results in ceramide formation. Sphingolipids, glycerophospholipids, and sterols constitute the primary lipid components of eukaryotic cell membranes (Chen et al., 2010; R. C. Dickson et al., 2006). As shown in the metabolic pathway diagrams (Figure 16), Sphingolipids metabolism pathway (L-Serine) is the is significantly enriched but shows a decrease in resistant isolates

Enrichment Overview (top 25)



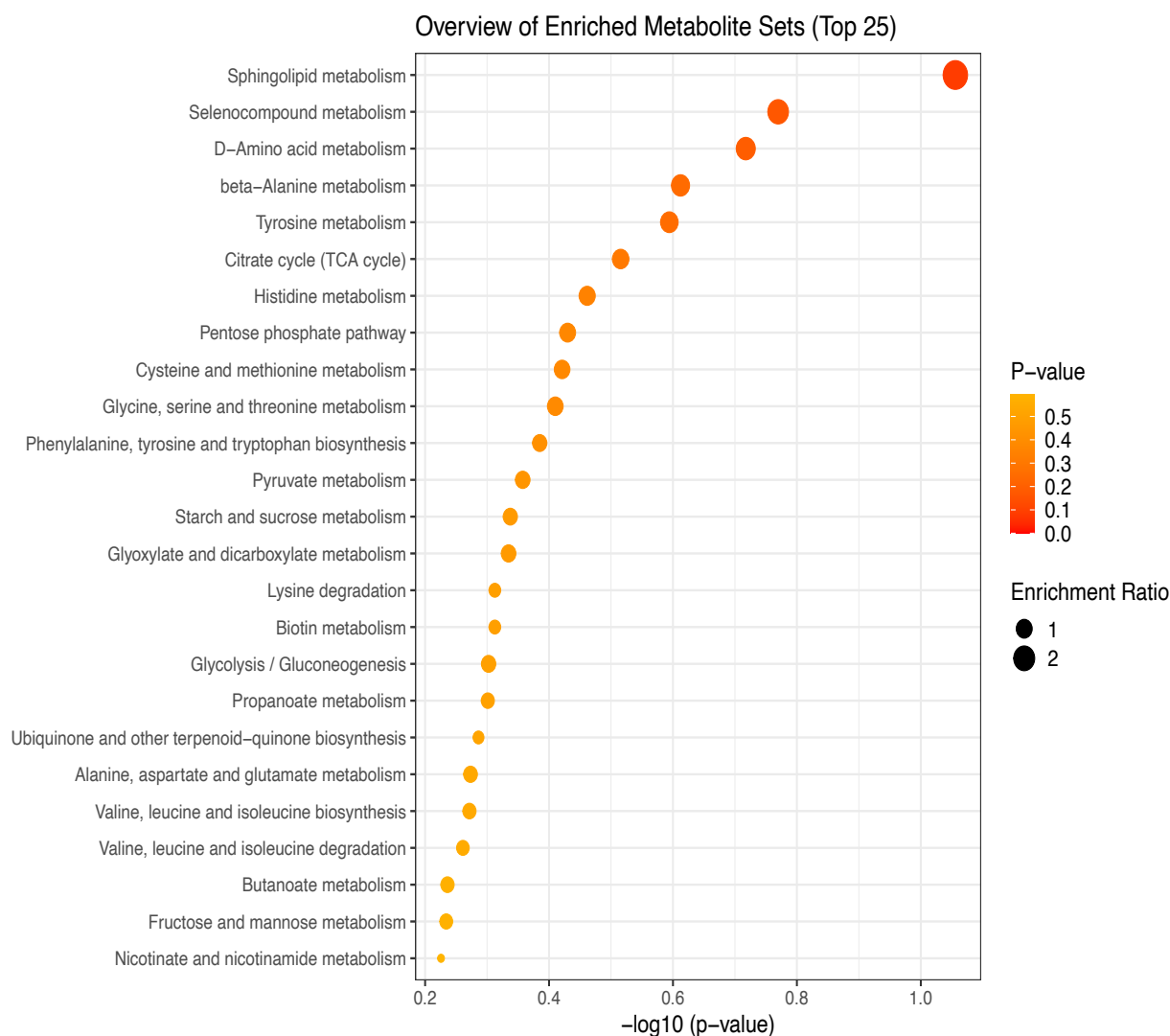


Figure 16: Enrichment overview of the top 25 metabolic pathways, illustrating carbon enrichment and pathway analysis. The bar chart reveals the selenocompound pathway as one of the most significantly altered pathways in resistant isolates. Pathway analysis compares the alterations between resistant and susceptible isolates, with all matched pathways displayed according to their p-values from pathway enrichment analysis (y-axis) and pathway impact values from pathway topology analysis (x-axis). The color gradient of each bar indicates p-values, with deeper red bars representing more significant pathway alterations. The length of each bar reflects the enrichment ratio per metabolite set, emphasizing the prominence of pathways such as sphingolipid metabolism alongside the selenocompound pathway.

3.3 Metabolic Variability Correlates with Genomic Diversity of Azole-Resistant *A. fumigatus* Isolates in Patients with Cystic Fibrosis and Other Respiratory Diseases.

3.3.1 *A. fumigatus* CF and non-CF isolates differ in their ability to metabolize and respond to stress conditions

To minimise the likelihood of capturing irrelevant metabolic substrates, A comparative untargeted metabolomics approach was employed using MetaboAnalyst 6.0v. First, a cohort of two groups for all four tested conditions was selected : patients with CF ($n = 20$) and patients with other respiratory diseases (collectively called non-CF) ($n = 20$) (Table 1). The data were analysed using a supervised multivariate classification technique (mean decrease accuracy) to distinguish metabolites that contributed significantly to the differentiation between the CF and non-CF groups against reference strains (Af293 and CEA10) (Table 8 and Figure 17). As shown in Figure 18C, the PCA score plots clearly differentiated between CF and non-CF groups, although some overlap was observed. In the CF group, $R^2X = 0.24$, $R^2Y = 0.98$, Q^2 (cum) = 0.68; in the non-CF group, $R^2X = 0.09$, $R^2Y = 0.86$, Q^2 (cum) = 0.45. Mean decrease accuracy is the measure of performance of the model without each metabolite ranked the top 15 discriminating carbon and nitrogen metabolites that contributed most to the separation of the two groups, with a score > 2.2 ($p < 0.05$) (Figure 18E). These metabolic substrates included L-histidine, lactulose, hydroxylamine, D-glutamic acid, L-cysteine, D-galactose, N-acetyl-D-glucosamine, α -ketoglutaric acid, acetic acid, putrescine, L-alanine, thymine, D-valine, asparagine, and D-asparagine (Figure 18E). The mean decrease accuracy model separating CF and non-CF patients identified 15 osmotic and pH stressors with a score > 1.5 ($p < 0.05$) included 50-100 mM sodium benzoate at pH5.2, 2-4% sodium sulfate, 6% urea, 6-9% NaCl and 80 mM sodium nitrate (Figure 18F). All relevant metabolites with mean decrease accuracy

scores higher than 0.0020 and/or significantly different among CF and non-CF patients are reported in Table 8 and Figure 18A, - 18F,



Figure 17 : a representative multi-panel dot plot illustrates the estimated parameters concerning the length of the raw area under the curve (AUC) data for both cystic fibrosis (CF) and non-CF isolates of *A. fumigatus* cultured on nitrogen plates. In this dot plot, each dot signifies the presence of *cyp51a* drug-resistance polymorphisms alongside wild-type alleles. This visual representation is crucial for understanding the distribution and impact of genetic variations on the phenotypic responses observed in the different isolates.

Table 8: Displaying the comparative analysis of various metabolites and stressors between cystic fibrosis (CF) and non-cystic fibrosis (non-CF) isolates. The columns represent **Metabolites**: The names of the metabolites evaluated, **CF**: The expression levels of metabolites in CF isolates, with negative values indicating lower levels compared to the baseline, **Non-CF**: The expression levels of metabolites in non-CF isolates, with positive values indicating higher levels, **AveExpr**: The average expression value calculated from both CF and non-CF isolates, with p-values indicating statistical significance, **p.value**: The raw p-values reflecting the significance of differences in metabolite levels between CF and non-CF groups and **adj.p.value**: The adjusted p-values accounting for multiple comparisons, indicating the likelihood that observed differences are due to chance.

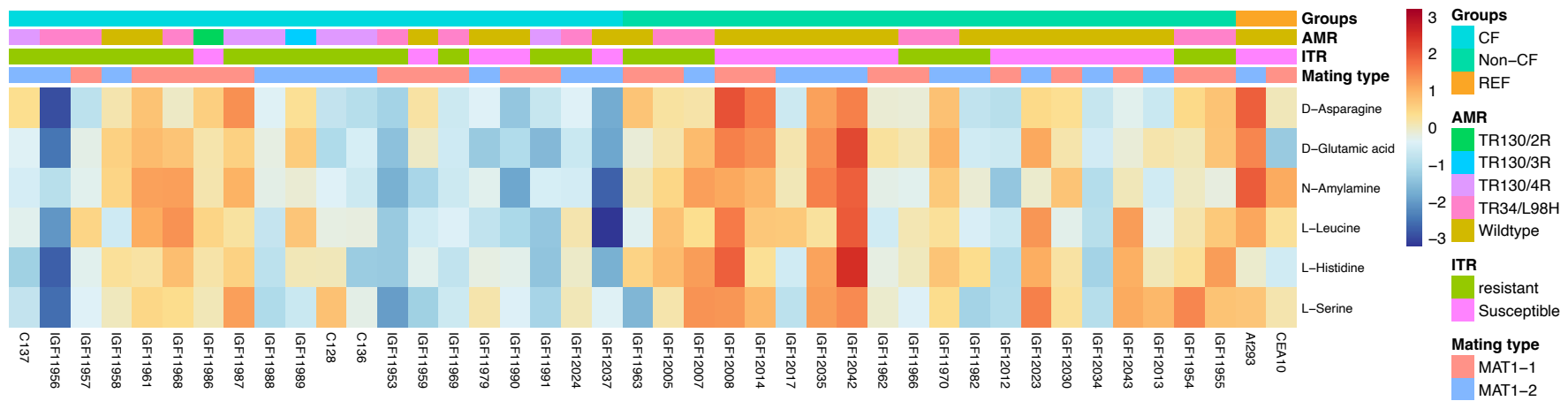
Metabolites	CF	Non.CF	AveExpr	p.value	adj.p.value
L-Histidine	-0.082188	0.38079	1.18e-16	0.0038235	0.50859
N-Amylamine	-0.84152	-0.55341	-3.47e-16	0.01161	0.50859
D-Glutamic acid	-0.24774	0.15711	-2.45e-16	0.015262	0.50859
D-Asparagine	-0.59696	-0.27714	5.30e-16	0.029525	0.50859
L-Leucine	-0.48558	-0.15265	-4.64e-16	0.03649	0.50859
L-Serine	-0.38352	-0.036891	-1.33e-16	0.037987	0.50859
Ethanolamine.	-0.54679	-0.25184	2.22e-16	0.050719	0.50859
DL-a-Amino-n-Caprylic acid	-0.78777	-0.66276	2.02e-16	0.053183	0.50859
L-Galactonic acid-g-Lactone	-0.21407	0.12004	1.97e-16	0.057709	0.50859
Ala-Gly	-0.24843	0.084125	1.88e-16	0.05799	0.50859
6% NaCl	0.95183	0.96375	-1.79e-16	0.022764	0.78582
3% Potassium Chloride	0.92706	0.84602	2.53e-16	0.034042	0.78582

Figure 18: Heatmap illustrating the expression profiles of various groups-based A) carbon and nitrogen metabolites B) osmolytes and pH stressors .

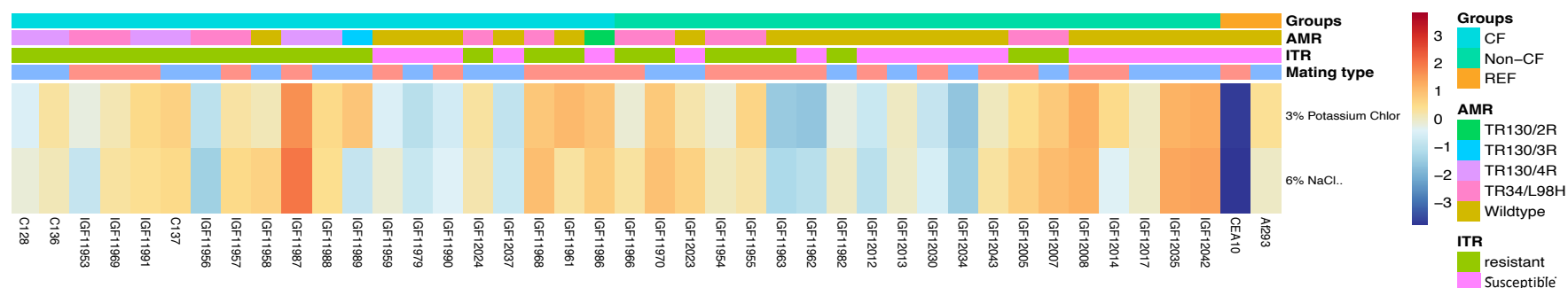
The rows represent different variables or parameters measured, while the columns categorize samples based on distinct characteristics: **Groups:** Identifies the experimental groups, including AMR (Antimicrobial Resistance), IS (Invasive Species), and others, indicated by color-coded bars at the top, **AMR Types:** Specifies the types of antimicrobial resistance present in the isolates, providing insights into their resistance mechanisms, and **ITR:** Displays the infection resistance factors associated with each group, contributing to understanding the isolates' pathogenic potential.

The colour gradient represents the level of expression, with warmer colours (e.g., red/orange) indicating higher expression levels and cooler colors (e.g., blue) representing lower expression levels. This visualization allows for quick identification of patterns and relationships among groups, facilitating comparative analysis of the expression profiles across different experimental conditions.

A

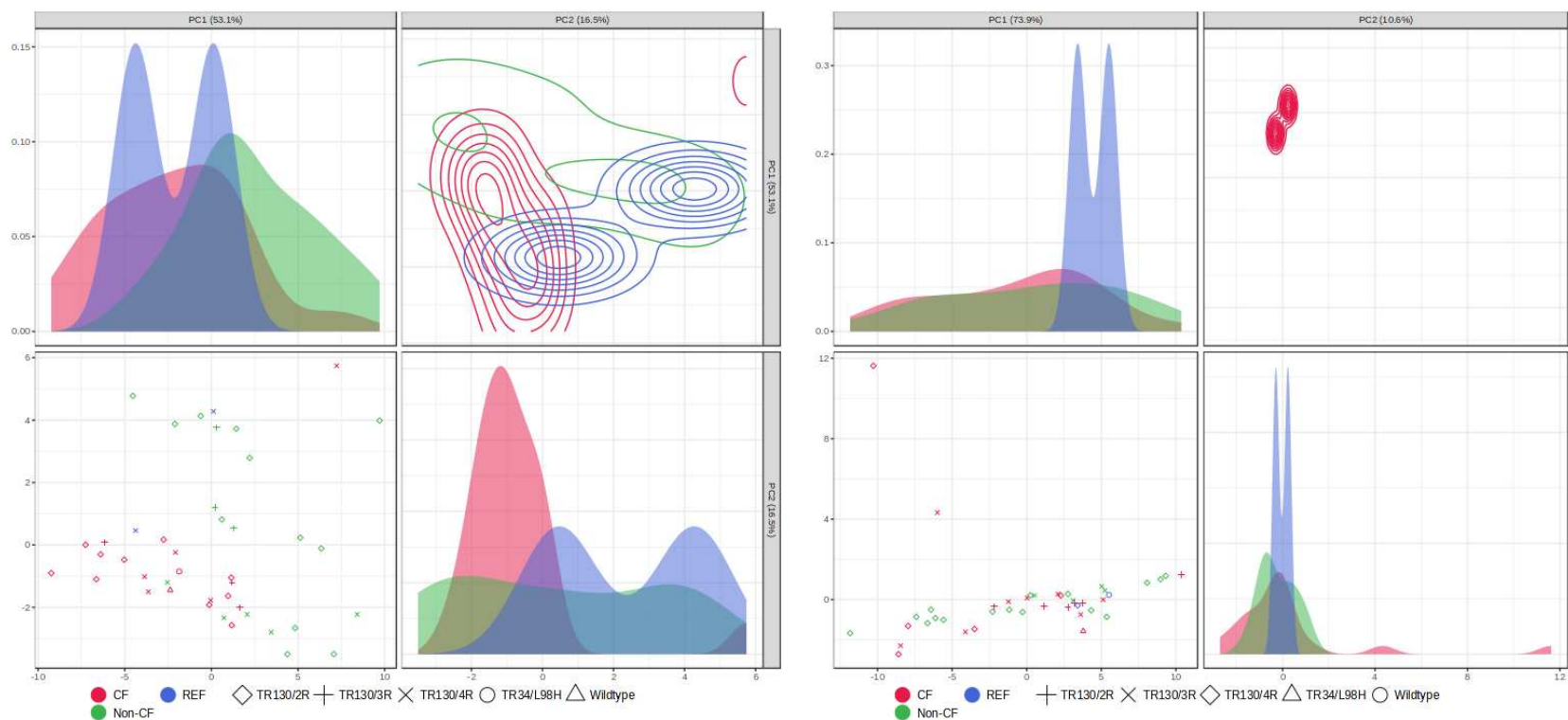


B



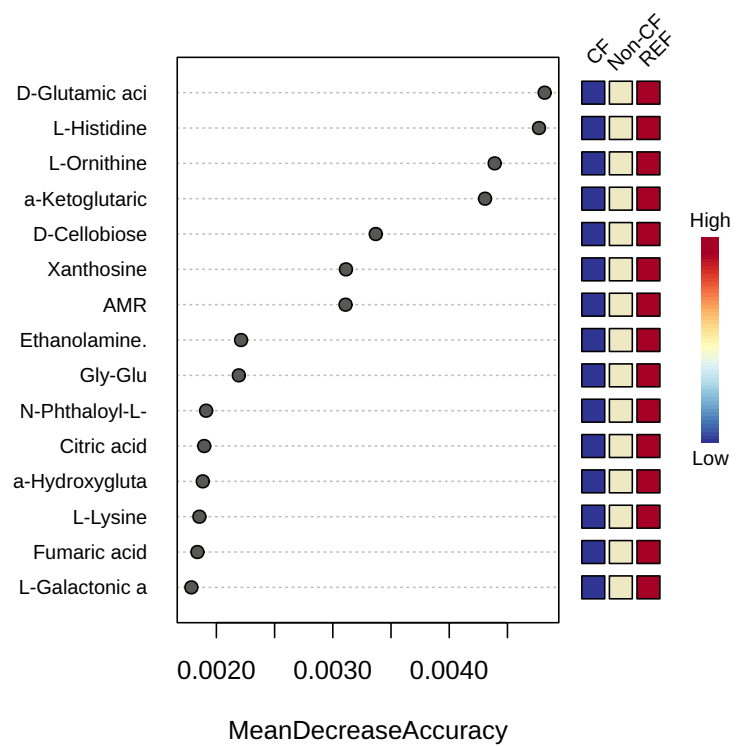
C): Carbon and Nitrogen D): Osmolytes and pH) plots illustrating the principal component analysis (PCA) results for various experimental conditions, highlighting the differences between cystic fibrosis (CF) and non-cystic fibrosis (Non-CF) isolates. **Top Left Plot:** Displays the distribution of the first principal component (PC1) across different groups. The peaks represent the density of samples within the respective categories: CF (green), REF (reference strain, blue), and Non-CF (red). The c D indicate the distribution patterns, allowing for visual comparisons of the groups. **Top Right Plot:** Shows the contour plot of the second principal component (PC2), illustrating the relationships between the groups. The contour lines indicate areas of concentration for each category, providing insights into the overlaps and distinctions in the data. **Bottom Left Plot:** Presents a scatter plot of the first two principal components (PC1 vs. PC2). Each point represents an individual sample, with colours denoting group categories: CF (green), Non-CF (red), TR130/2R (purple), TR130/3R (orange), TR134/0R (light blue), TR₃₄/L8H (pink), and Wildtype (blue). This visualization helps in assessing the clustering and separation of samples based on their principal component scores. **Bottom Right Plot:** Illustrates the density of PC2 similar to the top left, focusing on the distribution of samples across the second principal component. The overlapping areas among the groups indicate potential shared characteristics or responses.

C

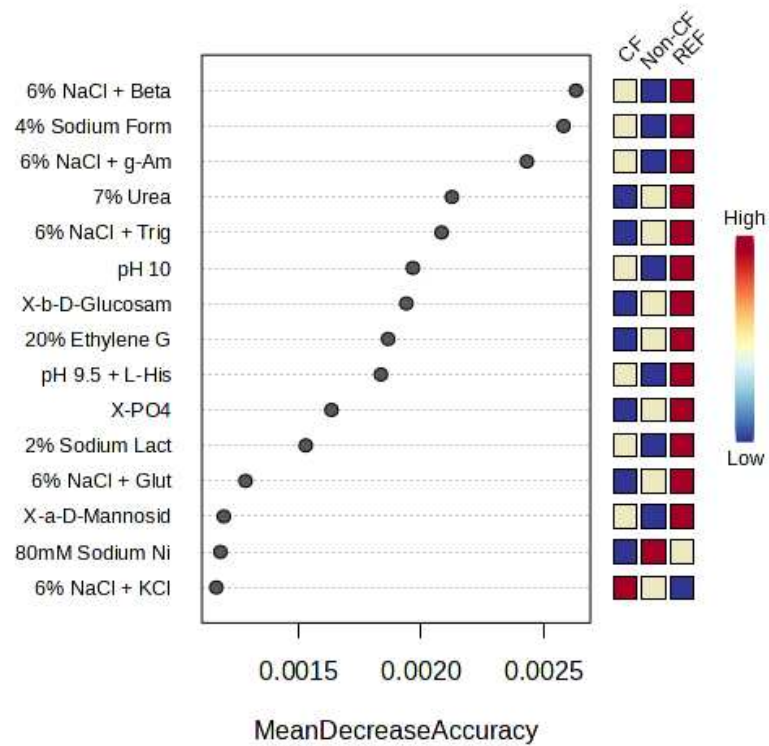


E): Carbon and Nitrogen , F): Osmolytes and pH) VIP score plot of the top 15 metabolites that differed in resistant vs susceptible isolates. Data were normalised to the control well, log-transformed and Pareto scaling (mean-centred and divided by the square root of standard deviation of each variable)

E



F



The relationship between differential metabolites and their metabolic pathways was assessed by conducting a metabolite set enrichment analysis (Figure 19). This analysis revealed that among the five metabolic pathways undergoing enrichment analysis, the metabolism of beta-alanine (L-aspartic acid, L-histidine) showed significant differences in patients with cystic fibrosis (CF) infected by *A. fumigatus* (Figure 19). A heatmap based on the p-value scores of the significant metabolites from the CF and non-CF groups infected with *A. fumigatus* visualizes the relationships and distinctions in the levels of carbon, nitrogen, osmotic, and pH metabolites among each sample, as shown in Figures 17A and 17B.

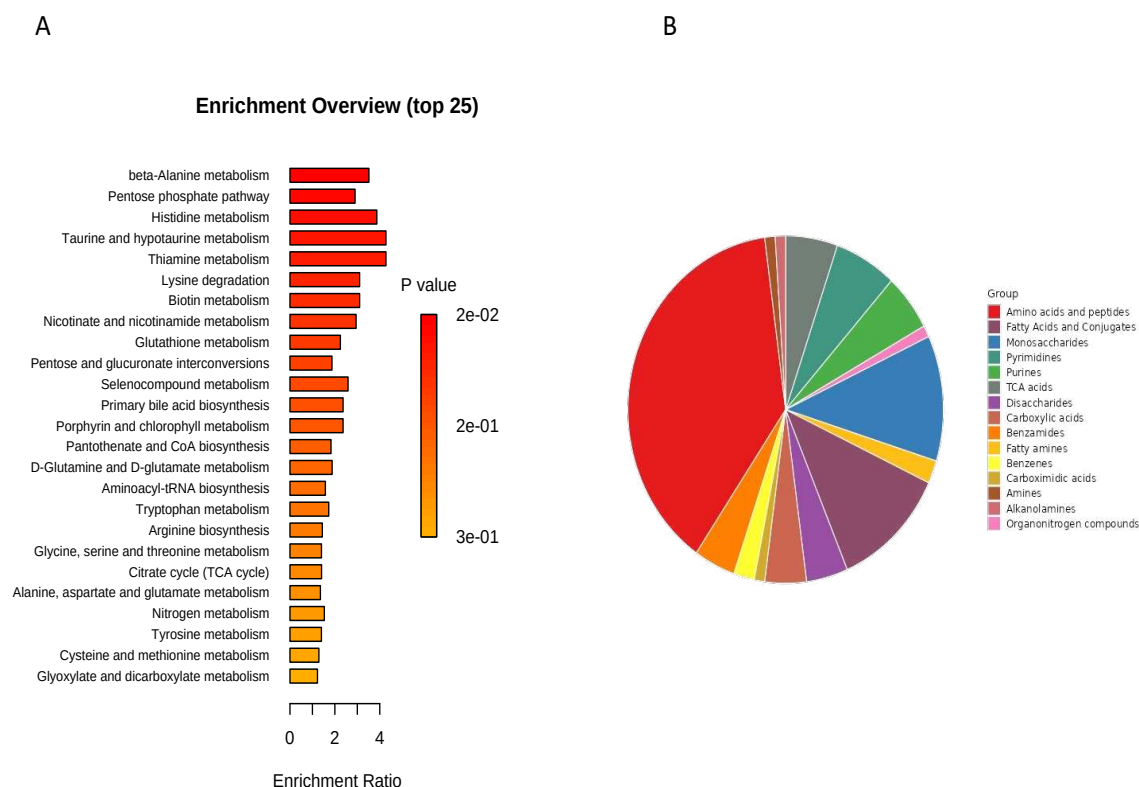


Figure 19: A) Bar chart of 379 enrichment and Pathway analysis reveals the beta Alanine pathway to be one of the most altered pathways. (B) Pie diagrams showing Pathway annotation category for 379 conditions. 10 out of 187 carbon and nitrogen sources tested were used by all isolates. Increase adaptation of *A. fumigatus* isolates in the presence of osmolytes

and pH. All *A. fumigatus* isolates tested had similar phenotypic traits and were highly resistance to osmotic stressors except in sodium salts, urea, and ethylene glycol. 9% out of the 379 conditions tested resulted in no growth.

3.3.2 Growth Media and Conditions for OmniLog validation

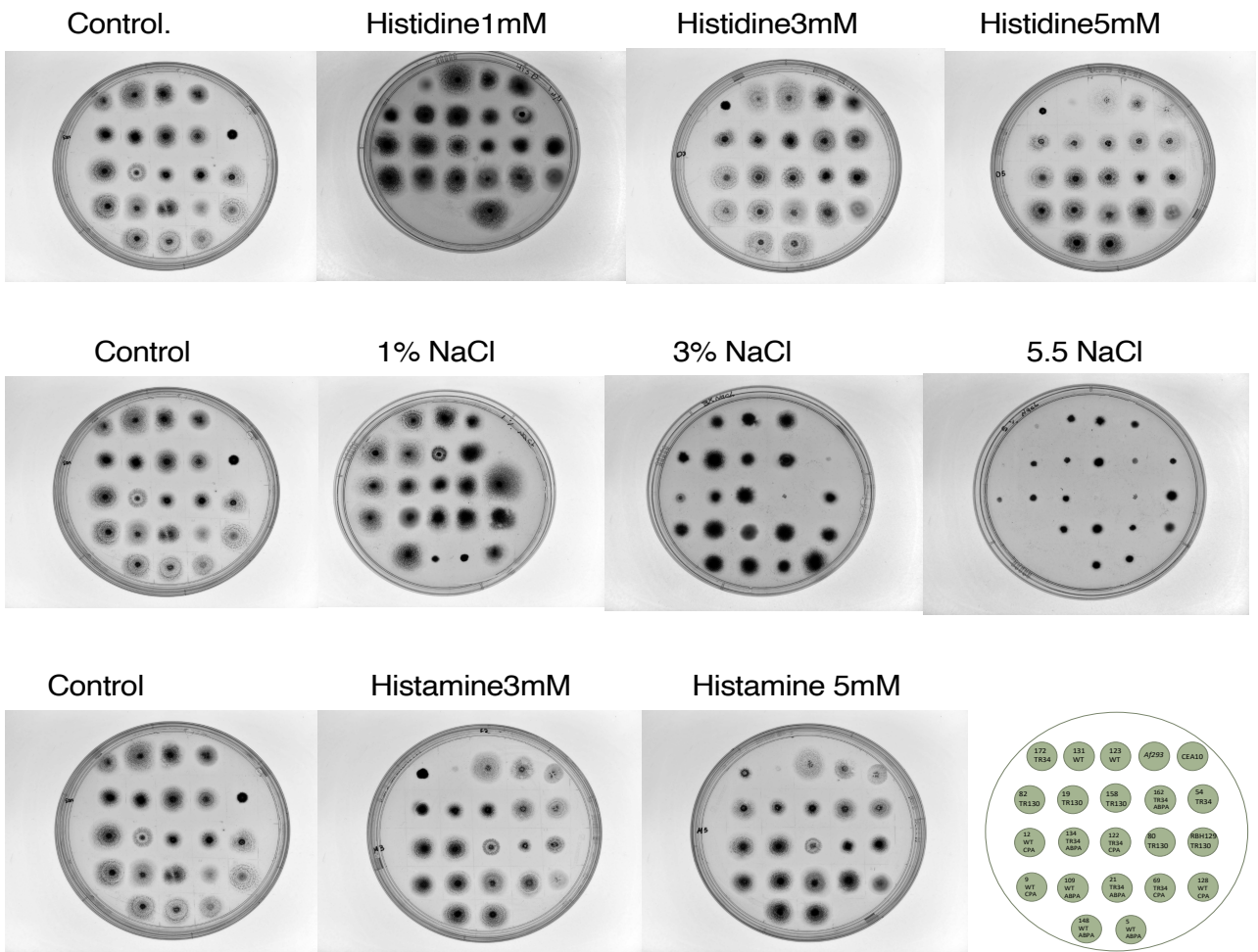
To establish the accuracy of the OmniLog system, twenty-two representative isolates were cultured on minimal media supplemented with three concentrations of histamine: 1 mM, 3 mM, and 5 mM. In parallel, minimal media supplemented with 1%, 3%, and 5.5% NaCl were also tested. These concentrations were evaluated to determine their relevance to the PM plates, as the exact concentrations were not known or provided by Biolog. The data were standardized and scaled for comparison purposes.

Supplementation with histidine and histamine resulted in increased growth across all isolates. Notably, histamine led to a significant increase in growth for non-CF isolates in both the OmniLog and radial growth assays. In the radial growth assays, 1 mM of histidine and 3 mM of histamine were identified as the concentrations most closely correlating with those in the PM plate. The correlation of fungal growth in histamine and histidine, as identified by the OmniLog system, was compared between liquid culture (OmniLog) and traditional solid agar culture. This comparison revealed a strong correlation in growth rates for both assays, as illustrated in Figure 20B.

In the case of NaCl, isolate-specific differences were observed, but no significant differences were noted between groups in general, whether in solid agar cultures or OmniLog assays, as shown in Figure 20. When comparing the two assays for the correlation of fungal growth in response to NaCl, growth was significantly higher on solid agar compared to OmniLog liquid media, presented in Figure 20C. This difference in growth may come from the preference of *Aspergillus* for growth in liquid culture, which may increase sensitivity to variations in oxygen

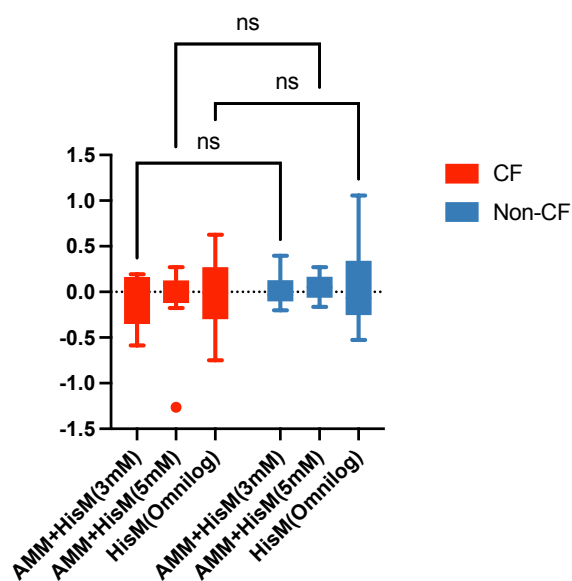
supply, nutrient contact, and shear forces, all of which can impact both metabolism and morphology.

A



B

Growth rate correlation between agar-based and OmniLog based approaches



C

Growth rate correlation between agar-based and OmniLog based approaches

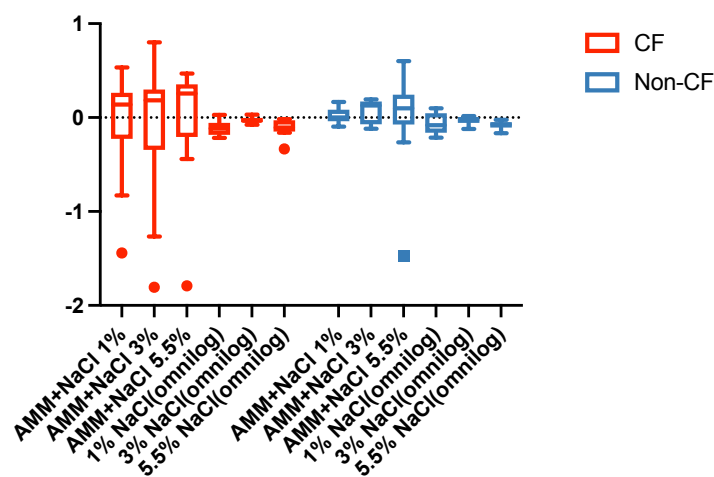


Figure 20: A) Radial growth assays were conducted to validate the Biolog system. Twenty-two selected *A. fumigatus* isolates were cultivated on Aspergillus minimal medium (AMM) agar plates supplemented with three concentrations of histamine: 1 mM, 3 mM, and 5 mM, respectively, for 48 hours at 37 °C. AMM supplemented with 1%, 3%, and 5.5% NaCl was also tested for 48 hours at 37 °C. B) Boxplot of the growth rate correlation between agar-based solid media and OmniLog-based liquid media. These two culture methods showed a strong correlation in growth rates across both assays, with no significant difference in growth rate (the mean was cantered and divided by the standard deviation for normalization; then an unpaired two-way ANOVA test was used). C) Boxplot of the growth rate correlation between agar-based solid media and OmniLog-based liquid media. The correlation of fungal growth in response to

NaCl indicated that growth was significantly higher on solid agar compared to the OmniLog liquid-based media.

3.3.3 Discussion

3.3.3.1 The Biolog system application for large-scale screening of phenotypic traits of azole resistant *A. fumigatus* isolates

The Biolog system has emerged as a vital tool in the characterization of microorganisms, particularly in elucidating the metabolic activities of *Aspergillus fumigatus* under varying conditions. In Chapter 3.2, we utilized a semi-high throughput approach to systematically examine the phenotypic characteristics of *A. fumigatus* isolates. This methodology enabled a robust functional interpretation of data derived from whole genome sequencing (WGS) (Blanco et al., 2018b; Singh, 2009a, 2009b). This standardized approach offers profound insights into the metabolic capabilities of the *A. fumigatus*, which are crucial for understanding its ecological and clinical behaviour.

The analysis categorized 42 *A. fumigatus* isolates into two primary groups: azole-resistant and susceptible. Within these categories, seven isolates were identified as outliers, exhibiting differential growth patterns that distinguished them from the majority of their respective groups. This finding corroborates earlier studies demonstrating significant genetic diversity among *A. fumigatus* isolates (Yourman, Jeffers and Dean, 2001; Launay and Dunbar, 2015; Kowalski et al., 2016b; Ries et al., 2019b). The observed heterogeneity suggests that variations in growth phenotypes may be indicative of underlying genetic variations, which may have critical implications for antifungal resistance. Such diversity underscores the necessity of tailoring therapeutic approaches based on the genetic profile of the isolates, as different strains may exhibit varying susceptibilities to antifungal agents.

Moreover, the presence of heterogeneity among clinical isolates in populations at risk implies that phylogenetic placement may not be a significant determinant of aspergillosis development

(Cunha et al., 2013; Kowalski et al., 2016b). In this chapter, we observed a notable relationship between azole susceptibility profiles and phenotypic variation among the selected *A. fumigatus* isolates. This finding is particularly significant as it hints at potential correlations between genotype diversity and phenotypic expression concerning patient disease endotypes. Previous research has established connections between the virulence and fitness of various clinically distinct *A. fumigatus* isolates, particularly under hypoxic conditions, reinforcing the relevance of the findings (Kowalski et al., 2016b).

The variability in azole susceptibility among *A. fumigatus* isolates, as highlighted in recent studies, underscores the complexity of fungal pathogens in clinical settings. For instance, dos (dos Santos et al., 2020b) provided compelling evidence of genomic and phenotypic heterogeneity among clinical isolates, identifying those differences in drug susceptibility correlate with specific genetic determinants, particularly in genes like *cyp51A*, which encodes for a key enzyme involved in azole resistance. This molecular insight suggests that the variations in antifungal susceptibility may not only stem from environmental factors but also reflect underlying genetic adaptations of the isolates themselves.

Moreover, the phenotypic variations observed—such as differences in growth rates, morphological traits, and virulence factors—further complicate the relationship between genotype and phenotype. (El-Kamand et al., 2022)(El-Kamand et al., 2022) noted that isolates from cases of proven invasive aspergillosis (IA) exhibited diverse traits, yet the differences did not always align with their clinical outcomes. This emphasizes the need for a more nuanced understanding of how these phenotypic traits translate to clinical virulence, especially in immunocompromised patients where the risk of IA is heightened.

However, it is essential to acknowledge that the correlation between phenotypes and phylogeny was not fully established in this analysis, primarily due to the limitations associated with the small sample size. This constraint may have hindered our ability to accurately detect and

interpret the intricate dynamics between genetic diversity and phenotypic traits. Therefore, future research endeavours should focus on expanding the population size to enhance the robustness of these correlations and allow for a more comprehensive assessment of growth rates and fitness among *A. fumigatus* isolates.

Future research with a larger population size is necessary to further elucidate the relationship between phenotypes and phylogeny and to assess the growth rates and fitness of the *A. fumigatus* population. Additionally, other influencing factors must be considered in future studies to deepen our understanding of these correlations. Additionally, other influencing factors, such as environmental conditions, nutrient availability, and host interactions, must be integrated into future studies. Understanding these multifaceted interactions will deepen our knowledge of the ecological and pathogenic potential of *A. fumigatus*, which is particularly critical in the context of increasing rates of azole resistance in clinical settings.

The distinct growth phenotypes exhibited by the *A. fumigatus* isolates Af293 and CEA10 under varying carbon and nitrogen sources provide crucial insights into the metabolic versatility and ecological adaptability of this opportunistic pathogen. Af293 demonstrates a pronounced growth advantage in the presence of amino acids and carbohydrates, suggesting that specific nutrient conditions significantly influence its metabolic activities. In contrast, both isolates show comparable resilience to osmotic and pH stress, aligning with findings from prior studies, which have established the importance of these stress responses in fungal virulence and adaptation (Beattie et al., 2017; Cunha et al., 2013). While previous research primarily focused on virulence assessments using murine models, this chapter emphasizes the relevance of nutrient availability in shaping the growth dynamics of these fungi, thereby offering a nuanced perspective on their potential virulence in natural environments.

The azole-resistant isolates, in particular, exhibited a remarkable capacity to utilize a diverse range of carbon sources, including glucose, fructose, galactose, and various organic acids such as citric and oxalic acids. The ability to thrive on both simple sugars and complex polysaccharides underscores the metabolic plasticity of *A. fumigatus*, a characteristic that is critical for its survival in fluctuating environments (Ries et al., 2019b). Notably, the robust growth observed on sucrose and trehalose implies the presence of specific extracellular enzymes, such as sucrases and trehalases, which facilitate the degradation of these carbohydrates (Beattie et al., 2017). This metabolic versatility not only supports the fungus' growth but also has implications for its pathogenic potential, highlighting the need to consider metabolic characteristics in understanding fungal infections.

Additionally, we observed that 24 isolates, including the reference strain Af293, demonstrated enhanced utilization of various nitrogen sources, such as ammonium, nitrate, L-histidine, and other amino acids. This finding suggests a potential positive correlation between nitrogen utilization capabilities and the environmental fitness of *A. fumigatus*. Disruptions in nitrogen metabolism could lead to decreased virulence, further complicating the pathogen's ability to establish infections. This intricate relationship between metabolic processes and virulence is paramount, as issues related to energy production, the synthesis of virulence factors, immune evasion, and cellular signalling are all affected by nitrogen availability (Krappmann et al., 2004b). Understanding these pathways can provide valuable insights for developing therapeutic strategies aimed at disrupting the metabolic processes that underpin *A. fumigatus*' pathogenicity.

Moreover, the results from our study indicate that *A. fumigatus* is expected to undergo nitrogen deprivation upon infection, as reflected by the increased expression of genes involved in amino acid transport and biosynthesis, which significantly contribute to the fungus' virulence (Krappmann et al., 2004b). This emphasizes the importance of nitrogen acquisition and

metabolism during infection, as corroborated by multiple studies that highlight the significance of nitrogen sources like ammonium and nitrate for *A. fumigatus* growth (Krappmann and Braus, 2005b; McDonagh et al., 2008; Cunha et al., 2013).

Our investigation into osmotic stress revealed that azole-resistant isolates harbouring TR₁₃₀-*cyp51A* polymorphisms were capable of growing in the presence of elevated concentrations of sodium benzoate (50 mM), while those with the TR₃₄/L98H polymorphism exhibited limited growth (up to 20 mM). Sodium benzoate is recognized for its antimicrobial properties, yet our findings imply that certain azole-resistant strains possess adaptations that confer advantages in high osmotic conditions (Zhelifonova et al., 2017a). Furthermore, the low pH at which sodium benzoate operates effectively (2.5–4.0) may challenge many microbes, but *A. fumigatus* can thrive within this range. The mechanisms underlying the efficacy of such preservatives encompass membrane disruption, inhibition of metabolic reactions, and the generation of reactive oxygen species, all of which contribute to cellular stress responses (P. Li et al., 2022a; Maj et al., 2024).

The implications of these findings are critical as they suggest that azole-resistant isolates may gain a competitive edge in environments characterized by higher osmotic pressures and salinity, particularly in light of climate change projections that could elevate ocean salinity levels. This ecological adaptability may enhance the survival and spread of resistant strains in marine ecosystems, thereby potentially altering community dynamics and impacting the prevalence of azole-resistant fungi in particular habitats.

Furthermore, when discussing the buffering of pH in Omnilog media, it is critical to note that the media is designed to maintain a stable environment for microbial growth. The buffering capacity is primarily achieved through the inclusion of specific salts and buffering agents that resist changes in pH as metabolic products accumulate during growth. Understanding the link between nitrogen sources and extracellular pH is particularly relevant, as nitrogen metabolism

can influence the acidity or alkalinity of the media. For instance, certain nitrogen sources, such as ammonium, can lower pH, while others, like nitrate, may lead to a more neutral or alkaline environment, depending on the metabolic pathways activated. This interplay is vital because pH can significantly affect enzyme activity, nutrient solubility, and ultimately, microbial growth and metabolism. Therefore, the choice of nitrogen source in experimental designs not only affects growth metrics but also the physiological state of the organism, which may contribute to variations in phenotypic expression observed in *A. fumigatus* isolates.

The assessment of pH tolerance using PM-10 plates revealed that azole-resistant isolates harbouring TR₁₃₀ exhibited greater tolerance to pH variation compared to their susceptible counterparts. This suggests that azole resistance could confer a selective advantage in environments with fluctuating pH levels, as high pH can induce significant stress on fungal cells by limiting nutrient acquisition and access to essential metals like iron and copper (Davis, 2009; Fernandes et al., 2017). The ability to regulate pH effectively may thus represent a critical factor underlying the differences in virulence among *A. fumigatus* isolates. Further research is warranted to validate these hypotheses and explore their therapeutic implications, as understanding the mechanisms of pH regulation in *A. fumigatus* could lead to innovative strategies for managing infections caused by various isolates (Tardi-Ovadia et al., 2017).

Finally, KEGG pathway analysis revealed that 25 pathways were differentially regulated in response to carbon and nitrogen sources among azole-resistant isolates, with seven pathways significantly enriched for sphingolipid and selenocompound metabolism. Notably, sphingolipid synthesis-related proteins, such as OrmA, have been implicated in azole susceptibility and virulence (Zhai et al., 2019). However, the potential interactions between sphingolipids and ergosterol biosynthesis—a primary target of azole drugs—remain underexplored and warrant further investigation.

Collectively, using the large-scale screening of phenotypic traits of *A. fumigatus* isolates, it was found MIC *azole resistant isolates and isolates harbouring the* TR₃₄/L98H polymorphism have a broader spectrum of nitrogen sources and a greater tolerance towards osmotic pressure (sodium benzoate) and alkali stress. This suggests a potential link between antifungal resistance and environmental stress tolerance. Specifically, azole-resistant fungal isolates, such as those harbouring the TR₁₃₀-*cyp51A* polymorphism, may possess adaptive traits that not only confer resistance to antifungal agents but also enhance their ability to survive in fluctuating and stressful environmental conditions. For instance, the capacity to thrive in environments with elevated osmotic pressures or extreme pH levels indicates that these isolates can effectively manage cellular stress

3.3.4 General limitations of the OmniLog technology

While the OmniLog Phenotype Microarray represents a valuable advancement in studying the metabolism of microorganisms, particularly fungi like *A. fumigatus*, it is essential to acknowledge several limitations that can impact the efficacy and interpretability of the data generated.

One significant limitation is the software's reliance on the 8×12-well plate configuration for Phenotype Microarray (PM) measurements. This design restricts the ability to perform comprehensive visual comparisons of kinetic curves, especially when analyzing more than two curves simultaneously. The PM software employs a parametric analysis approach that computes parameters describing curve kinetics but lacks sophisticated modeling or curve-fitting methods. Moreover, it does not provide confidence intervals (CIs), which are crucial for assessing the reliability of the data. This limitation means that while the assay offers valuable insights into the metabolic capabilities of *A. fumigatus*, it fails to illuminate the underlying genetic mechanisms governing these processes or to establish correlations between them.

Consequently, the current tools available do not fully facilitate an in-depth analysis of the data, hindering our understanding of the complex interactions between genotype and phenotype in *A. fumigatus* isolates.

Another critical limitation of the PM method lies in the preparation of a representative, homogeneous, and viable inoculum when working with fungi. The quality and quantity of the inoculum can be significantly influenced by external conditions, such as temperature, humidity, and nutrient availability. Fungi, including *A. fumigatus*, exhibit considerable phenotypic flexibility in response to their immediate growing environment, leading to variations in growth rates and nutrient requirements. Additionally, the choice of substrate for fungal growth can profoundly impact the characteristics of the inoculum produced, which in turn can affect the metabolic assessments performed using the OmniLog system.

These limitations underscore the urgent need for enhancements in both the software capabilities and the methodologies employed for inoculum preparation. Improving the software to incorporate advanced modeling techniques and visualization tools would enable a more comprehensive analysis of metabolic data, thereby facilitating a better understanding of the relationships between metabolic activities and genetic factors in *A. fumigatus*. Furthermore, developing standardized protocols for inoculum preparation would ensure consistency in the results, thereby increasing the reliability of metabolic assessments.

Addressing these challenges is crucial for maximizing the potential of the OmniLog Phenotype Microarray in fungal research, particularly in the context of understanding the ecological and pathogenic behavior of *A. fumigatus*. Enhancing the capabilities of the OmniLog system will not only improve the accuracy of metabolic assessments but will also facilitate a deeper exploration of the intricate interplay between genetic mechanisms and metabolic functions, ultimately advancing our knowledge of antifungal resistance and therapeutic strategies.

3.3.5 Metabolic Traits Suggesting Attribution in Differentiating Patients with Cystic Fibrosis from Patients with Other Respiratory Diseases in Azole-Resistant *A. fumigatus* Isolates.

As *A. fumigatus* establishes infection within the CF lung environment, it undergoes significant metabolic adaptations to thrive. Studies have shown that while the lung is rich in nutrients, *A. fumigatus* must navigate a complex environment where not all available resources are easily accessible (Dickson, Robert; Erb-Downward, Jonhn; Martines, Fernando; Gaby, 2017; Lachnit et al., 2019). This necessitates a reprogramming of its metabolic pathways to convert these nutrients into forms necessary for growth (Dagenais & Keller, 2009b; Hartmann et al., 2011). Recent findings indicate that CF isolates of *A. fumigatus* exhibit a notable reduction in mean growth rates compared to non-CF isolates, likely due to selective pressures in the CF lung, which may drive microevolution and phenotypic regulation of virulence factors (Breuer et al., 2019; Reece et al., 2019). To ensure that these differences are not confounded by growth efficiency variations, the data has been standardized against basal growth rates calculated for each isolate.

Phenotypic analyses have highlighted that persistent CF isolates demonstrate enhanced fitness related to nitrogen sources and osmotic stress, with TR₁₃₀ potentially serving as a differentiating factor between CF and non-CF groups. These adaptations suggest that specific *A. fumigatus* genotypes may persistently infect CF patients, acquiring mutations that promote survival in the unique lung environment (Huang et al., 2019; Verweij, Chowdhary, et al., 2016b; J. Zhang et al., 2021a). Moreover, the high-osmolarity glycerol (HOG) pathway has emerged as a promising target for antifungal therapies, underscoring its potential relevance in treating CF patients infected with *A. fumigatus* (Ross et al., 2021).

As we consider the contemporary CF population, the advent of CFTR modulators has significantly altered the landscape of CF management and patient outcomes. These modulators,

such as Ivacaftor and the combination therapies like elexacaftor/tezacaftor/ivacaftor, have demonstrated substantial improvements in lung function and quality of life (Heijerman et al., 2019; Middleton et al., 2019). By restoring some functionality of the CFTR protein, these therapies enhance mucociliary clearance, which may reduce the burden of both bacterial and fungal infections, including *A. fumigatus* (Frost et al., 2019; Heltshe et al., 2015).

However, while these improvements are promising, they also introduce complexities in the management of fungal infections. As life expectancy increases among CF patients, there is a concern regarding the potential for prolonged exposure to inhaled antibiotics, which may lead to an increased risk of fungal colonization and infection in individuals with structural lung changes (Chesshyre et al., 2025). Furthermore, there is a need for ongoing research to understand how the introduction of CFTR modulators affects the prevalence and clinical implications of *A. fumigatus* colonization and infection, particularly in pediatric populations who are often underrepresented in these studies (Bercusson et al., 2021)

In conclusion, the metabolic traits of *A. fumigatus* provide essential insights into its pathogenicity in CF patients, highlighting significant differences from those with other respiratory diseases. The impact of CFTR modulators on fungal infections remains an area ripe for exploration, necessitating longitudinal studies to fully elucidate their role in modifying the disease landscape in cystic fibrosis.

3.3.6 Growth Dynamics of Non-CF Isolates in Response to Histidine, Histamine, and Sodium Chloride: A Comparative Analysis of OmniLog and Solid Agar Culture Systems

The results demonstrate a significant enhancement in the growth rates of non-CF isolates when supplemented with histidine and histamine, suggesting that these compounds serve as critical growth factors. The optimal concentrations were identified as 1 mM histidine and 3 mM histamine, which aligns with previous findings that highlight the role of amino acids and biogenic amines in microbial growth (Banicod et al., 2025; Saha Turna et al., 2024). The

correlation observed between the OmniLog system and solid agar cultures further validates the reliability of the OmniLog system in measuring growth dynamics. This is supported by (Goel & Nath Mishra, 2007), who emphasized the advantages of liquid culture systems in understanding microbial physiology.

In contrast, the impact of NaCl was less straightforward. The absence of significant differences among groups suggests that the effects of NaCl are complex and dependent on the specific characteristics of each isolate. Previous studies, such as those by (Musa et al., 2018; Tiwari et al., 2024), have indicated that salt stress induces variable responses in different fungal species, influenced by their tolerance mechanisms. The finding that growth was significantly higher on solid agar compared to OmniLog media suggests that solid substrates provide unique environmental conditions that optimize nutrient availability and oxygen diffusion.

The preference of *Aspergillus sp.* for liquid culture highlights the intricate dynamics of microbial growth, where variations in oxygen supply, nutrient contact, and shear forces significantly influence metabolic pathways and morphological development (Miyazawa et al., 2022). The observed differences between the two culture methods underscore the importance of employing both approaches to achieve a comprehensive understanding of fungal physiology. Overall, this showed the importance of a multifaceted approach in assessing microbial growth responses to various substrates and environmental conditions. The significant findings regarding the enhancement of growth by histidine and histamine, along with the complex interactions with NaCl, contribute to a broader understanding of microbial ecology.

3.4 Identify genomic variations associated with phenotypes differences and identified overlap gene sets using GWAS coupled with transcriptomic analysis.

3.4.1 Preface:

Aspergillus fumigatus is an opportunistic human fungal pathogen that thrives in a wide array of environmental niches and is capable of surviving under diverse conditions. A powerful method for investigating the relationships between genetic variants and the complex traits of *A. fumigatus* involves microbial genome-wide association studies (GWAS) coupled with transcriptome analysis. Most existing research has concentrated on identifying genetic variants associated with triazole-resistant phenotypes, focusing on mutations linked to triazole resistance and their underlying molecular mechanisms. In this chapter, we conducted a GWAS that included 246,253 high-confidence SNPs across 42 *A. fumigatus* isolates. The analysis was performed under both standard conditions and two experimental conditions identified as important adaptive traits through PM analysis (Chapter 3.2): specifically, histamine and sodium chloride (NaCl). For the histamine condition, we identified a total of 140 SNPs that were significantly associated with the growth response. In contrast, under the NaCl condition, 22 SNPs were found to be significantly associated.

Additionally, we explored the transcriptomic responses of *A. fumigatus* to these conditions. Under histamine treatment, we identified 8,274 differentially expressed genes (DEGs), of which 48 were significantly upregulated and 9 downregulated. Among these, nineteen genes passed the filter threshold of 0.5 counts per million (CPM). For the NaCl condition, a total of 9,741 DEGs were identified, with 1,175 significantly upregulated and 1,580 downregulated; twenty-one of these genes met the stricter filter threshold of 0.05 CPM.

Overall, our findings highlight the complex genetic and transcriptional responses of *A. fumigatus* to varying environmental conditions, providing valuable insights into the adaptive traits of this important pathogen.

Genome-Wide Association Study of Histamine and NaCl Phenotypes Coupled with Transcriptome Results

In the quest to understand the genetic underpinnings of histamine metabolism in *Aspergillus fumigatus*, a comprehensive analysis was conducted utilizing whole-genome sequencing (WGS) and phenotype microarray results. From 42 distinct *A. fumigatus* isolates, a robust dataset was generated, comprising 246,253 high-quality single nucleotide polymorphisms (SNPs). To avoid the risk of false positives, a false discovery rate (FDR) threshold of 0.01 was applied, leading to the identification of 140 significant SNPs through genome-wide association studies (GWAS), outlined in Table 9.

The GWAS findings revealed a nuanced landscape of genetic associations. Significant loci were discerned through various scoring methods, with the terminal score yielding 69 loci at a significance threshold of 0.27 and 102 loci at a threshold of 0.12. Notably, the simultaneous score of association did not identify any significant loci. The SNP density plots generated during this analysis exhibited no systematic inflation across the samples, reinforcing the integrity of the results. A breakdown of the significant SNPs showed that 47.1% resided in intergenic regions, while 52% were located within protein-coding regions. The most notable concentration of SNPs associated with the histamine phenotype was observed on chromosome 2, encompassing 102 SNPs. The genes influenced by these SNPs played pivotal roles in central metabolic pathways, signalling, cell wall biosynthesis, pigment production, and the regulation of secondary metabolite synthesis, as detailed in Table 6A.

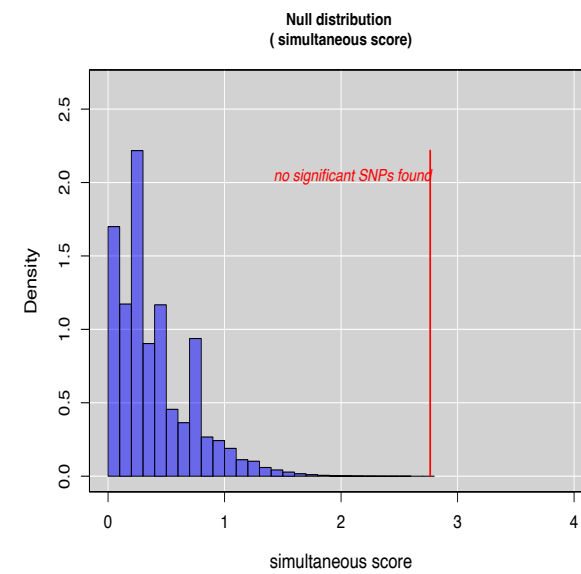
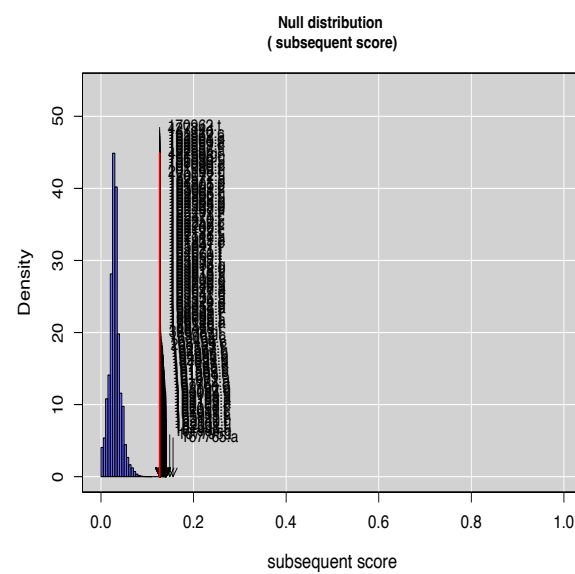
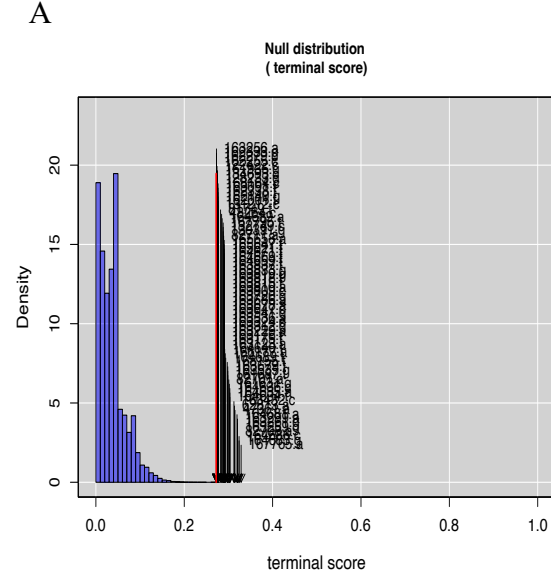
In addition to the histamine-related SNPs, a total of 22 SNPs were significantly associated with NaCl tolerance. The analysis again demonstrated the utility of the terminal score of association, identifying 14 significant loci at a threshold of 0.27 and 2 loci at 0.12, with a single locus emerging from the simultaneous score of association.

To further elucidate the connection between genomic variations and phenotypic expressions, RNA isolation and sequencing were performed. This effort yielded an impressive 56 million reads, with each sample presenting between 18.72 and 22.87 million clean reads, indicating high-quality RNA-seq data. The reads were aligned against the *A. fumigatus* genome, achieving an average unique mapping rate of 94%. This meticulous alignment facilitated the identification of 9,750 differentially expressed genes (DEGs), with nine genes exhibiting downregulation and 48 showing upregulation (as summarized in Table 11). Filtering criteria set at 0.5 counts per million (CPM) highlighted 19 genes for further analysis.

Applying DESeq2 for differential expression analysis revealed a striking 9,806 significantly differentially expressed genes ($FDR \leq 0.05$ and $\log_2FC \geq 2$). Among these, only 19 genes (7.94%) were upregulated during growth on histamine, as depicted in Figures 21 and 22. The remaining DEGs, totalling 9,731, comprised 1,175 significantly upregulated and 1,580 downregulated genes. With a further filter of 0.05 CPM, 21 genes were identified for deeper exploration (Figures 23 and 24).

Figure 21: SNP density plots derived from Genome-Wide Association Studies (GWAS) results are presented for three distinct tests of association: terminal, subsequent, and simultaneous. The analysis focuses on the relationships between genetic variations and phenotypes related to histamine and sodium chloride (NaCl) tolerance. **Panel A** illustrates the SNP density plots for histamine, demonstrating the distribution of single nucleotide polymorphisms across the genome. The left section highlights the raw data distribution, which shows a pronounced skew with a significant majority of SNPs clustered near zero, indicating a high density of associations in specific genomic regions. The middle section presents the transformed data, where the skewness is reduced, facilitating a clearer visualization of the underlying genetic architecture associated with histamine tolerance. The right section reveals the residuals from the model, displaying the differences between observed phenotypic responses and predicted values. The red vertical line in this panel denotes the mean of the residuals, serving as a reference point for evaluating the model's predictive accuracy. **Panel B** focuses on the NaCl tolerance phenotypes, with a similar structure to the previous panel. The left section shows the raw SNP distribution, providing insights into the genetic factors influencing NaCl tolerance. The middle section illustrates the transformed data, highlighting key areas of genetic variation that correlate with phenotypic responses. Finally, the right section again displays the residuals, offering a perspective on how well the model captures the variability in NaCl tolerance, marked by the red vertical line indicating the mean of the residuals.

A



B

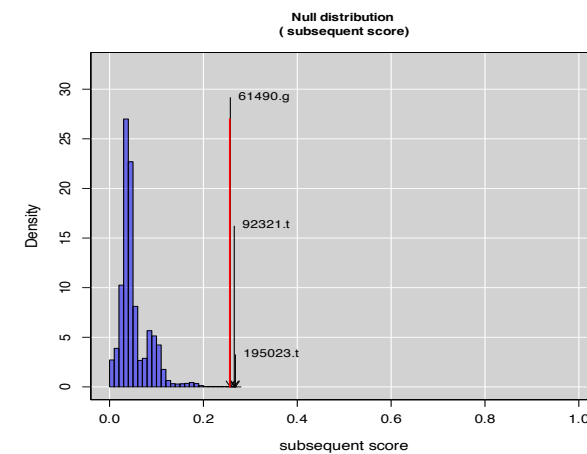
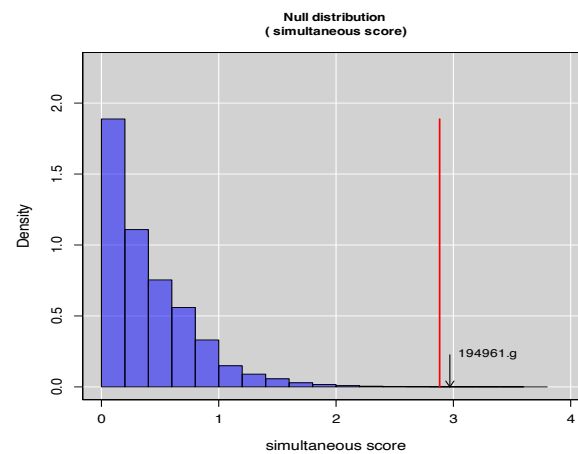
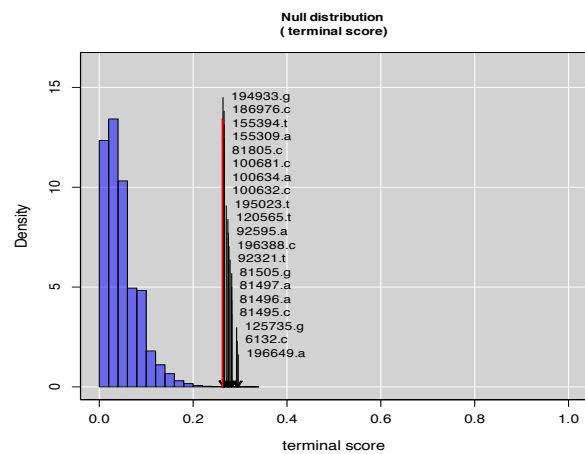


Table 9: Summary of GWAS Analysis Identifying Loci Associated with Histamine Tolerance Phenotype. This table presents a comprehensive overview of 140 identified loci linked to histamine tolerance across 42 *A. fumigatus* isolates. Each entry details the chromosome (CHR), specific genomic positions, associated p-values, amino acid changes resulting from identified single nucleotide polymorphisms (SNPs), gene IDs, and the type of genomic position (CDS, intronic, or intergenic). The analysis underlines significant genetic variations that may contribute to the phenotypic expression of histamine tolerance, providing a foundation for further research into the underlying mechanisms of tolerance in this species. For a detailed description of the implicated genes, refer to Table 6A.

Abbreviations: CHR - Chromosome; SNP - Single Nucleotide Polymorphism; p-value - Analytical Permutation p-values; CDS - Coding Sequence; NA - Not Applicable.

CHR	Positions	p-value	Amino acid change	Gene ID	Position type
III	3182777	0.00E+00	Alanine- GCG to GCA	AFUA_3G12120	CDS
II	163254	0.00E+00	Leucine-CTC to CTT	AFUA_2G00730	CDS
II	1135001	4.08E-07	Arginine- AGG to AGA	AFUA_2G04140	CDS
II	1184783	0.00E+00	Valine (GTA) to Isoleucine (ATA)	AFUA_2G04290	CDS
II	1194088	0.00E+00	NA	AFUA_2G04330	intergenic
II	1194839	0.00E+00	NA	AFUA_2G04330	intergenic
II	1194996	0.00E+00	NA	AFUA_2G04330	intergenic

II	1207904	0	Arginine-CGG to CGC	AFUA_2G04390	CDS
II	1215468	0.00E+00	Glycine (GGG) to Arginine (AGG)	AFUA_2G04420	CDS
II	1216821	0	NA	AFUA_2G04420	CDS
II	1217544	0.00E+00	NA	AFUA_2G04840	CDS
II	1244312	0.00E+00	NA	AFUA_2G04535	intergenic
II	1250469	0.00E+00	Histidine- CAC to CAT	AFUA_2G04560	CDS
II	1282164	0.00E+00	NA	AFUA_2G04690	intergenic
II	1293950	0.00E+00	NA	AFUA_2G04720	intron
II	1296789	0.00E+00	Isoleucine-ATC to ATT	AFUA_2G04740	CDS
II	1297569	0.00E+00	NA	AFUA_2G04740	intergenic
II	1299550	0.00E+00	Glutamine(CAG) to Proline (CCG)	AFUA_2G04750	CDS
II	1302059	0.00E+00	Arginine (CGT) to Histidine (CAT)	AFUA_2G04760	CDS
II	1309077	0.00E+00	NA	AFUA_2G04760	intergenic
II	1309179	0.00E+00	NA	AFUA_2G04760	intergenic
II	1310065	0.00E+00	Alanine-GCA to GCA	AFUA_2G04770	CDS
II	1311243	0.00E+00	Proline- CCG to CCA	AFUA_2G04770	CDS
II	1312634	0.00E+00	Glutamate (AGG) to Alanine (GCA)	AFUA_2G04770	CDS
II	1318106	0.00E+00	Threonine (ACC) to Serine (TCC)	AFUA_2G04790	CDS
II	1321513	0.00E+00	Proline (CCG) to Proline (CCC)	AFUA_2G04790	CDS

II	1321898	0.00E+00	NA	AFUA_2G04790	intergenic
II	1322487	0.00E+00	NA	AFUA_2G04790	intergenic
II	1322500	0.00E+00	NA	AFUA_2G04790	intergenic
II	1329865	0.00E+00	Arginine (AGA) to Methionine (ATG)	AFUA_2G04840	CDS
II	1330683	0.00E+00	NA	AFUA_2G04840	intergenic
II	1330911	0	Aspartate (GAT) to Tyrosine (TAT)	AFUA_2G04850	CDS
II	1333359	0	Leucine (CTG) to Proline (CCG)	AFUA_2G04860	CDS
II	1341167	0	NA	AFUA_2G04880	intergenic
II	1341655	0.00E+00	NA	AFUA_2G04880	intergenic
II	1355684	0.00E+00	Aspartate (GAC) to Glycine (GGC)	,AFUA_2G04920	CDS
II	1361156	0.00E+00	NA	AFUA_2G04950	intergenic
II	1361224	0.00E+00	NA	AFUA_2G04950	intergenic
II	1365917	0.00E+00	Leucine (TTA) to Leucine (CTA)	AFUA_2G04960	CDS
II	1365952	0.00E+00	Asparagine (AAC) to Serine (AGC)	AFUA_2G04960	CDS
II	1369263	0.00E+00	NA	AFUA_2G04970	intergenic
II	1374086	4.08E-07	Lysine (AAA) to Asparagine (AAC)	AFUA_2G05000	CDS
II	1374381	4.08E-07	Glycine (GGC) to Cysteine (TGC)	AFUA_2G05000	CDS
II	1375105	4.08E-07	NA	AFUA_2G05000	intergenic
II	1375181	4.08E-07	NA	AFUA_2G05000	intergenic
II	1375248	4.08E-07	Glycine-GGC to GGT	AFUA_2G05010	CDS

II	1375257	4.08E-07	Phenylalanine- TTT to TTC	AFUA_2G05010	CDS
II	1375766	0.00E+00	Histidine (CAC) to Glutamine (CAA)	,AFUA_2G05010	CDS
II	1376811	0.00E+00	NA	AFUA_2G05010	intergenic
II	1377296	0.00E+00	NA	AFUA_2G05020	introns
II	1377445	0.00E+00		AFUA_2G05020	introns
II	1377822	0.00E+00		AFUA_2G05021	introns
II	1379071	0.00E+00	Glutamate- GAA to GAG	AFUA_2G05020	CDS
II	1424503	0.00E+00	Proline- CCT to CCC	AFUA_2G05150	CDS
II	1439708	0.00E+00	Methionine (ATG) to Isoleucine (ATC)	AFUA_2G05180	CDS
II	1443944	0.00E+00	NA	AFUA_2G05190	introns
II	1452551	0.00E+00	NA	AFUA_2G05220	intergenic
II	1455654	0.00E+00	NA	AFUA_2G05230	intergenic
II	1458585	0.00E+00	Threonine- ACC to ACT	AFUA_2G05250	CDS
II	1460918	0.00E+00	NA	AFUA_2G05250	intergenic
II	1461247	0.00E+00	NA	AFUA_2G05250	intergenic
II	1469376	0	NA	AFUA_2G05280	intergenic
II	1484677	0	NA	AFUA_2G05330	intergenic
II	1484991	0	NA	AFUA_2G05330	intergenic
II	1498850	0	NA	AFUA_2G05380	CDS
II	1504873	0	Leucine- CTG to TTG	AFUA_2G05390	CDS
II	1505979	0	Serine- TCG to TCA	AFUA_2G05390	CDS

II	1506661	0	Proline (CCT) to Serine (TCT)	AFUA_2G05390	CDS
II	1519718	0	Isoleucine - ATT to ATC	AFUA_2G05420	CDS
II	1526156	0	Glycine- GGC to GGG	AFUA_2G05450	CDS
II	1529488	0	Histidine-CAC to CAT	AFUA_2G05470	CDS
II	1567065	0	Glutamine- CAA to CAG	AFUA_2G05620	CDS
II	1596098	0	NA	AFUA_2G05690	intergenic
II	1596192	0	NA	AFUA_2G05690	intergenic
II	1597265	0	NA	AFUA_2G05690	intergenic
II	1627637	0	NA	AFUA_2G05780	intergenic
II	1640019	0	NA	AFUA_2G05830	intergenic
II	1640186	0	NA	AFUA_2G05830	intergenic
II	1642028	0	NA	AFUA_2G05840	CDS
II	1651485	0	NA	AFUA_2G05860	intergenic
II	1668110	0	Aspartate- GAT to GAC	AFUA_2G05930	CDS
II	1668602	0	Arginine (CGC) to Histidine (CAC)	AFUA_2G05930	CDS
II	1670453	0	NA	AFUA_2G05940	intergenic
II	1671033	0	NA	AFUA_2G05940	intergenic
II	1672069	0	NA	AFUA_2G05950	intergenic
II	1678433	0	Glutamine (CAA) to Proline (CCA)	AFUA_2G05970	CDS

II	1680273	0	Proline-CCC to CCT	AFUA_2G05980	CDS
II	1803513	0	NA	AFUA_2G06260	CDS
II	1803614	0	NA	AFUA_2G06260	CDS
II	1803634	0	NA	AFUA_2G06260	CDS
II	1806135	0	Phenylalanine-TTC to TTT	AFUA_2G06270	CDS
II	1815526	0	Threonine (ACC) to Serine (AGC)	AFUA_2G06320	CDS
II	1817072	0	NA	AFUA_2G06320	intergenic
II	1817490	0	NA	AFUA_2G06320	intergenic
II	1821086	0	NA	AFUA_2G06330	intergenic
II	1821420	0	NA	AFUA_2G06330	intergenic
II	1821936	0	NA	AFUA_2G06330	intergenic
II	1822034	0	NA	AFUA_2G06330	intergenic
II	1822034	0	NA	AFUA_2G06330	intergenic
II	1948512	0	Aspartate (GAT) to Asparagine (AAT)	AFUA_2G07640	CDS
II	1957527	0	NA	AFUA_2G07670	intergenic
II	1960918	0	Serine -TCC to TCA	AFUA_2G07680	CDS
II	2124773	0	NA	AFUA_2G08230	CDS
II	2582254	0.00E+00	Alanine- GCC to GCT	AFUA_2G10080	CDS
II	2584827	0.00E+00	NA	AFUA_2G10090	intergenic
II	2584844	0	NA	AFUA_2G10090	CDS
II	2590619	0	Leucine – TTG to CTG	AFUA_2G10130	CDS
II	2711008	0	NA	AFUA_2G10590	intron

II	2708280	0	NA	AFUA_2G10560	intergenic
II	3322202	0.00E+00	Aspartate (GAC) to Asparagine (AAC)	AFUA_2G12940	CDS
II	3620629	4.08E-07	NA	AFUA_2G13860	intergenic
II	4819684	0.00E+00	NA	AFUA_2G18070	intergenic
I	566887	0.00E+00	NA	AFUA_1G01910	intergenic
I	584671	0.00E+00	NA	AFUA_1G01960	intergenic
I	608916	0.00E+00	Valine (GTT) to Alanine (GCT)	AFUA_1G02060	CDS
I	632419	0.00E+00	Isoleucine (ATT)to Methionine (ATG)	AFUA_1G02140	CDS
I	634219	0.00E+00	Histidine (CAC) to Glutamine (CAG)	AFUA_1G02140	CDS
I	1695919	0.00E+00	NA	AFUA_1G05860	intergenic
I	4694647	0.00E+00	Methionine (ATG) to Threonine (ACG)	AFUA_1G17200	CDS
I	4695332	0.00E+00	Phenylalanine - TTC to TTT	AFUA_1G17200	CDS
VI	97451	0.00E+00	Glycine- GGG to GGA	AFUA_7G08510	CDS
VI	628196	4.08E-07	NA	AFUA_6G03020	intergenic
VI	1648599	0	NA	AFUA_6G07310	intergenic
VI	1819970	0	Glutamine (CAG) to Histidine (CAT)	AFUA_6G07890	CDS
VI	1820504	0.00E+00	Isoleucine- ATC to ATA	AFUA_6G07890	CDS
VI	3760340	0	NA	AFUA_6G14640	intergenic
VI	3764327	0	NA	AFUA_4G14300	intergenic
VI	3769860	0	NA	AFUA_4G14310	intergenic

VI	3769876	0	NA	AFUA_4G14310	intergenic
VI	3769886	0	NA	AFUA_4G14310	intergenic
V	2385523	0.00E+00	Phenylalanine-TTC to TTT	AFUA_5G09260	CDS
V	3738790	0.00E+00	Isoleucine (ATC) to Leucine (CTC)	AFUA_5G14340	CDS
V	3738790	0.00E+00	Isoleucine (ATC) to Leucine (CTC)	AFUA_5G14340	CDS
V	3769222	0.00E+00	Cysteine (TGC) to Arginine (CGC)	AFUA_5G14530	CDS
III	678955	0	NA	AFUA_5G06820	intergenic
III	1024668	0	NA	AFUA_3G03720	intergenic
III	1046035	0	NA	AFUA_3G03760	intergenic
III	1050415	0	NA	AFUA_3G03760	intergenic
III	1059841	0	NA	AFUA_3G03760	intergenic
III	1071065	0	NA	AFUA_3G03760	intergenic

3.4.2 Growth under histamine and NaCl conditions induces expression of genes involved in nutrient acquisition, stress responses, and secondary metabolite biosynthesis

To gain insight into the functional categories associated with the differentially expressed genes (DEGs), a comprehensive gene ontology (GO) enrichment analysis was conducted, encompassing three pivotal categories: biological processes, cellular components, and molecular functions. The findings from this analysis, detailed in Table 6A and Table 11, reveal a significant upregulation of genes in the control group compared to those under histamine conditions.

The majority of these DEGs are engaged in processes related to cell communication, signal transduction, and metabolic pathways. Noteworthy genes such as AFUA_2G07670 (*akr1*), AFUA_2G05010 (*mon1*), and AFUA_2G04740 (*vps27*) were identified for their involvement in transmembrane transport, indicating a robust response to environmental stimuli. The cellular components highlighted in this analysis include membrane-bound organelles, endolysosomes, cytoplasmic structures, and the extracellular region, as presented in Table 6A and Table 10. This suggests a complex interaction between gene expression and cellular architecture, essential for the organism's adaptive mechanisms.

In terms of molecular functions, the analysis delineates critical subcategories, with binding and catalytic activities emerging as prominent themes. The transmembrane transport function is particularly vital when contrasting conditions involving pure acetate. A significant upregulation of the *sidA* gene was observed, which plays a crucial role in iron acquisition and siderophore biosynthesis, thereby supporting the organism's survival in varying nutrient environments (Table 6A).

Among the upregulated genes, *aspf2* stands out due to its regulatory role in zinc uptake, which is essential for fungal growth under alkaline conditions and in zinc-limiting media, as explored

by Amich et al. (2010b). Furthermore, *xanB* and *icsB* were both found to be transcriptionally responsive to external copper levels, highlighting their potential in metal ion metabolism. The production of metabolites linked to *xanB* is notably enhanced during copper starvation, as discussed by Lim et al. (2018). The gene *nrps5*, responsible for nonribosomal peptide synthetases, facilitates the synthesis of peptides and amino acids, underscoring its importance in fungal development (Stack et al., 2007). Additionally, AFUA_2G07670 (*akr1*) has been implicated in VCZ resistance in clinical isolates of *A. flavus*, while *pyr1* is involved in the biosynthesis of pyripyropene A, demonstrating the adaptive strategies of these organisms (Itoh et al., 2010).

Moreover, three genes—AFUA_3G00680, AFUA_3G00765, and AFUA_7G04180—were identified as crucial for copper amine oxidase activity and copper uptake, functions that are vital for maintaining metal homeostasis. This is particularly relevant as various metal ions are critical for fungal survival and pathogenesis, as shown in Tables 11 and 12. The expression of these genes was notably heightened during growth under histamine and NaCl conditions, suggesting a shared requirement for micronutrient intake across these environments. This might imply a broader influence on metabolic processes, enhancing our understanding of how these organisms adapt to fluctuating conditions.

Interestingly, approximately 20% of the 161 genes associated with transmembrane transport were linked to amino acid transport, indicating a significant metabolic engagement in nutrient acquisition. The mean normalized probe counts for all assayed genes remained strikingly similar across both conditions, suggesting a consistent metabolic profile despite the variations in environmental challenges.

Table 10: A comprehensive GWAS analysis identifies loci linked to the NaCl phenotype across 42 *A. fumigatus* isolates. The subsequent phenotype and Gene Ontology (GO) term analysis indicate a significant overrepresentation of these loci in pertinent biological processes. Identified genes were elucidated using the Motif Pattern tool provided by FungiDB (Basenko et al., 2018).

SNP.locus	Chromosome	Position	p.value	Gene ID	Position type	Description	Gene Name	GO annotation (if available)	
6130	VIII	576816	1.63E-05	AFUA_8G02220	CDS	Ortholog of <i>A. niger</i> CBS 513.88 : An11g02940, <i>Neosartorya fischeri</i> NRRL 181 : NFIA_095850, <i>Aspergillus versicolor</i> : Aspvel1_			
61000	VI	3699273	7.99E-05	AFUA_6G14520	intergenic	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_000250			
80900	IV	3708027	2.85E-05	AFUA_4G14190	CDS	Ortholog of <i>Aspergillus glaucus</i> : Aspgl1_0029891, Aspgl1_0057413, <i>Aspergillus wentii</i> : Aspwe1_0039585, <i>Aspergillus fumig</i>			

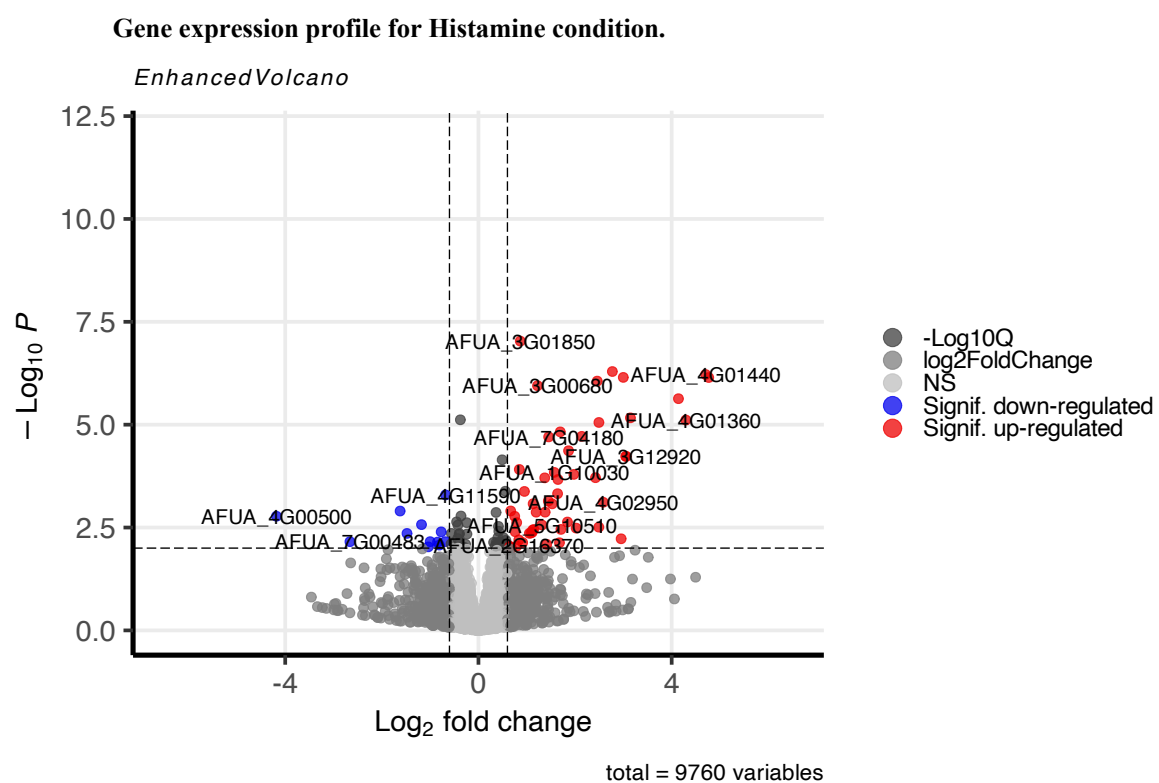
80900	IV	37081 37	2.85E -05	AFUA_ 4G1419 0	CDS	Ortholog of <i>Aspergillus glaucus</i> : Aspgl1_0029891, Aspgl1_0057413, <i>Aspergillus wentii</i> : Aspwe1_0039585, <i>Aspergillus fumig</i>			
80900	IV	37081 74	2.85E -05	AFUA_ 4G1419 0	CDS	Ortholog of <i>Aspergillus glaucus</i> : Aspgl1_0029891, Aspgl1_0057413, <i>Aspergillus wentii</i> : Aspwe1_0039585, <i>Aspergillus fumig</i>			
80900	IV	37096 97	2.85E -05	AFUA_ 4G1419 0	interg enic	Ortholog of <i>Aspergillus glaucus</i> : Aspgl1_0029891, Aspgl1_0057413, <i>Aspergillus wentii</i> : Aspwe1_0039585, <i>Aspergillus fumig</i>			
81100	IV	37654 69	8.07E -05	AFUA_ 4G1430 0	interg enic	Has domain (s) with predicted GTP binding, GTPase activity			
91500	V	36767 6	3.59E -05	AFUA_ 5G0140 0	interg enic	Has domain (s) with predicted GTP binding, GTPase activity			
91800	V	39195 0	4.65E -05	AFUA_ 5G0151 0	interg enic	Ortholog (s) have L- proline transmembrane			

						transporter activity, role in proline transport and fungal- type vacuole, plasma membrane localization			
99800	V	22358 04	7.95E -05	AFUA_ 5G0876 0	CDS	Ortholog of <i>Aspergillus</i> <i>fumigatus</i> A1163 : AFUB_056290			
99800	V	22364 94	7.95E -05	AFUA_ 5G0876 0	interg enic	Ortholog of <i>Aspergillus</i> <i>fumigatus</i> A1163 : AFUB_056291			
99900	V	22460 18	7.95E -05	AFUA_ 5G0876 0	interg enic	Ortholog of <i>Aspergillus</i> <i>fumigatus</i> A1163 : AFUB_056292			
120000	III	60561 9	5.01E -05	AFUA_ 3G0243 0	interg enic	Ortholog of <i>A. niger</i> CBS 513.88 : An02g10610, <i>Aspergillus</i> <i>tubingensis</i> : Asptu1_0074938, <i>Aspergillus</i> <i>brasiliensis</i> : Aspbr1_00			
125000	III	98722 2	1.71E -05	AFUA_ 3G0366 0	CDS	Siderophore triacylfusarinine C esterase	estB	EstB- mediated hydrolysis of the siderophore	siderophore -dependent iron import into cell

								triacetylfusarinine C optimizes iron uptake of <i>Aspergillus fumigatus</i> .	
155000	III	4042287	8.07E-05	AFUA_3G15350	intergenic	Has domain (s) with predicted oxidoreductase activity and role in metabolic process			
155000	III	4052294	8.07E-05	AFUA_3G15350	intergenic	Has domain (s) with predicted oxidoreductase activity and role in metabolic process			
186000	I	393676	8.07E-05	AFUA_1G01090	intergenic	protein of unknown function			
194000	I	2635602	9.25E-05	AFUA_1G10240	intergenic	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_009649			
194000	I	2642658	7.22E-05	AFUA_1G10240	intergenic	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_009650			
194000	I	2668789	5.99E-05	AFUA_1G10355	intergenic	Ortholog of <i>A. nidulans</i> FGSC A4 : AN1245, <i>A. niger</i> CBS 513.88 : An08g02270, <i>Aspergillus wentii</i> : Aspwe1_0036569, <i>Aspergillus sydowii</i> : Aspsy1_0053120 and <i>Aspergillus terreus</i> NIH2624 : ATET_00225			

195000	I	2932677	4.12E-05	AFUA_1G1120	intergenic	Has domain (s) with predicted cytoplasm, nucleus localization		
196000	I	2998187	1.35E-05	AFUA_1G11370	intergenic	Has domain (s) with predicted flavin adenine dinucleotide binding, oxidoreductase activity, acting on CH-OH group of donors activity and role in oxidation-reduction process		

Figure 22. Volcano plot of gene expression profiles for histamine exposure compared with the control group. The plot displays log₂-fold change on the x-axis and False Discovery Rate (FDR)-adjusted p-values on the y-axis. Each dot represents an individual gene. Eight genes are significantly upregulated (red) in the control group relative to the histamine condition, while five genes are significantly downregulated (blue). Genes exhibiting greater than 3-fold change are labeled with their gene IDs.



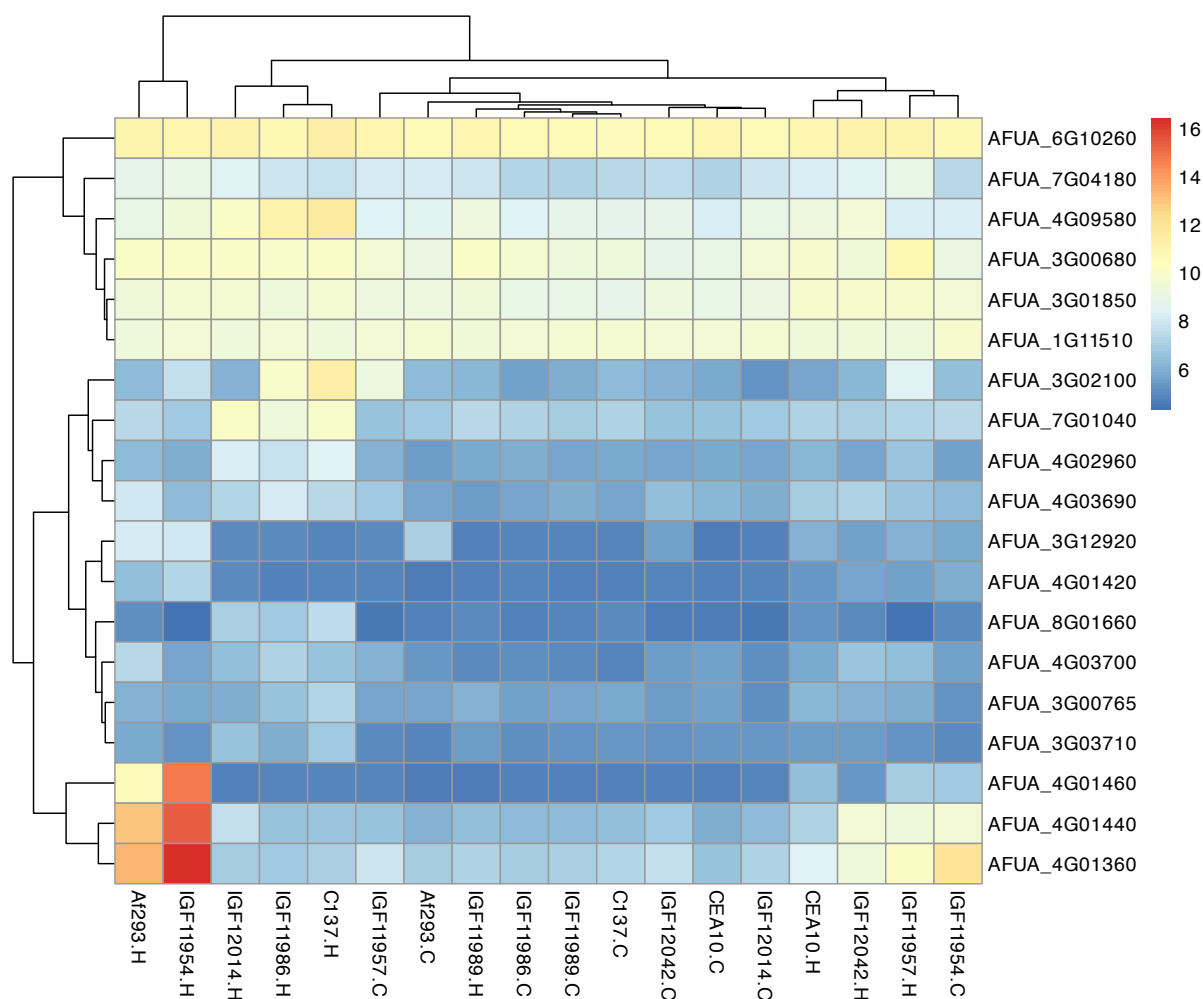


Figure 23: Gene expression profiles for the histamine condition compared with the control group are presented. A heatmap clustering of all the differentially expressed genes (DEGs) demonstrates their expression abundance. The various colors indicate different levels of expression abundance, with the color bar reflecting the range of expression ratios in the heatmap. Nineteen genes with significantly differential expression were identified and can be found in Table 9. the color bar indicating the range of expression ratios: red for higher expression and blue for down expression .

- **Table 11: Gene expression list for the histamine condition compared to the control group.**
- **baseMean:** The average normalized count value across all samples, adjusted for library size differences.
- **log2FoldChange:** The estimated change in gene expression between the histamine and control conditions, expressed on a log base 2 scale.
- **lfcSE:** The standard error of the log2 fold change estimate.
- **stat:** The test statistic value used to assess differential expression.
- **pvalue:** The p-value indicating the significance of the differential expression test.
- **padj:** The p-value adjusted for multiple testing to control false discovery rate.

Gene ID	Gene Name	Description	logFC	baseMean	lfcSE	stat	p.Value	adj.p.val
AFUA_4G01440		Predicted glutathione S transferase	4.7049	725.34	0.94375	4.9853	6.19e-07	0.0013324
AFUA_4G02960		predicted hydrolase activity and role in nucleotide catabolic process	2.7629	144.82	0.55585	4.9705	6.68e-07	0.0013324
AFUA_7G01040		predicted hydrolase activity, zinc ion binding activity	2.9838	404.47	0.60416	4.9388	7.86e-07	0.0013324
AFUA_4G09580	aspf2	Allergen Aspf2	2.4472	1022.5	0.49592	4.9348	8.02e-07	0.0013324
AFUA_4G01460		Ortholog of Neosartorya fischeri NRRL	4.765	84.97	0.97552	4.8845	1.04e-06	0.0014089
AFUA_3G00680		Copper amine oxidase	1.2234	929.72	0.25186	4.8577	1.19e-06	0.0014089

AFUA_3G02100		predicted oxidoreductase activity and role in metabolic process	4.1206	423.02	0.87614	4.7031	2.56e-06	0.0026598
AFUA_4G01360		predicted role in transmembrane transport and integral component of membrane localization	4.2954	1006.9	0.96185	4.4657	7.98e-06	0.0073628
AFUA_4G03700			2.4873	90.881	0.57082	4.3573	1.32e-05	0.0099385
AFUA_4G03690		predicted oxidoreductase activity, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor activity and role in oxidation-reduction process	2.1317	140.06	0.50641	4.2094	2.56e-05	0.017715
AFUA_7G04180		Ortholog(s) have cytosol, nuclear periphery localization	1.4525	310.75	0.34717	4.1837	2.87e-05	0.018321
AFUA_3G00765		Copper amine oxidase	1.1877	57.231	0.29946	3.966	7.31e-05	0.036457
AFUA_3G12920	nrps5	Nonribosomal peptide synthase (NRPS)	3.0531	112.95	0.7702	3.9641	7.37e-05	0.036457
AFUA_4G01420	icsB	Isocyanide synthase B, putative	3.0851	38.915	0.77888	3.961	7.46e-05	0.036457
AFUA_1G10030		Ortholog of Aspergillus tubingensis	1.5659	1270.1	0.40735	3.8442	0.00012096	0.055804
AFUA_4G03710		predicted catalytic activity, glutamate-ammonia ligase activity	1.9696	158.83	0.52737	3.7347	0.0001879	0.078018

		and role in nitrogen compound metabolic process						
AFUA_2G17730		predicted role in transmembrane transport and integral component of membrane localization	1.3688	357.25	0.3699	3.7005	0.00021514	0.085073
AFUA_1G03352		Has domain(s) with predicted hydrolase activity	2.4096	118.34	0.65815	3.6611	0.00025111	0.094782
AFUA_4G00400		predicted methyltransferase activity and role in metabolic process	1.6367	70.527	0.45342	3.6096	0.00030669	0.10798
AFUA_5G00920		Ortholog(s) have intracellular localization	1.6277	635.48	0.46684	3.4866	0.00048928	0.16252
AFUA_6G04360	atrF	Putative ABC transporter	1.5152	808.9	0.45638	3.3201	0.00089997	0.2669
AFUA_4G03680		Putative oxidoreductase, short-chain dehydrogenase/reductase family	1.1288	244.46	0.34277	3.2932	0.00099054	0.27377
AFUA_8G02445			1.4412	30.247	0.43986	3.2765	0.0010512	0.27377
AFUA_6G14460		2-haloalkanoic acid dehalogenase	1.3731	531.79	0.42751	3.2118	0.0013191	0.33195
AFUA_7G01050		predicted FAD binding, oxidoreductase activity and role in metabolic process	1.1856	64.551	0.38187	3.1048	0.0019039	0.44892

AFUA_5G10510		Ortholog(s) have role in secondary metabolite biosynthetic process	1.3069	2590.7	0.43365	3.0138	0.00258	0.54935
AFUA_7G04960		predicted N-acetyltransferase activity	1.8397	169.45	0.61213	3.0055	0.0026518	0.55051
AFUA_1G16440		predicted ATP binding, ATPase activity, nucleoside-triphosphatase activity, nucleotide binding activity	2.0123	426.89	0.6861	2.9329	0.0033576	0.62746
AFUA_1G13950		Has domain(s) with predicted oxidoreductase activity and role in metabolic process	1.7114	325.83	0.58825	2.9093	0.0036224	0.64361
AFUA_5G11180		Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_058750	-1.1869	40.582	0.40822	-2.9075	0.0036428	0.64361
AFUA_2G17360		Ortholog(s) have role in cellular response to drug	1.048	267.89	0.3692	2.8385	0.0045328	0.67393
AFUA_5G00790		predicted ATP binding, ATPase activity, ATPase activity, coupled to transmembrane movement of substances, nucleoside-triphosphatase activity, nucleotide binding activity and role in transport	1.1045	283.16	0.38969	2.8343	0.0045921	0.67393

AFUA_5G13180		Putative agmatinase, phoB-regulated	1.1476	86.158	0.4053	2.8316	0.0046314	0.67393
AFUA_2G12790		Predicted role in transmembrane transport and integral component of membrane localization	1.1206	56.054	0.39646	2.8264	0.0047072	0.67393
AFUA_2G12650		predicted carbon-sulfur lyase activity and role in metabolic process	1.0763	136.81	0.38298	2.8104	0.0049482	0.69643
AFUA_4G11320		predicted role in transmembrane transport and integral component of membrane localization	-1.0033	2475	0.37091	-2.7049	0.0068332	0.81333
AFUA_4G01450		predicted 3-deoxy-7-phosphoheptulonate synthase activity, catalytic activity and role in aromatic amino acid family biosynthetic process, biosynthetic process	2.9458	48.487	1.0968	2.6857	0.0072375	0.81333
AFUA_4G01400		ThiJ/PfpI family protein	1.4147	277.26	0.53778	2.6307	0.0085212	0.85223
AFUA_6G00770		Putative extracellular arabinanase	-1.8879	263.51	0.74126	-2.5468	0.01087	0.92725
AFUA_3G12900	hasB	Putative transporter	1.5026	4100.9	0.59499	2.5255	0.011554	0.94179
AFUA_4G01140		Putative multidrug resistance protein	1.3286	1121.2	0.52615	2.525	0.011568	0.94179
AFUA_6G00290		predicted catalytic activity, pyridoxal	1.022	379.44	0.41101	2.4866	0.012896	0.98322

		phosphate binding activity and role in biosynthetic process						
AFUA_5G02660	xanB	Isocyanide synthase xanB	1.8075	176.03	0.74391	2.4297	0.015112	0.99978
AFUA_8G06840		Ortholog of Aspergillus fumigatus A1163 : AFUB_080930	1.331	175.39	0.5605	2.3747	0.017563	0.99978
AFUA_6G09880		predicted role in transmembrane transport and integral component of membrane localization	1.2336	120.99	0.53103	2.323	0.020181	0.99978
AFUA_2G01280		predicted NAD binding, oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor activity and role in oxidation-reduction process	1.2004	39.914	0.51889	2.3135	0.020697	0.99978
AFUA_5G07902		Ortholog of A. nidulans FGSC A4 : AN11018, A. niger CBS 513.88 : An03g03930, A. oryzae RIB40 : AO090701000702,	-1.065	185.6	0.4646	-2.2923	0.021886	0.99978
AFUA_8G01560		Putative aldo-keto reductase	1.9012	1044.8	0.83114	2.2875	0.022169	0.99978
AFUA_7G06600		Ortholog(s) have NAD(P)H dehydrogenase	1.1484	237.01	0.50472	2.2753	0.02289	0.99978

		(quinone) activity, transcription factor binding activity and role in apoptotic process, cellular response to oxidative stress, cytoplasmic sequestering of transcription factor						
AFUA_5G06880		predicted role in transmembrane transport and integral component of membrane localization	-1.5916	132.05	0.73285	-2.1719	0.029866	0.99978
AFUA_2G17900		Ortholog of <i>A. oryzae</i> RIB40 : AO090003000473, <i>Neosartorya fischeri</i> NRRL 181 : NFIA_003780, NFIA_093330, <i>Aspergillus niger</i>	-1.5241	186.5	0.70912	-2.1493	0.031608	0.99978
AFUA_6G00430		Ortholog of <i>A. nidulans</i> FGSC A4 : AN0362, <i>A.</i> <i>fumigatus</i> Af293	-1.314	2398.3	0.62135	-2.1147	0.034457	0.99978
AFUA_3G00510		Ortholog of <i>Aspergillus</i> <i>fumigatus</i> A1163 : AFUB_047940	-1.3538	326.77	0.64619	-2.095	0.036168	0.99978
AFUA_5G07490		Ortholog of <i>A. nidulans</i> FGSC A4 : AN4151, AN5421, <i>A. niger</i> CBS 513.88	1.1362	69.59	0.54517	2.084	0.037156	0.99978

AFUA_1G13110		4-coumarate-CoA ligase	1.0111	195.16	0.48924	2.0666	0.038769	0.99978
AFUA_6G13920	pyr1	Nicotinic acid-CoA ligase pyr1	-1.0132	437.75	0.51176	-1.9799	0.047713	0.99978
AFUA_6G11850		protein of unknown function	1.189	149.21	0.60627	1.9612	0.049856	0.99978

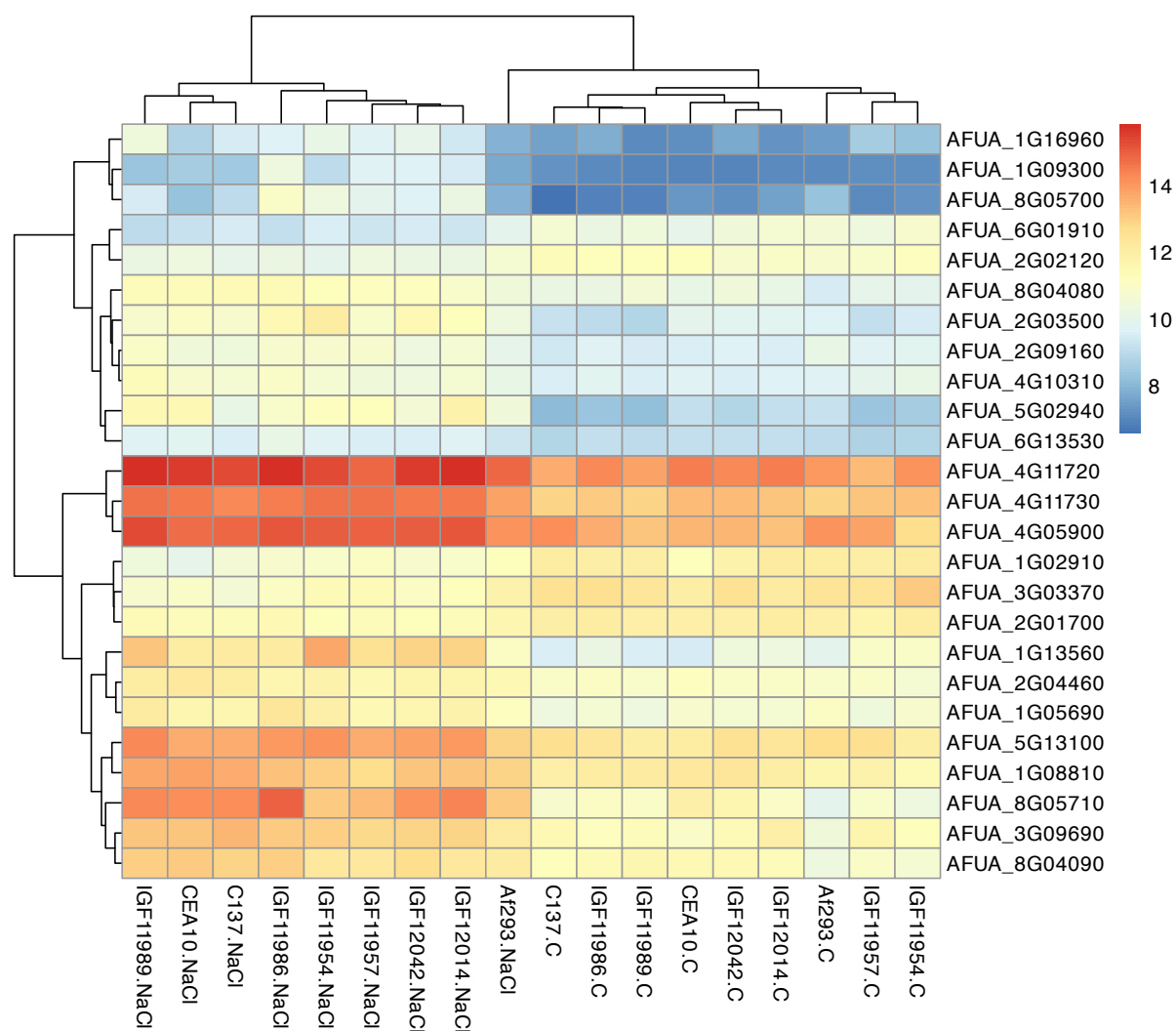


Figure 24: Gene expression profile for NaCl condition against the control group. Heatmap clustering of all the DEGs according to their expression abundance. The different colours indicate different levels of expression abundance. The colour bar indicates the range of the expression ratio in the heatmap. Top10 Gene with significantly differentiates expression are shown in Table 11.

Figure 25: Volcano plots of differentially expressed genes for the NaCl condition compared to the control group. The volcano plot depicts the log₂-fold change on the x-axis and False Discovery Rate-adjusted p-values on the y-axis. Individual genes are represented as dots. Eleven genes were upregulated (in red) in the control relative to the NaCl condition, while nine genes were downregulated (in blue). Genes with greater than 3-fold change are labelled with their gene IDs

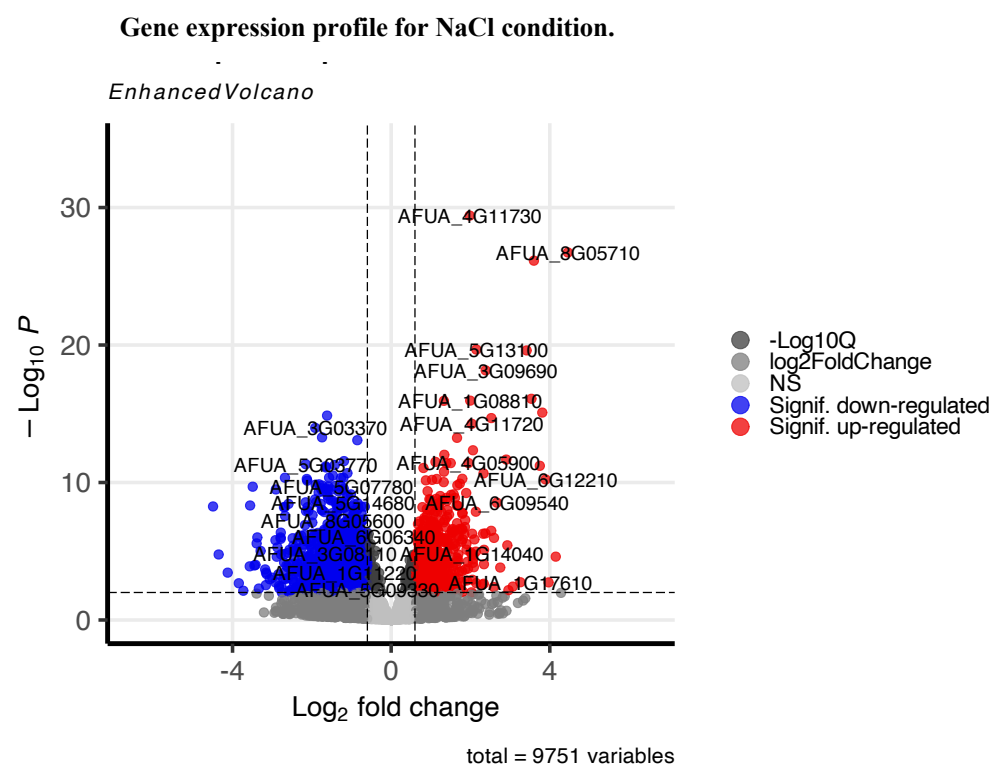


Table 12: Gene Expression List (Adjusted p-value < 0.05) for NaCl Condition Compared to the Control Group

- **baseMean:** The average normalized count value across all samples, adjusted for library size differences.
- **log2FoldChange:** The estimated change in gene expression between the histamine and control conditions, expressed on a log base 2 scale.
- **lfcSE:** The standard error of the log2 fold change estimate.
- **stat:** The test statistic value used to assess differential expression.
- **pvalue:** The p-value indicating the significance of the differential expression test.
- **padj:** The p-value adjusted for multiple testing to control false discovery rate.

Additionally, overlap candidate genes associated with histamine and NaCl phenotypes are highlighted in red.

Gene ID	Gene name	Description	Base mean	log2FC	lfcSE	stat	p-value	Adjusted p-value
AFUA_3G01850		predicted protein-arginine deiminase activity and role in putrescine biosynthetic process	716.501992	0.86597193	0.16218275	28.1126738	1.14e-07	0.0011149
AFUA_3G00680		Copper amine oxidase	928.608028	1.2227248	0.25119781	23.0357033	1.59e-06	0.0075668
AFUA_4G02960		predicted hydrolase activity	145.619836	2.77175096	0.55190592	22.2645352	2.38e-06	0.0075668

		and role in nucleotide catabolic process						
AFUA_4G09580	aspf2	Allergen Asp f 2	1028.26066	2.45834776	0.49983774	21.7491981	3.11e-06	0.0075668
AFUA_7G01040		Has domain(s) with predicted hydrolase activity, zinc ion binding activity	406.967051	2.9981959	0.60464002	21.1799118	4.18e-06	0.00814589
AFUA_1G11510		Ortholog of A. nidulans FGSC A4	769.632808	-0.3747124	0.08371931	19.9818368	7.82e-06	0.01269273
AFUA_4G01460		Ortholog of Neosartorya fischeri NRRL 181	84.5910301	4.76699751	0.96163801	18.3210728	1.87e-05	0.02279997
AFUA_4G01440		Predicted glutathione S transferase	722.53975	4.70212172	0.94226253	18.3147724	1.87e-05	0.02279997
AFUA_3G00765		predicted N-acetyltransferase activity	73.8463135	1.69296159	0.39104739	17.9264314	2.30e-05	0.02328407
AFUA_4G03700		Ortholog(s) have cytosol, nuclear periphery localization	90.9185086	2.49304765	0.56101187	17.8499021	2.39e-05	0.02328407
AFUA_7G04180		Copper amine oxidase	309.462082	1.45291249	0.34051529	17.5171093	2.85e-05	0.02366854
AFUA_8G01660		Ortholog of Aspergillus fumigatus A1163 : AFUB_084940	81.8317175	3.14533518	0.69909225	17.3655883	3.08e-05	0.02366854

AFUA_3G02100		predicted oxidoreductase activity and role in metabolic process	426.001417	4.14000668	0.8767423	17.3198378	3.16e-05	0.02366854
AFUA_4G03690		predicted oxidoreductase activity, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor activity and role in oxidation-reduction process	140.258016	2.13839853	0.50035959	16.8876279	3.97e-05	0.02759433
AFUA_3G03710		Ortholog(s) have aminopeptidase activity and role in cellular protein catabolic process	58.8856953	1.86523481	0.45594942	15.8544931	6.84e-05	0.04442151
AFUA_6G10260	akr1	Putative aldehyde reductase	1935.22512	0.49025716	0.12344042	15.702617	7.41e-05	0.04512612
AFUA_4G01360		predicted role in transmembrane transport and integral component of membrane localization	1002.54841	4.29439471	0.96007922	15.2818022	9.26e-05	0.0530623

AFUA_6G10790		Ortholog(s) have endoplasmic reticulum localization	144.713411	0.84579628	0.22019465	14.6049255	0.00013257	0.07174117
AFUA_4G01420	icsB	Isocyanide synthase B, putative	38.8393255	3.0587839	0.76157682	14.0262577	0.00018028	0.09242443
AFUA_3G12920	nrps5	Nonribosomal peptide synthase (NRPS)	111.887727	3.04810491	0.75875813	13.8842172	0.00019442	0.09469436
AFUA_1G10030		Ortholog of Aspergillus tubingensis	1275.77283	1.56917236	0.41290964	13.7875196	0.00020469	0.09494761

3.4.3 Overlap candidate genes found to be associated with histamine and NaCl phenotypes

The combined analysis of GWAS and DEG data provided insight into the molecular mechanisms underlying *A. fumigatus*'s response to histamine and salt stress. The identification of 15 overlapping candidate genes suggests the existence of shared pathways that confer tolerance or adaptation to these distinct but potentially related stressors.

Among these, several genes stand out due to their significant differential expression and potential functional roles in stress response pathways: **AFUA_3G01850** and **AFUA_4G02960** exhibited the highest baseMean values and substantial log2 fold changes, indicating robust expression changes under stress conditions. These genes may encode proteins involved in osmoprotection or detoxification processes essential for surviving environmental stress.

AFUA_4G01460 and **AFUA_4G01440** also showed marked upregulation, highlighting their possible roles in cellular defense mechanisms, such as regulation of ion channels, osmolyte synthesis, or stress signalling pathways.

AFUA_3G00765 and **AFUA_3G02100** with moderate to high fold changes may encode enzymes or regulatory proteins that modulate gene expression in response to stress stimuli.

Additionally , Functionally, many of these genes could participate in pathways such as:

Osmoregulation: Maintaining cellular ion balance under high salt or histamine exposure.

Detoxification: Managing reactive compounds or osmolytes produced during stress. **Signal**

transduction: Activating downstream stress response cascades.

The high statistical significance (low p-values and adjusted p-values) of these genes suggests that their differential expression is unlikely due to random variation, reinforcing their potential roles in mediating stress responses.

The overlap of candidate genes shared between histamine and NaCl conditions emphasizes the possibility of convergent molecular pathways that underlie *A. fumigatus*'s resilience to diverse environmental challenges. These findings could inform future functional studies aimed at validating gene roles and elucidating the mechanisms of stress tolerance, with potential implications for developing targeted antifungal strategies.

Table 13: Summary of Overlapping Candidates Associated with Histamine and NaCl Phenotypes

The combined results of differentially expressed genes (DEGs) from RNA sequencing and genome-wide association studies (GWAS) highlight candidate genes associated with histamine and NaCl phenotypes (highlighted in red).

- **baseMean:** The average normalized count value across all samples, adjusted for library size differences.
- **log2FoldChange:** The estimated change in gene expression between the histamine and control conditions, expressed on a log base 2 scale.
- **lfcSE:** The standard error of the log2 fold change estimate.
- **stat:** The test statistic value used to assess differential expression.
- **pvalue:** The p-value indicating the significance of the differential expression test.
- **padj:** The p-value adjusted for multiple testing to control false discovery rate.

Genes ID	Base Mean	log2 Fold Change	lfcSE	stat	p.value	adj.p_val
AFUA_3G01850	716.501992	0.86597193	0.16218275	28.1126738	1.14e-07	0.0011149
AFUA_3G00680	928.608028	1.2227248	0.25119781	23.0357033	1.59e-06	0.0075668
AFUA_4G02960	145.619836	2.77175096	0.55190592	22.2645352	2.38e-06	0.0075668
AFUA_4G09580	1028.26066	2.45834776	0.49983774	21.7491981	3.11e-06	0.0075668
AFUA_7G01040	406.967051	2.9981959	0.60464002	21.1799118	4.18e-06	0.00814589
AFUA_1G11510	769.632808	-0.3747124	0.08371931	19.9818368	7.82e-06	0.01269273
AFUA_4G01460	84.5910301	4.76699751	0.96163801	18.3210728	1.87e-05	0.02279997
AFUA_4G01440	722.53975	4.70212172	0.94226253	18.3147724	1.87e-05	0.02279997

AFUA_3G00765	73.8463135	1.69296159	0.39104739	17.9264314	2.30e-05	0.02328407
AFUA_4G03700	90.9185086	2.49304765	0.56101187	17.8499021	2.39e-05	0.02328407
AFUA_7G04180	309.462082	1.45291249	0.34051529	17.5171093	2.85e-05	0.02366854
AFUA_8G01660	81.8317175	3.14533518	0.69909225	17.3655883	3.08e-05	0.02366854
AFUA_3G02100	426.001417	4.14000668	0.8767423	17.3198378	3.16e-05	0.02366854
AFUA_4G03690	140.258016	2.13839853	0.50035959	16.8876279	3.97e-05	0.02759433
AFUA_3G03710	58.8856953	1.86523481	0.45594942	15.8544931	6.84e-05	0.04442151
AFUA_6G10260	1935.22512	0.49025716	0.12344042	15.702617	7.41e-05	0.04512612
AFUA_4G01360	1002.54841	4.29439471	0.96007922	15.2818022	9.26e-05	0.0530623
AFUA_6G10790	144.713411	0.84579628	0.22019465	14.6049255	0.00013257	0.07174117
AFUA_4G01420	38.8393255	3.0587839	0.76157682	14.0262577	0.00018028	0.09242443
AFUA_3G12920	111.887727	3.04810491	0.75875813	13.8842172	0.00019442	0.09469436
AFUA_1G10030	1275.77283	1.56917236	0.41290964	13.7875196	0.00020469	0.09494761
AFUA_2G05620	895.253972	-0.5832096	0.17710791	-3.2929622	0.00099138	0.22571144

3.4.4 Discussion

The combined results of GWAS and DEG analyses have identified a set of 22 candidate genes potentially involved in the adaptive response of *A. fumigatus* to environmental stresses, notably salt and histamine exposure. Among these, 15 genes were found to be common to both conditions, indicating shared molecular pathways that underpin the ability of *A. fumigatus* to withstand diverse environmental challenges (highlighted in Table 13). These overlapping genes suggest core mechanisms of phenotypic plasticity, stress response, and virulence regulation that are activated during exposure to both salt stress and histamine.

Several of these candidate genes demonstrate significant differential expression, with notable upregulation under stress conditions. For example, AFUA_3G01850 and AFUA_4G02960 exhibited high baseMean expression levels and substantial fold changes, implying their vital roles in cellular adaptation. Although their exact functions are still being elucidated, preliminary evidence indicates they may encode proteins involved in osmoregulation, detoxification, or signal transduction pathways critical for maintaining cellular homeostasis during osmotic and chemical stress. Supporting this, homologous genes in other fungi have been implicated in stress resilience and pathogenicity (Tepšič et al., 2006; Rinu & Pandey, 2010).

In addition to their roles in stress tolerance, some candidate genes are linked to metabolic and regulatory pathways that influence virulence. Notably, AFUA_3G00680, AFUA_3G00765, and AFUA_7G04180 are involved in copper uptake and N-acetyltransferase activity—functions that are crucial for protein regulation through histone modifications, affecting gene expression during environmental adaptation (Kusuya et al., 2017; Wiemann et al., 2017). These processes are well-known to impact fungal pathogenicity: copper-dependent enzymes such as superoxide dismutases are essential for neutralizing oxidative stress, a key component of host immune defenses (Yin et al., 2014). The upregulation of these genes

suggests that *A. fumigatus* actively modulates metal homeostasis to survive in hostile host environments and potentially enhances its virulence.

A particularly noteworthy candidate is AFUA_2G05620, which emerged as a key regulator in histamine metabolism. Given histamine's well-established role in immune signaling and inflammation—particularly in the lung, where it mediates bronchoconstriction, edema, and immune cell recruitment (Valerius et al., 2001b; Carlos et al., 2009)—the ability of *A. fumigatus* to regulate histamine levels could be instrumental in immune evasion or immune modulation. Investigating AFUA_2G05620 may reveal how *A. fumigatus* influences host immune responses, especially in histamine-rich environments such as allergic asthma or during invasive lung infections. Modulating histamine pathways could be a fungal strategy to dampen immune activation or facilitate tissue invasion.

Furthermore, the expression profiles of these candidate genes during growth under histamine and NaCl conditions closely mirror transcriptional responses observed during invasive growth within host tissues, such as the lung (Chiang et al., 2008). This suggests that *A. fumigatus* employs conserved molecular mechanisms to adapt to the host environment, including nutrient limitation, osmotic stress, and immune challenges. Activation of pathways involved in metal ion homeostasis, effector production, and stress signalling underscores its capacity for phenotypic plasticity and adaptive virulence.

Considering these findings, exploring the epigenetic regulation of these key genes—such as histone modifications and DNA methylation—may further elucidate mechanisms by which environmental cues induce heritable changes in gene expression, thereby contributing to pathogenicity and resistance (Dubey & Jeon, 2017; Mhatre et al., 2008). Environmental factors, including pH, temperature, and nutrient status, are known to influence epigenetic marks, enabling rapid adaptation to host defenses and stress conditions (Yu & Keller, 2005; Duran et al., 2010).

In conclusion, the integration of GWAS and transcriptomic data highlights a core set of genes that are likely pivotal regulators of the stress response in *A. fumigatus*, virulence, and adaptation. These findings provide a foundation for future functional validation studies, which could lead to novel targeted therapies aimed at disrupting these adaptive pathways, ultimately improving strategies to combat invasive fungal infections.

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4 General Conclusions

This thesis aimed to enhance our understanding of the diversity and population structure of *Aspergillus fumigatus*, particularly in relation to phenotypic variation, genetic variation, and their implications for the fungus's transcriptome and metabolism. By investigating these factors, we gain valuable insights into the evolution and ecology of *A. fumigatus* during infections.

In Chapter 3.1, we explored the genetic diversity present among *A. fumigatus* isolates. Utilizing whole genome sequencing and phylogenomic analyses, we identified two distinct clades, A and B, each characterized by unique genetic traits linked to antifungal resistance. The clustering of isolates with the TR₃₄/L98H polymorphism within Clade A indicates a shared evolutionary path and subsequent clonal expansion under selective pressure, underscoring the importance of genomic surveillance to track the emergence of resistant strains, particularly in vulnerable populations like cystic fibrosis individuals. The observed genetic variability within isolates—especially those sourced from a single patient—highlights the rapid adaptability of *A. fumigatus*, involve a thorough understanding of its population dynamics to inform clinical interventions.

In Chapter 3.2, we employed BIOLOG™ Phenotype MicroArrays to assess the metabolic profiles of azole-resistant and susceptible *A. fumigatus* isolates under varying carbon and nitrogen conditions. The results showed significant phenotypic heterogeneity, illustrating a complex relationship between genetic background and metabolic capability. Notably, the growth responses of isolates carrying the TR₃₄/L98H polymorphism indicated that genetic changes may provide both advantages and limitations in nutrient utilization. This chapter emphasizes the crucial role of metabolic adaptability in *A. fumigatus* and highlights high-throughput phenotyping as a valuable method for elucidate the metabolic complexities of this opportunistic pathogen

Throughout Chapters 3.1, 3.2, and 3.3, we observed substantial genetic heterogeneity among the isolates. This finding reinforces the importance of metabolomics in studying vital metabolites within living organisms, contributing to biomarker screening and revealing regulatory pathways. Additionally, the phenotypic microarray proves to be a rapid and reliable tool for characterizing fungal metabolism. The capacity of *A. fumigatus* to metabolize diverse carbon and nitrogen sources indicates its ability to thrive in various environments. Our research highlights the significance of nitrogen acquisition and metabolism during infections with azole-resistant *A. fumigatus*, especially in individuals with cystic fibrosis and other respiratory diseases.

The findings reveal that *A. fumigatus* isolates from cystic fibrosis patients exhibit notable differences in phenotypic traits compared to non-CF isolates. This adaptability is vital for successful colonization of specific environmental niches, and the observed responses in CF isolates may reflect the biological costs of coping with challenging conditions characteristic of CF lungs. In cystic fibrosis patients, the disruption of electrolyte balance and osmolality due to mutations in the cystic fibrosis transmembrane conductance regulator can lead to mucoid secretions, facilitating the colonization of opportunistic pathogens like *A. fumigatus*. Consequently, CF isolates may experience more significant challenges compared to those from other clinical environments, leading to the potential development of a CF-specific phenotype. Understanding the molecular pathways underlying these differences can help researchers identify new targets for effective strategies to combat *A. fumigatus* infections.

In Chapter 3.4, we integrated genomic and transcriptomic data to analyse how *A. fumigatus* adapts to nitrogen and salt stress conditions. This multifaceted approach allowed us to identify critical regulatory networks and pathways activated in response to environmental stressors. We found that genes related to osmoregulation, nutrient uptake, and stress responses are integral to the fungus' adaptation to these challenges. Additionally, genes significantly upregulated in

response to histamine underscore their importance in histamine metabolism and related stress responses. Specifically, genes associated with iron acquisition and zinc uptake play vital roles in the physiological adaptations of *A. fumigatus* in adverse environments. Our analysis of differential gene expression indicates that certain transcription factors may be crucial in mediating these responses, shedding light on how *A. fumigatus* prioritizes metabolic processes under stress.

The interplay between these regulatory elements is key to understanding how the fungus maintains resilience in nutrient-poor or saline environments, enabling its success as an opportunistic pathogen. Moreover, elucidating the pathways and key genes involved in these stress responses enhances our comprehension of *A. fumigatus* biology and presents potential targets for novel antifungal strategies. Identifying these vulnerabilities can inform the design of specific inhibitors aimed at disrupting critical adaptive pathways, thereby improving therapeutic outcomes against this opportunistic pathogen.

In conclusion, our research demonstrates that increased fitness and adaptability to metabolic and stress conditions drive the evolution of *A. fumigatus* in the lung environment. Furthermore, we emphasize the importance of employing diverse animal models to accurately assess strain-specific virulence variations in future research.

The integration of diverse datasets in this concluding chapter reveals the significant role of environmental stressors in shaping adaptive responses to *A. fumigatus*. Our findings illustrate how genetic factors, and environmental challenges combine to drive the evolution of phenotypic traits associated with virulence and resistance. The identification of key stress response pathways and their corresponding gene networks enhances our understanding of how *A. fumigatus* sustains its fitness as an opportunistic pathogen in immunocompromised individuals.

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In summary, this thesis advances our understanding of the genetic diversity, metabolic adaptations, and phenotypic variations of *A. fumigatus*. Through detailed genomic analyses and innovative phenotypic characterization, we have illuminated the complexities of the biology of this opportunistic pathogen and its clinical ramifications. Our findings underscore the urgent need for continuous surveillance of *A. fumigatus* populations and the development of targeted antifungal strategies to combat the rising threat of azole resistance. Moving forward, fostering interdisciplinary collaborations and integrating clinical and environmental data will be essential in addressing the challenges posed by this resilient fungus and improving outcomes for patients affected by its infections.

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6 Appendices:

Raw and normalised data for the PM analysis, GWAS and transcriptome raw count read data, and all genes found before and after adjusting p -value can be downloaded at <https://github.com/renad122/aljohani-re.git>

Figure 1A : Phylogenetic analysis of all 145 *A. fumigatus* isolates with bootstrap support over 1000 replicates performed on WGS SNP data to generate a maximum-likelihood phylogeny. On the branches of the tree are reported the branch length. The branches of the tree with 90% bootstrap support are shown. The scale bar indicates the number of substitutions per site.

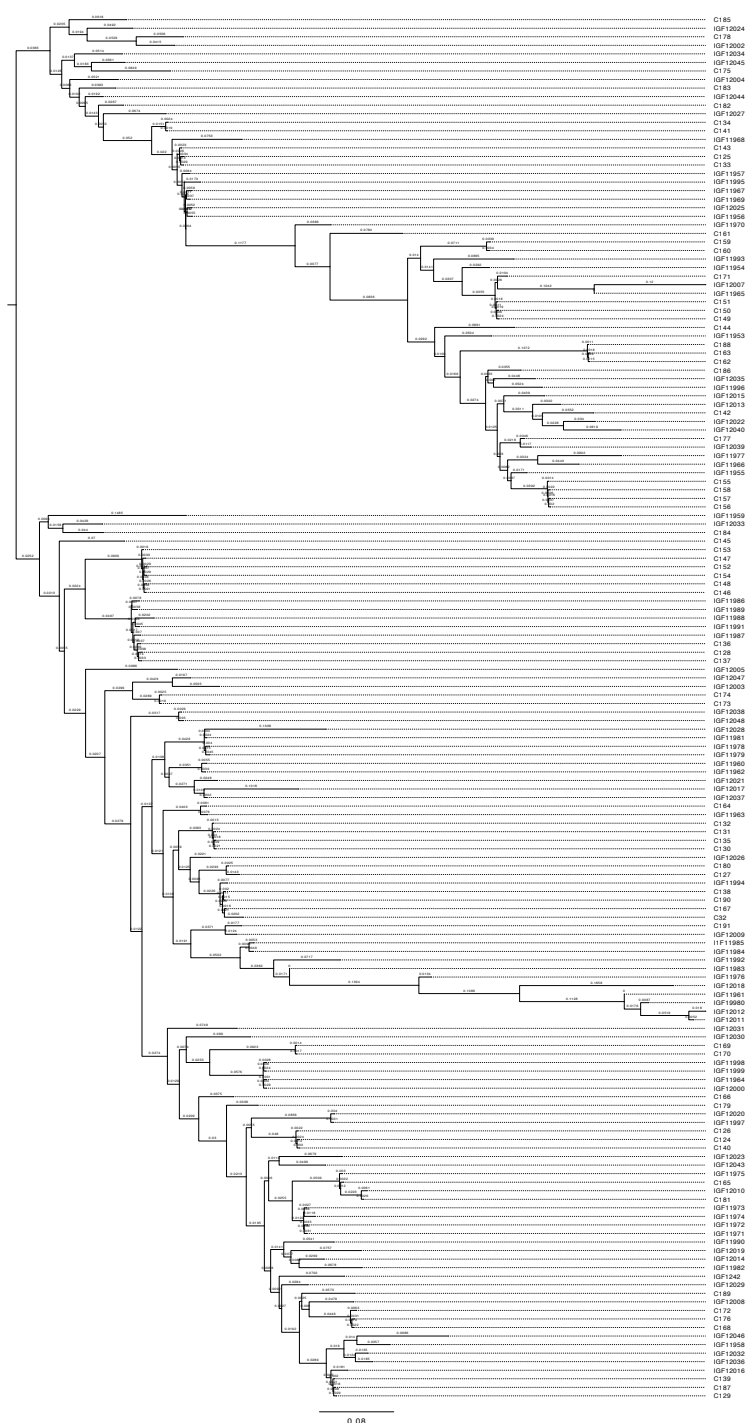


Table 1A: List of Substrates in Biolog PM1, PM3B, PM9, and PM10 Microplates Used in PM Assays for Profiling 42 *A. fumigatus* Clinical

Isolates: Locations of Substrates on the Plate, Chemical Abstracts Service (CAS) IDs, KEGG IDs, and Mechanisms of Action (MoA)

Plate	Location	Chemical	CAS ID	KEGG ID	Mechanism of Action (MoA)
PM01	A01	Negative Control	Negative Control		C-Source, negative control
PM01	A02	L-Arabinose	CAS 87-72-9	C00259	C-Source, carbohydrate
PM01	A03	N-Acetyl-D-Glucosamine	CAS 7512-17-6	C00140	C-Source, carbohydrate
PM01	A04	D-Saccharic acid	CAS 576-42-1	C00818	C-Source, carboxylic acid
PM01	A05	Succinic acid	CAS 6106-21-4	C00042	C-Source, carboxylic acid
PM01	A06	D-Galactose	CAS 59-23-4	C00124	C-Source, carbohydrate
PM01	A07	L-Aspartic acid	CAS 3792-50-5	C00049	C-Source, amino acid
PM01	A08	L-Proline	CAS 147-85-3	C00148	C-Source, amino acid
PM01	A09	D-Alanine	CAS 338-69-2	C00133	C-Source, amino acid
PM01	A10	D-Trehalose	CAS 6138-23-4	C01083	C-Source, carbohydrate
PM01	A11	D-Mannose	CAS 3458-28-4	C00159	C-Source, carbohydrate
PM01	A12	Dulcitol	CAS 608-66-2	C01697	C-Source, carbohydrate
PM01	B01	D-Serine	CAS 312-84-5	C00740	C-Source, amino acid
PM01	B02	D-Sorbitol	CAS 50-70-4	C00794	C-Source, carbohydrate
PM01	B03	Glycerol	CAS 56-81-5	C00116	C-Source, carbohydrate
PM01	B04	L-Fucose	CAS 2438-80-4	C01019	C-Source, carbohydrate
PM01	B05	D-Glucuronic acid	CAS 14984-34-0	C00191	C-Source, carboxylic acid
PM01	B06	D-Gluconic acid	CAS 527-07-1	C00257	C-Source, carboxylic acid
PM01	B07	DL- α -Glycerol Phosphate	CAS 3325-00-6	C00093	C-Source, carbohydrate
PM01	B08	D-Xylose	CAS 58-86-6	C00181	C-Source, carbohydrate
PM01	B09	L-Lactic acid	CAS 312-85-6	C00186	C-Source, carboxylic acid

PM01	B10	Formic acid	CAS 141-53-7	C00058	C-Source, carboxylic acid
PM01	B11	D-Mannitol	CAS 69-65-8	C00392	C-Source, carbohydrate
PM01	B12	L-Glutamic acid	CAS 142-47-2	C00025	C-Source, amino acid
PM01	C01	D-Glucose-6-Phosphate	CAS 3671-99-6	C00092	C-Source, carbohydrate
PM01	C02	D-Galactonic acid-g-Lactone	CAS 2782-07-2	C03383	C-Source, carboxylic acid
PM01	C03	DL-Malic acid	CAS 6915-15-7	C00711	C-Source, carboxylic acid
PM01	C04	D-Ribose	CAS 50-69-1	C00121	C-Source, carbohydrate
PM01	C05	Tween 20	CAS 9005-64-5	C11624	C-Source, fatty acid
PM01	C06	L-Rhamnose	CAS 3615-41-6	C00507	C-Source, carbohydrate
PM01	C07	D-Fructose	CAS 57-48-7	C00095	C-Source, carbohydrate
PM01	C08	Acetic acid	CAS 127-09-3	C00033	C-Source, carboxylic acid
PM01	C09	a-D-Glucose	CAS 50-99-7	C00031	C-Source, carbohydrate
PM01	C10	Maltose	CAS 6363-53-7	C00208	C-Source, carbohydrate
PM01	C11	D-Melibiose	CAS 585-99-9	C05402	C-Source, carbohydrate
PM01	C12	Thymidine	CAS 50-89-5	C00214	C-Source, carbohydrate
PM01	D01	L-Asparagine	CAS 70-47-3	C00152	C-Source, amino acid
PM01	D02	D-Aspartic acid	CAS 1783-96-6	C00402	C-Source, amino acid
PM01	D03	D-Glucosaminic acid	CAS 3646-68-2	C03752	C-Source, carboxylic acid
PM01	D04	1,2-Propanediol	CAS 57-55-6	C00583	C-Source, alcohol
PM01	D05	Tween 40	CAS 9005-66-7		C-Source, fatty acid
PM01	D06	a-Ketoglutaric acid	CAS 22202-68-2	C00026	C-Source, carboxylic acid
PM01	D07	a-Ketobutyric acid	CAS 2013-26-5	C00109	C-Source, carboxylic acid
PM01	D08	a-Methyl D-Galactoside	CAS 3396-99-4		C-Source, carbohydrate
PM01	D09	a-D-Lactose	CAS 5989-81-1	C00243	C-Source, carbohydrate
PM01	D10	Lactulose	CAS 4618-18-2	C07064	C-Source, carbohydrate
PM01	D11	Sucrose	CAS 57-50-1	C00089	C-Source, carbohydrate
PM01	D12	Uridine	CAS 58-96-8	C00299	C-Source, carbohydrate

PM01	E01	L-Glutamine	CAS 56-85-9	C00064	C-Source, amino acid
PM01	E02	m-Tartaric acid	CAS 147-73-9	C00552	C-Source, carboxylic acid
PM01	E03	D-Glucose-1-Phosphate	CAS 56401-20-8	C00103	C-Source, carbohydrate
PM01	E04	D-Fructose-6-Phosphate	CAS 26177-86-6	C00085	C-Source, carbohydrate
PM01	E05	Tween 80	CAS 9005-65-6	C11625	C-Source, fatty acid
PM01	E06	a-Hydroxyglutaric acid-g-Lactone	CAS 21461-84-7		C-Source, carboxylic acid
PM01	E07	a-Hydroxybutyric acid	CAS 19054-57-0	C05984	C-Source, carboxylic acid
PM01	E08	b-Methyl D-Glucoside	CAS 709-50-2		C-Source, carbohydrate
PM01	E09	Adonitol	CAS 488-81-3	C00474	C-Source, carbohydrate
PM01	E10	Maltotriose	CAS 1109-28-0	C01835	C-Source, carbohydrate
PM01	E11	2'-Deoxyadenosine	CAS 16373-93-6	C00559	C-Source, carbohydrate
PM01	E12	Adenosine	CAS 58-61-7	C00212	C-Source, carbohydrate
PM01	F01	Gly-Asp	CAS 4685-12-5	C02871	C-Source, amino acid
PM01	F02	Citric acid	CAS 6132-04-3	C00158	C-Source, carboxylic acid
PM01	F03	m-Inositol	CAS 87-89-8	C00137	C-Source, carbohydrate
PM01	F04	D-Threonine	CAS 632-20-2	C00820	C-Source, amino acid
PM01	F05	Fumaric acid	CAS 17013-01-3	C00122	C-Source, carboxylic acid
PM01	F06	Bromosuccinic acid	CAS 923-06-8		C-Source, carboxylic acid
PM01	F07	Propionic acid	CAS 137-40-6	C00163	C-Source, carboxylic acid
PM01	F08	Mucic acid	CAS 526-99-8	C01807	C-Source, carboxylic acid
PM01	F09	Glycolic acid	CAS 79-14-1	C00160	C-Source, carboxylic acid
PM01	F10	Glyoxylic acid	CAS 563-96-2	C00048	C-Source, carboxylic acid
PM01	F11	D-Cellobiose	CAS 528-50-7	C00185	C-Source, carbohydrate
PM01	F12	Inosine	CAS 58-63-9	C00294	C-Source, carbohydrate
PM01	G01	Gly-Glu	CAS 7412-78-4		C-Source, amino acid
PM01	G02	Tricarballic acid	CAS 99-14-9		C-Source, carboxylic acid
PM01	G03	L-Serine	CAS 56-45-1	C00065	C-Source, amino acid

PM01	G04	L-Threonine	CAS 72-19-5	C00188	C-Source, amino acid
PM01	G05	L-Alanine	CAS 56-41-7	C00041	C-Source, amino acid
PM01	G06	Ala-Gly	CAS 687-69-4		C-Source, amino acid
PM01	G07	Acetoacetic acid	CAS 3483-11-2	C00164	C-Source, carboxylic acid
PM01	G08	N-Acetyl-D-Mannosamine	CAS 7772-94-3	C00645	C-Source, carbohydrate
PM01	G09	Mono-methyl Succinate	CAS 3878-55-5		C-Source, carboxylic acid
PM01	G10	Methyl Pyruvate	CAS 600-22-6		C-Source, ester
PM01	G11	D-Malic acid	CAS 636-61-3	C00497	C-Source, carboxylic acid
PM01	G12	L-Malic acid	CAS 138-09-0	C00149	C-Source, carboxylic acid
PM01	H01	Gly-Pro	CAS 704-15-4		C-Source, amino acid
PM01	H02	p-Hydroxyphenyl Acetic acid	CAS 156-38-7	C00642	C-Source, carboxylic acid
PM01	H03	m-Hydroxyphenyl Acetic acid	CAS 621-37-4	C05593	C-Source, carboxylic acid
PM01	H04	Tyramine	CAS 60-19-5	C00483	C-Source, amine
PM01	H05	D-Psicose	CAS 551-68-8	C06468	C-Source, carbohydrate
PM01	H06	L-Lyxose	CAS 1949-78-6	C01508	C-Source, carbohydrate
PM01	H07	Glucuronamide	CAS 3789-97-7	D01791	C-Source, amide
PM01	H08	Pyruvic acid	CAS 113-24-6	C00022	C-Source, carboxylic acid
PM01	H09	L-Galactonic acid-g-Lactone	CAS 1668-08-2	C01115	C-Source, carboxylic acid
PM01	H10	D-Galacturonic acid	CAS 91510-62-2	C00333	C-Source, carboxylic acid
PM01	H11	b-Phenylethylamine	CAS 156-28-5	C05332	C-Source, amine
PM01	H12	Ethanolamine	CAS 2002-24-6	C00189	C-Source, alcohol
PM03 B	A01	Negative Control	Negative Control		N-Source, Negative control
PM03 B	A02	Ammonia	CAS 540-69-2	C00014	N-Source, inorganic
PM03 B	A03	Nitrite	CAS 7632-00-0	C00088	N-Source, inorganic

PM03 B	A04	Nitrate	CAS 7631-99-4	C00244	N-Source, inorganic
PM03 B	A05	Urea	CAS 57-13-6	C00086	N-Source, other
PM03 B	A06	Biuret	CAS 108-19-0	C06555	N-Source, other
PM03 B	A07	L-Alanine	CAS 56-41-7	C00041	N-Source, amino acid
PM03 B	A08	L-Arginine	CAS 1119-34-2	C00062	N-Source, amino acid
PM03 B	A09	L-Asparagine	CAS 70-47-3	C00152	N-Source, amino acid
PM03 B	A10	L-Aspartic acid	CAS 3792-50-5	C00049	N-Source, amino acid
PM03 B	A11	L-Cysteine	CAS 7048-04-6	C00097	N-Source, amino acid
PM03 B	A12	L-Glutamic acid	CAS 142-47-2	C00025	N-Source, amino acid
PM03 B	B01	L-Glutamine	CAS 56-85-9	C00064	N-Source, amino acid
PM03 B	B02	Glycine	CAS 6000-43-7	C00037	N-Source, amino acid
PM03 B	B03	L-Histidine	CAS 5934-29-2	C00135	N-Source, amino acid
PM03 B	B04	L-Isoleucine	CAS 73-32-5	C00407	N-Source, amino acid
PM03 B	B05	L-Leucine	CAS 61-90-5	C00123	N-Source, amino acid
PM03 B	B06	L-Lysine	CAS 657-27-2	C00047	N-Source, amino acid
PM03 B	B07	L-Methionine	CAS 63-68-3	C00073	N-Source, amino acid

PM03 B	B08	L-Phenylalanine	CAS 63-91-2	C00079	N-Source, amino acid
PM03 B	B09	L-Proline	CAS 147-85-3	C00148	N-Source, amino acid
PM03 B	B10	L-Serine	CAS 56-45-1	C00065	N-Source, amino acid
PM03 B	B11	L-Threonine	CAS 72-19-5	C00188	N-Source, amino acid
PM03 B	B12	L-Tryptophan	CAS 73-22-3	C00078	N-Source, amino acid
PM03 B	C01	L-Tyrosine	CAS 69847-45-6	C00082	N-Source, amino acid
PM03 B	C02	L-Valine	CAS 72-18-4	C00183	N-Source, amino acid
PM03 B	C03	D-Alanine	CAS 338-69-2	C00133	N-Source, amino acid
PM03 B	C04	D-Asparagine	CAS 5794-24-1	C01905	N-Source, amino acid
PM03 B	C05	D-Aspartic acid	CAS 1783-96-6	C00402	N-Source, amino acid
PM03 B	C06	D-Glutamic acid	CAS 6893-26-1	C00217	N-Source, amino acid
PM03 B	C07	D-Lysine	CAS 923-27-3	C00739	N-Source, amino acid
PM03 B	C08	D-Serine	CAS 312-84-5	C00740	N-Source, amino acid
PM03 B	C09	D-Valine	CAS 640-68-6	C06417	N-Source, amino acid
PM03 B	C10	L-Citrulline	CAS 372-75-8	C00327	N-Source, amino acid
PM03 B	C11	L-Homoserine	CAS 672-15-1	C00263	N-Source, amino acid

PM03 B	C12	L-Ornithine	CAS 3184-13-2	C00077	N-Source, amino acid
PM03 B	D01	N-Acetyl-L-Glutamic acid	CAS 1188-37-0	C00624	N-Source, amino acid
PM03 B	D02	N-Phthaloyl-L-Glutamic acid	CAS 340-90-9		N-Source, amino acid
PM03 B	D03	L-Pyroglutamic acid	CAS 98-79-3	C02238	N-Source, amino acid
PM03 B	D04	Hydroxylamine	CAS 5470-11-1	C00192	N-Source, other
PM03 B	D05	Methylamine	CAS 593-51-1	C00218	N-Source, other
PM03 B	D06	N-Amylamine	CAS 110-58-7		N-Source, other
PM03 B	D07	N-Butylamine	CAS 109-73-9		N-Source, other
PM03 B	D08	Ethylamine	CAS 75-04-7	C00797	N-Source, other
PM03 B	D09	Ethanolamine	CAS 2002-24-6	C00189	N-Source, other
PM03 B	D10	Ethylenediamine	CAS 333-18-6	C12511	N-Source, other
PM03 B	D11	Putrescine	CAS 333-93-7	C00134	N-Source, other
PM03 B	D12	Agmatine	CAS 2482-00-0	C00179	N-Source, other
PM03 B	E01	Histamine	CAS 56-92-8	C00388	N-Source, other
PM03 B	E02	b-Phenylethylamine	CAS 156-28-5	C05332	N-Source, other
PM03 B	E03	Tyramine	CAS 60-19-5	C00483	N-Source, other

PM03 B	E04	Acetamide	CAS 60-35-5	C06244	N-Source, other
PM03 B	E05	Formamide	CAS 75-12-7	C00488	N-Source, other
PM03 B	E06	Glucuronamide	CAS 3789-97-7	D01791	N-Source, other
PM03 B	E07	DL-Lactamide	CAS 65144-02-7		N-Source, other
PM03 B	E08	D-Glucosamine	CAS 66-84-2	C00329	N-Source, other
PM03 B	E09	D-Galactosamine	CAS 1772-03-8	C02262	N-Source, other
PM03 B	E10	D-Mannosamine	CAS 5505-63-5	C03570	N-Source, other
PM03 B	E11	N-Acetyl-D-Glucosamine	CAS 7512-17-6	C00140	N-Source, other
PM03 B	E12	N-Acetyl-D-Galactosamine	CAS 14215-68-0	C01132	N-Source, other
PM03 B	F01	N-Acetyl-D-Mannosamine	CAS 7772-94-3	C00645	N-Source, other
PM03 B	F02	Adenine	CAS 6055-72-7	C00147	N-Source, other
PM03 B	F03	Adenosine	CAS 64503-27-1	C00212	N-Source, other
PM03 B	F04	Cytidine	CAS 6018-48-0	C00475	N-Source, other
PM03 B	F05	Cytosine	CAS 71-30-7	C00380	N-Source, other
PM03 B	F06	Guanine	CAS 635-39-2	C00242	N-Source, other
PM03 B	F07	Guanosine	CAS 118-00-3	C00387	N-Source, other

PM03 B	F08	Thymine	CAS 65-71-4	C00178	N-Source, other
PM03 B	F09	Thymidine	CAS 50-89-5	C00214	N-Source, other
PM03 B	F10	Uracil	CAS 66-22-8	C00106	N-Source, other
PM03 B	F11	Uridine	CAS 58-96-8	C00299	N-Source, other
PM03 B	F12	Inosine	CAS 58-63-9	C00294	N-Source, other
PM03 B	G01	Xanthine	CAS 1196-43-6	C00385	N-Source, other
PM03 B	G02	Xanthosine	CAS 5968-90-1	C01762	N-Source, other
PM03 B	G03	Uric acid	CAS 1198-77-2	C00366	N-Source, other
PM03 B	G04	Alloxan	CAS 2244-11-3	C07599	N-Source, other
PM03 B	G05	Allantoin	CAS 97-59-6	C01551	N-Source, other
PM03 B	G06	Parabanic acid	CAS 120-89-8		N-Source, other
PM03 B	G07	DL-a-Amino-n-Butyric acid	CAS 2835-81-6	C02261	N-Source, other
PM03 B	G08	g-Amino-n-Butyric acid	CAS 56-12-2	C00334	N-Source, other
PM03 B	G09	e-Amino-n-Caproic acid	CAS 60-32-2	C02378	N-Source, other
PM03 B	G10	DL-a-Amino-n-Caprylic acid	CAS 644-90-6		N-Source, other
PM03 B	G11	d-Amino-n-Valeric acid	CAS 627-95-2	C00431	N-Source, other

PM03 B	G12	a-Amino-n-Valeric acid	CAS 6600-40-4	C01826	N-Source, other
PM03 B	H01	Ala-Asp	CAS 20727-65-5	C02871	N-Source, peptide
PM03 B	H02	Ala-Gln	CAS 39537-23-0		N-Source, peptide
PM03 B	H03	Ala-Glu	CAS 13187-90-1		N-Source, peptide
PM03 B	H04	Ala-Gly	CAS 687-69-4		N-Source, peptide
PM03 B	H05	Ala-His	CAS 3253-17-6		N-Source, peptide
PM03 B	H06	Ala-Leu	CAS 3303-34-2	C05035	N-Source, peptide
PM03 B	H07	Ala-Thr	CAS 24032-50-6		N-Source, peptide
PM03 B	H08	Gly-Asn	CAS 1999-33-3		N-Source, peptide
PM03 B	H09	Gly-Gln	CAS 13115-71-4		N-Source, peptide
PM03 B	H10	Gly-Glu	CAS 7412-78-4		N-Source, peptide
PM03 B	H11	Gly-Met	CAS 554-94-9		N-Source, peptide
PM03 B	H12	Met-Ala	CAS 3061-96-9		N-Source, peptide
PM09	A01	1% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A02	2% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A03	3% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A04	4% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A05	5% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl

PM09	A06	5.5% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A07	6% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A08	6.5% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A09	7% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A10	8% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A11	9% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	A12	10% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl
PM09	B01	6% NaCl	CAS 7647-14-5	C13563	osmotic sensitivity, NaCl control
PM09	B02	6% NaCl + Betaine	CAS 107-43-7	C00719	osmolyte, betaine
PM09	B03	6% NaCl + N,N Dimethyl Glycine	CAS 1118-68-9	C01026	osmolyte, dimethylglycine
PM09	B04	6% NaCl + Sarcosine	CAS 107-97-1	C00213	osmolyte, sarcosine
PM09	B05	6% NaCl + Dimethyl Sulphonyl Propionate	Dimethyl Sulphonyl Propionate		osmolyte, dimethyl sulphonyl propionate
PM09	B06	6% NaCl + MOPS	CAS 1132-61-2		osmolyte, MOPS
PM09	B07	6% NaCl + Ectoine	CAS 96702-03-3	C06231	osmolyte, ectoine
PM09	B08	6% NaCl + Choline	CAS 77-91-8	C00114	osmolyte, choline
PM09	B09	6% NaCl + Phosphorylcholine	CAS 4826-71-5	C00588	osmolyte, phosphorylcholine
PM09	B10	6% NaCl + Creatine	CAS 57-00-1	C00300	osmolyte, creatine
PM09	B11	6% NaCl + Creatinine	CAS 19230-81-0	C00791	osmolyte, creatinine
PM09	B12	6% NaCl + L-Carnitine	CAS 44985-71-9	C00318	osmolyte, carnitine
PM09	C01	6% NaCl + KCl	CAS 7447-40-7	C13567	osmolyte, KCl
PM09	C02	6% NaCl + L-Proline	CAS 147-85-3	C00148	osmolyte, proline
PM09	C03	6% NaCl + N-Acetyl-L-Glutamine	CAS 35305-74-9		osmolyte, acetyl glutamine
PM09	C04	6% NaCl + b-Glutamic acid	CAS 1948-48-7		osmolyte, b-glutamate
PM09	C05	6% NaCl + g-Amino-b-Butyric acid	CAS 56-12-2	C00334	osmolyte, g-amino butyric acid
PM09	C06	6% NaCl + Glutathione	CAS 70-18-8	C00051	osmolyte, glutathione
PM09	C07	6% NaCl + Glycerol	CAS 56-81-5	C00116	osmolyte, glycerol

PM09	C08	6% NaCl + Trehalose	CAS 99-20-7	C01083	osmolyte, trehalose
PM09	C09	6% NaCl + Trimethylamine N-oxide	CAS 1184-78-7	C01104	osmolyte, trimethylamine-N-oxide
PM09	C10	6% NaCl + Trimethylamine	CAS 75-50-3	C00565	osmolyte, trimethylamine
PM09	C11	6% NaCl + Octopine	CAS 34522-32-2	C04137	osmolyte, octopine
PM09	C12	6% NaCl + Trigonelline	CAS 535-83-1	C01004	osmolyte, trigonelline
PM09	D01	3% Potassium Chloride	CAS 7447-40-7	C13567	osmotic sensitivity, KCl
PM09	D02	4% Potassium Chloride	CAS 7447-40-7	C13567	osmotic sensitivity, KCl
PM09	D03	5% Potassium Chloride	CAS 7447-40-7	C13567	osmotic sensitivity, KCl
PM09	D04	6% Potassium Chloride	CAS 7447-40-7	C13567	osmotic sensitivity, KCl
PM09	D05	2% Sodium Sulfate	CAS 7757-82-6	C13199	osmotic sensitivity, Na ₂ SO ₄
PM09	D06	3% Sodium Sulfate	CAS 7757-82-6	C13199	osmotic sensitivity, Na ₂ SO ₄
PM09	D07	4% Sodium Sulfate	CAS 7757-82-6	C13199	osmotic sensitivity, Na ₂ SO ₄
PM09	D08	5% Sodium Sulfate	CAS 7757-82-6	C13199	osmotic sensitivity, Na ₂ SO ₄
PM09	D09	5% Ethylene Glycol	CAS 107-21-1	C01380	osmotic sensitivity, ethylene glycol
PM09	D10	10% Ethylene Glycol	CAS 107-21-1	C01380	osmotic sensitivity, ethylene glycol
PM09	D11	15% Ethylene Glycol	CAS 107-21-1	C01380	osmotic sensitivity, ethylene glycol
PM09	D12	20% Ethylene Glycol	CAS 107-21-1	C01380	osmotic sensitivity, ethylene glycol
PM09	E01	1% Sodium Formate	CAS 141-53-7	C00058	osmotic sensitivity, sodium formate
PM09	E02	2% Sodium Formate	CAS 141-53-7	C00058	osmotic sensitivity, sodium formate
PM09	E03	3% Sodium Formate	CAS 141-53-7	C00058	osmotic sensitivity, sodium formate
PM09	E04	4% Sodium Formate	CAS 141-53-7	C00058	osmotic sensitivity, sodium formate
PM09	E05	5% Sodium Formate	CAS 141-53-7	C00058	osmotic sensitivity, sodium formate
PM09	E06	6% Sodium Formate	CAS 141-53-7	C00058	osmotic sensitivity, sodium formate
PM09	E07	2% Urea	CAS 57-13-6	C00086	osmotic sensitivity, urea
PM09	E08	3% Urea	CAS 57-13-6	C00086	osmotic sensitivity, urea
PM09	E09	4% Urea	CAS 57-13-6	C00086	osmotic sensitivity, urea
PM09	E10	5% Urea	CAS 57-13-6	C00086	osmotic sensitivity, urea

PM09	E11	6% Urea	CAS 57-13-6	C00086	osmotic sensitivity, urea
PM09	E12	7% Urea	CAS 57-13-6	C00086	osmotic sensitivity, urea
PM09	F01	1% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F02	2% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F03	3% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F04	4% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F05	5% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F06	6% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F07	7% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F08	8% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F09	9% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F10	10% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F11	11% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	F12	12% Sodium Lactate	CAS 72-17-3	C13960	osmotic sensitivity, sodium lactate
PM09	G01	20mM Sodium Phosphate pH 7	CAS 7601-54-9		osmotic sensitivity, sodium phosphate
PM09	G02	50mM Sodium Phosphate pH 7	CAS 7601-54-9		osmotic sensitivity, sodium phosphate
PM09	G03	100mM Sodium Phosphate pH 7	CAS 7601-54-9		osmotic sensitivity, sodium phosphate
PM09	G04	200mM Sodium Phosphate pH 7	CAS 7601-54-9		osmotic sensitivity, sodium phosphate
PM09	G05	20mM Sodium Benzoate pH 5.2	CAS 532-32-1	D02277	toxicity, benzoate
PM09	G06	50mM Sodium Benzoate pH 5.2	CAS 532-32-1	D02277	toxicity, benzoate
PM09	G07	100mM Sodium Benzoate pH 5.2	CAS 532-32-1	D02277	toxicity, benzoate
PM09	G08	200mM Sodium Benzoate pH 5.2	CAS 532-32-1	D02277	toxicity, benzoate
PM09	G09	10mM Ammonium Sulfate pH 8	CAS 7783-20-2	D08853	toxicity, ammonia
PM09	G10	20mM Ammonium Sulfate pH 8	CAS 7783-20-2	D08853	toxicity, ammonia

PM09	G11	50mM Ammonium Sulfate pH 8	CAS 7783-20-2	D08853	toxicity, ammonia
PM09	G12	100mM Ammonium Sulfate pH 8	CAS 7783-20-2	D08853	toxicity, ammonia
PM09	H01	10mM Sodium Nitrate	CAS 7631-99-4	C00244	toxicity, nitrate
PM09	H02	20mM Sodium Nitrate	CAS 7631-99-4	C00244	toxicity, nitrate
PM09	H03	40mM Sodium Nitrate	CAS 7631-99-4	C00244	toxicity, nitrate
PM09	H04	60mM Sodium Nitrate	CAS 7631-99-4	C00244	toxicity, nitrate
PM09	H05	80mM Sodium Nitrate	CAS 7631-99-4	C00244	toxicity, nitrate
PM09	H06	100mM Sodium Nitrate	CAS 7631-99-4	C00244	toxicity, nitrate
PM09	H07	10mM Sodium Nitrite	CAS 7632-00-0	C00088	toxicity, nitrite
PM09	H08	20mM Sodium Nitrite	CAS 7632-00-0	C00088	toxicity, nitrite
PM09	H09	40mM Sodium Nitrite	CAS 7632-00-0	C00088	toxicity, nitrite
PM09	H10	60mM Sodium Nitrite	CAS 7632-00-0	C00088	toxicity, nitrite
PM09	H11	80mM Sodium Nitrite	CAS 7632-00-0	C00088	toxicity, nitrite
PM09	H12	100mM Sodium Nitrite	CAS 7632-00-0	C00088	toxicity, nitrite
PM10	A01	pH 3.5	pH		pH, growth at 3.5
PM10	A02	pH 4	pH		pH, growth at 4
PM10	A03	pH 4.5	pH		pH, growth at 4.5
PM10	A04	pH 5	pH		pH, growth at 5
PM10	A05	pH 5.5	pH		pH, growth at 5.5
PM10	A06	pH 6	pH		pH, growth at 6
PM10	A07	pH 7	pH		pH, growth at 7
PM10	A08	pH 8	pH		pH, growth at 8
PM10	A09	pH 8.5	pH		pH, growth at 8.5
PM10	A10	pH 9	pH		pH, growth at 9
PM10	A11	pH 9.5	pH		pH, growth at 9.5
PM10	A12	pH 10	pH		pH, growth at 10
PM10	B01	pH 4.5	pH		pH, decarboxylase control

PM10	B02	pH 4.5 + L-Alanine	CAS 56-41-7	C00041	pH, decarboxylase
PM10	B03	pH 4.5 + L-Arginine	CAS 1119-34-2	C00062	pH, decarboxylase
PM10	B04	pH 4.5 + L-Asparagine	CAS 70-47-3	C00152	pH, decarboxylase
PM10	B05	pH 4.5 + L-Aspartic acid	CAS 3792-50-5	C00049	pH, decarboxylase
PM10	B06	pH 4.5 + L-Glutamic acid	CAS 142-47-2	C00025	pH, decarboxylase
PM10	B07	pH 4.5 + L-Glutamine	CAS 56-85-9	C00064	pH, decarboxylase
PM10	B08	pH 4.5 + Glycine	CAS 56-40-6	C00037	pH, decarboxylase
PM10	B09	pH 4.5 + L-Histidine	CAS 5934-29-2	C00135	pH, decarboxylase
PM10	B10	pH 4.5 + L-Isoleucine	CAS 73-32-5	C00407	pH, decarboxylase
PM10	B11	pH 4.5 + L-Leucine	CAS 61-90-5	C00123	pH, decarboxylase
PM10	B12	pH 4.5 + L-Lysine	CAS 657-27-2	C00047	pH, decarboxylase
PM10	C01	pH 4.5 + L-Methionine	CAS 63-68-3	C00073	pH, decarboxylase
PM10	C02	pH 4.5 + L-Phenylalanine	CAS 63-91-2	C00079	pH, decarboxylase
PM10	C03	pH 4.5 + L-Proline	CAS 147-85-3	C00148	pH, decarboxylase
PM10	C04	pH 4.5 + L-Serine	CAS 56-45-1	C00065	pH, decarboxylase
PM10	C05	pH 4.5 + L-Threonine	CAS 72-19-5	C00188	pH, decarboxylase
PM10	C06	pH 4.5 + L-Tryptophan	CAS 73-22-3	C00078	pH, decarboxylase
PM10	C07	pH 4.5 + L-Citrulline	CAS 372-75-8	C00327	pH, decarboxylase
PM10	C08	pH 4.5 + L-Valine	CAS 72-18-4	C00183	pH, decarboxylase
PM10	C09	pH 4.5 + Hydroxy-L-Proline	CAS 51-35-4	C01015	pH, decarboxylase
PM10	C10	pH 4.5 + L-Ornithine	CAS 3184-13-2	C00077	pH, decarboxylase
PM10	C11	pH 4.5 + L-Homoarginine	CAS 1483-01-8		pH, decarboxylase
PM10	C12	pH 4.5 + L-Homoserine	CAS 672-15-1	C00263	pH, decarboxylase
PM10	D01	pH 4.5 + Anthranilic acid	CAS 118-92-3	C00108	pH, decarboxylase
PM10	D02	pH 4.5 + L-Norleucine	CAS 327-57-1	C01933	pH, decarboxylase
PM10	D03	pH 4.5 + L-Norvaline	CAS 6600-40-4	C01826	pH, decarboxylase
PM10	D04	pH 4.5 + DL- α -Amino-n-Butyric acid	CAS 2835-81-6		pH, decarboxylase

PM10	D05	pH 4.5 + p-Aminobenzoate	CAS 555-06-6	C00568	pH, decarboxylase
PM10	D06	pH 4.5 + L-Cysteic acid	CAS 23537-25-9	C00506	pH, decarboxylase
PM10	D07	pH 4.5 + D-Lysine	CAS 923-27-3	C00739	pH, decarboxylase
PM10	D08	pH 4.5 + 5-Hydroxy-L-Lysine	CAS 1190-94-9	C01211	pH, decarboxylase
PM10	D09	pH 4.5 + 5-Hydroxy-L-Tryptophan	CAS 4350-09-8		pH, decarboxylase
PM10	D10	pH 4.5 + DL- α , ϵ -Diamino-Pimelic acid	CAS 2577-62-0	C00680	pH, decarboxylase
PM10	D11	pH 4.5 + Trimethylamine N-oxide	CAS 1184-78-7	C01104	pH, decarboxylase
PM10	D12	pH 4.5 + Urea	CAS 57-13-6	C00086	pH, decarboxylase
PM10	E01	pH 9.5	pH		pH, deaminase control
PM10	E02	pH 9.5 + L-Alanine	CAS 56-41-7	C00041	pH, deaminase
PM10	E03	pH 9.5 + L-Arginine	CAS 1119-34-2	C00062	pH, deaminase
PM10	E04	pH 9.5 + L-Asparagine	CAS 70-47-3	C00152	pH, deaminase
PM10	E05	pH 9.5 + L-Aspartic acid	CAS 3792-50-5	C00049	pH, deaminase
PM10	E06	pH 9.5 + L-Glutamic acid	CAS 142-47-2	C00025	pH, deaminase
PM10	E07	pH 9.5 + L-Glutamine	CAS 56-85-9	C00064	pH, deaminase
PM10	E08	pH 9.5 + Glycine	CAS 56-40-6	C00037	pH, deaminase
PM10	E09	pH 9.5 + L-Histidine	CAS 5934-29-2	C00135	pH, deaminase
PM10	E10	pH 9.5 + L-Isoleucine	CAS 73-32-5	C00407	pH, deaminase
PM10	E11	pH 9.5 + L-Leucine	CAS 61-90-5	C00123	pH, deaminase
PM10	E12	pH 9.5 + L-Lysine	CAS 657-27-2	C00047	pH, deaminase
PM10	F01	pH 9.5 + L-Methionine	CAS 63-68-3	C00073	pH, deaminase
PM10	F02	pH 9.5 + L-Phenylalanine	CAS 63-91-2	C00079	pH, deaminase
PM10	F03	pH 9.5 + L-Proline	CAS 147-85-3	C00148	pH, deaminase
PM10	F04	pH 9.5 + L-Serine	CAS 56-45-1	C00065	pH, deaminase
PM10	F05	pH 9.5 + L-Threonine	CAS 72-19-5	C00188	pH, deaminase
PM10	F06	pH 9.5 + L-Tryptophan	CAS 73-22-3	C00078	pH, deaminase
PM10	F07	pH 9.5 + L-Tyrosine	CAS 60-18-4	C00082	pH, deaminase

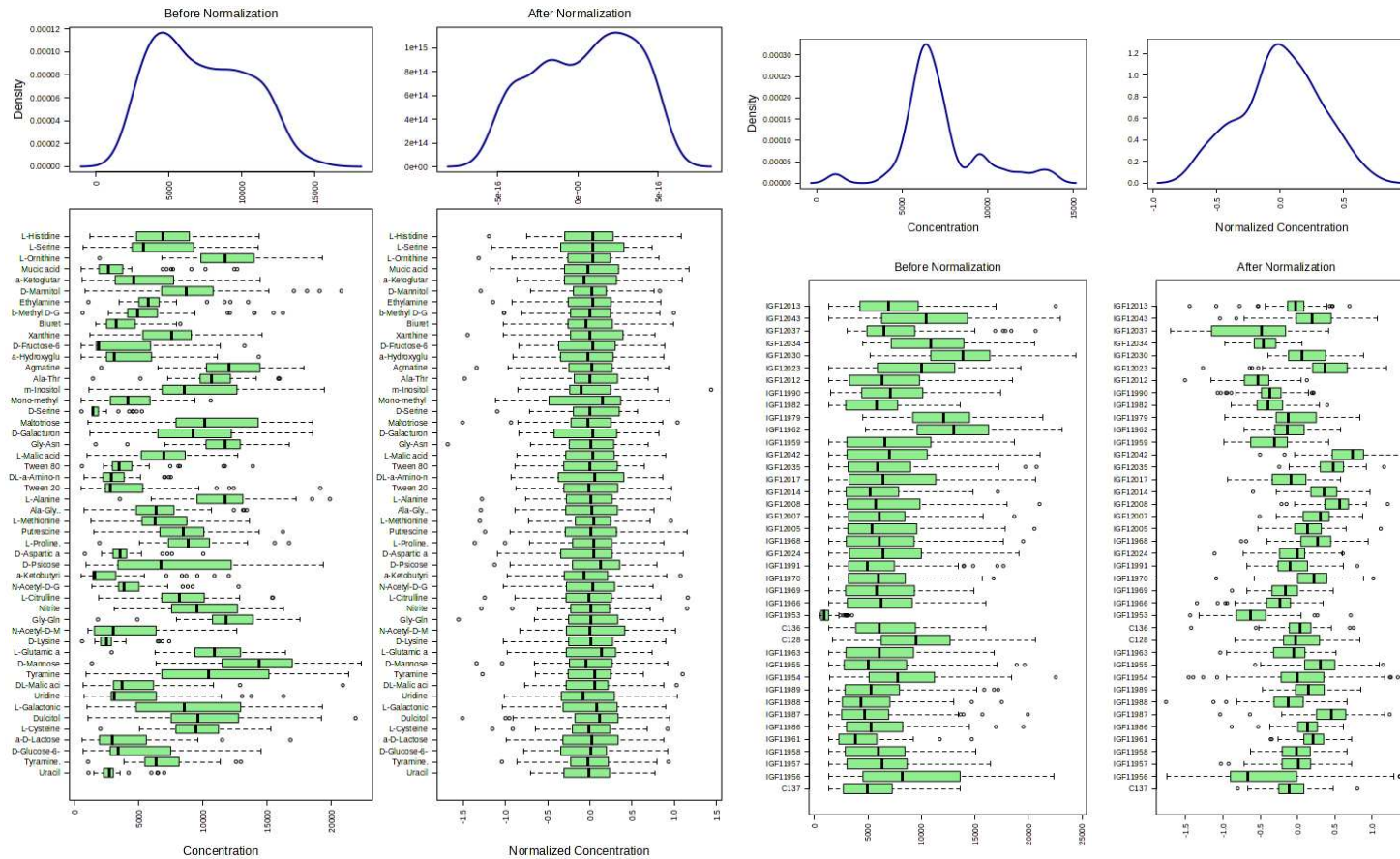
PM10	F08	pH 9.5 + L-Valine	CAS 72-18-4	C00183	pH, deaminase
PM10	F09	pH 9.5 + Hydroxy-L-Proline	CAS 51-35-4	C01015	pH, deaminase
PM10	F10	pH 9.5 + L-Ornithine	CAS 3184-13-2	C00077	pH, deaminase
PM10	F11	pH 9.5 + L-Homoarginine	CAS 1483-01-8		pH, deaminase
PM10	F12	pH 9.5 + L-Homoserine	CAS 672-15-1	C00263	pH, deaminase
PM10	G01	pH 9.5 + Anthranilic acid	CAS 118-92-3	C00108	pH, deaminase
PM10	G02	pH 9.5 + L-Norleucine	CAS 327-57-1	C01933	pH, deaminase
PM10	G03	pH 9.5 + L-Norvaline	CAS 6600-40-4	C01826	pH, deaminase
PM10	G04	pH 9.5 + Agmatine	CAS 2482-00-0	C00179	pH, deaminase
PM10	G05	pH 9.5 + Cadaverine	CAS 462-94-2	C01672	pH, deaminase
PM10	G06	pH 9.5 + Putrescine	CAS 333-93-7	C00134	pH, deaminase
PM10	G07	pH 9.5 + Histamine	CAS 56-92-8	C00388	pH, deaminase
PM10	G08	pH 9.5 + b-Phenylethylamine	CAS 156-28-5	C05332	pH, deaminase
PM10	G09	pH 9.5 + Tyramine	CAS 60-19-5	C00483	pH, deaminase
PM10	G10	pH 9.5 + Creatine	CAS 57-00-1	C00300	pH, deaminase
PM10	G11	pH 9.5 + Trimethylamine N-oxide	CAS 1184-78-7	C01104	pH, deaminase
PM10	G12	pH 9.5 + Urea	CAS 57-13-6	C00086	pH, deaminase
PM10	H01	X-Caprylate	CAS 129541-42-0		caprylate esterase
PM10	H02	X-a-D-Glucoside	CAS 108789-36-2		a-D-glucosidase
PM10	H03	X-b-D-Glucoside	CAS 15548-60-4		b-D-glucosidase
PM10	H04	X-a-D-Galactoside	CAS 107021-38-5		a-D-galactosidase
PM10	H05	X-b-D-Galactoside	CAS 7240-90-6		b-D-galactosidase
PM10	H06	X-a-D-Glucuronide	X-a-D-Glucuronide		a-D-glucuronidase
PM10	H07	X-b-D-Glucuronide	X-b-D-Glucuronide		b-D-glucuronidase
PM10	H08	X-b-D-Glucosaminide	X-b-D-Glucosaminide		b-D-glucosaminidase
PM10	H09	X-b-D-Galactosaminide	X-b-D-Galactosaminide		b-D-galactosaminidase
PM10	H10	X-a-D-Mannoside	CAS 125229-64-3		a-D-mannosidase

PM10	H11	X-PO4	CAS 102185-33-1		aryl phosphatase
PM10	H12	X-SO4	CAS 6578-07-0		aryl sulfatase

[illegible]

[illegible]

[illegible]



B

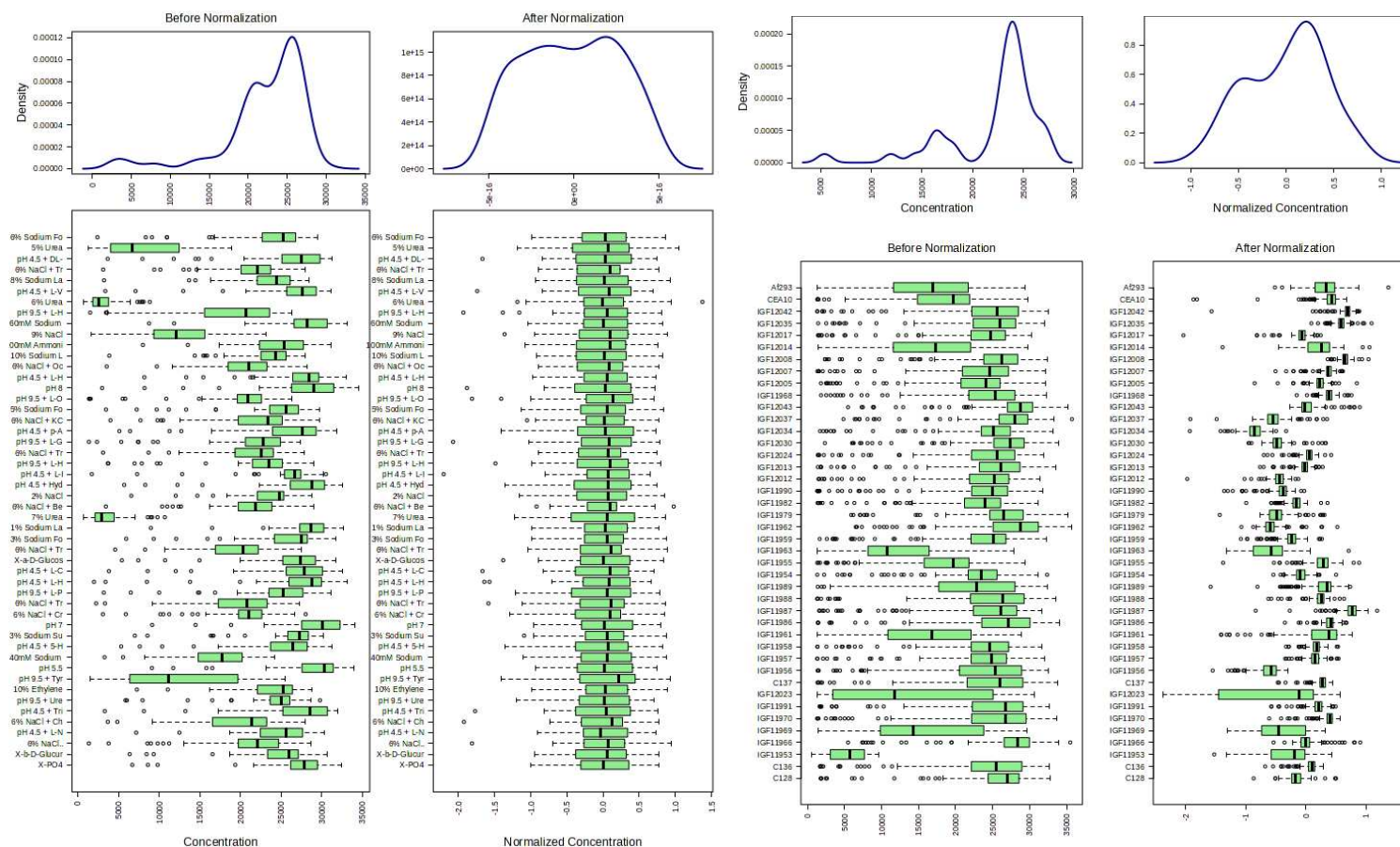


Figure 6A: Box plots and kernel density plots before and after normalization patients with two groups and four variables CF vs. non-CF and resistant vs. susceptible. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: Normalization by a reference feature; Data transformation: Log10 Normalization; Data scaling: Pareto Scaling. **A)** kernel density plots for carbon and nitrogen conditions. **B)** Kernel density plots for osmolytes and pH conditions

Figure 7A: Violin plots of significant metabolites that differentiated between 42 of azole-resistant and azole-susceptible *A. fumigatus* isolates.

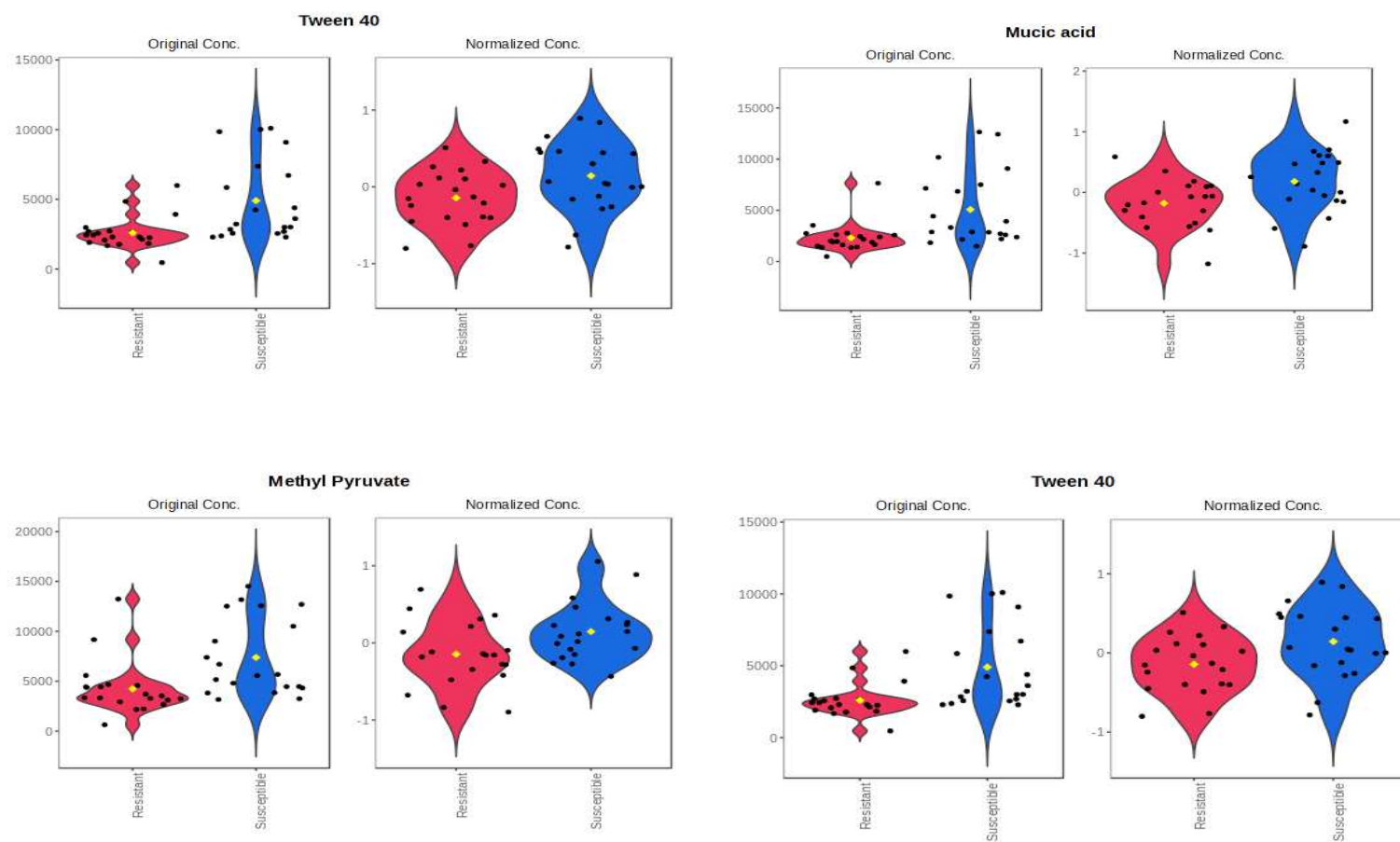
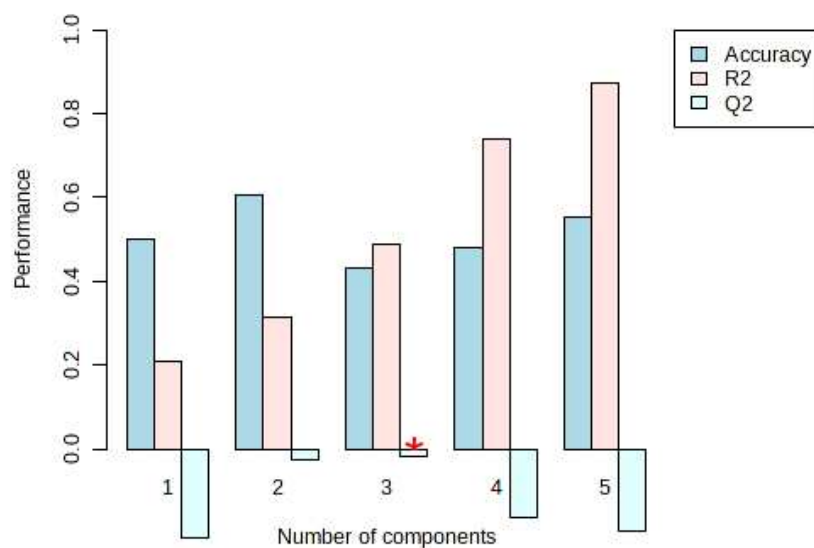


Figure 8A: Cross validation of Partial Least Squares Discriminant Analysis (PLS-DA). Q2 is an estimate of the predictive ability of the model and is calculated *via* cross-validation (CV). In each CV, the predicted data are compared with the original data, and the sum of squared errors is calculated. The prediction error is then summed over all samples (Predicted Residual Sum of Squares or PRESS). For convenience, the PRESS is divided by the initial sum of squares and subtracted from 1 to resemble the scale of the R2. Good predictions will have low PRESS or high Q2. It is possible to have negative Q2, which means that your model is not at all predictive or is overfitted (Szymańska et al., 2012). A) for carbon and nitrogen conditions B) for osmolytes and pH conditions between resistant and susceptible groups.

A



B

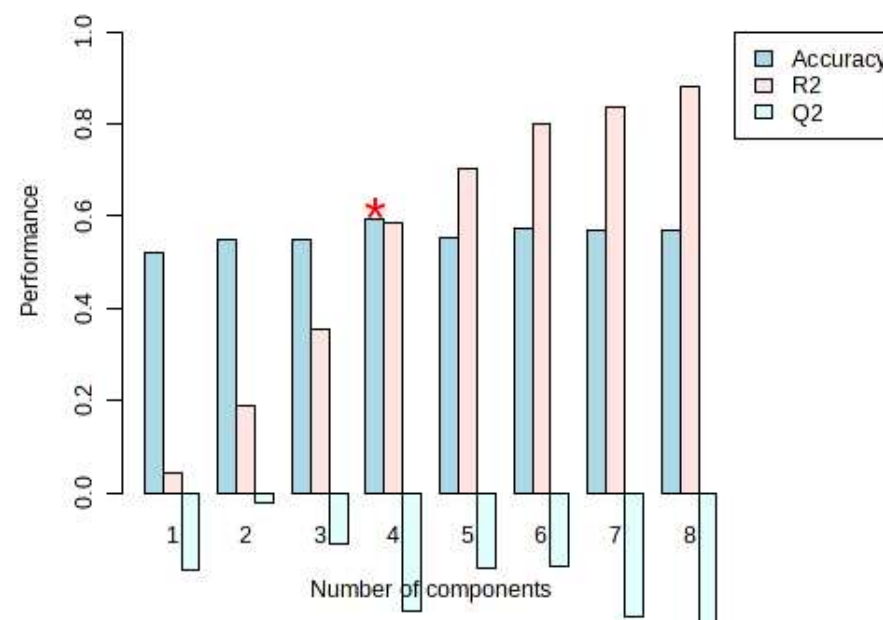


Figure 9A: Phylogenetic reconstruction all 42 *A. fumigatus* isolates with bootstrap support over 1000 replicates performed on WGS SNP data to generate a maximum-likelihood phylogeny.

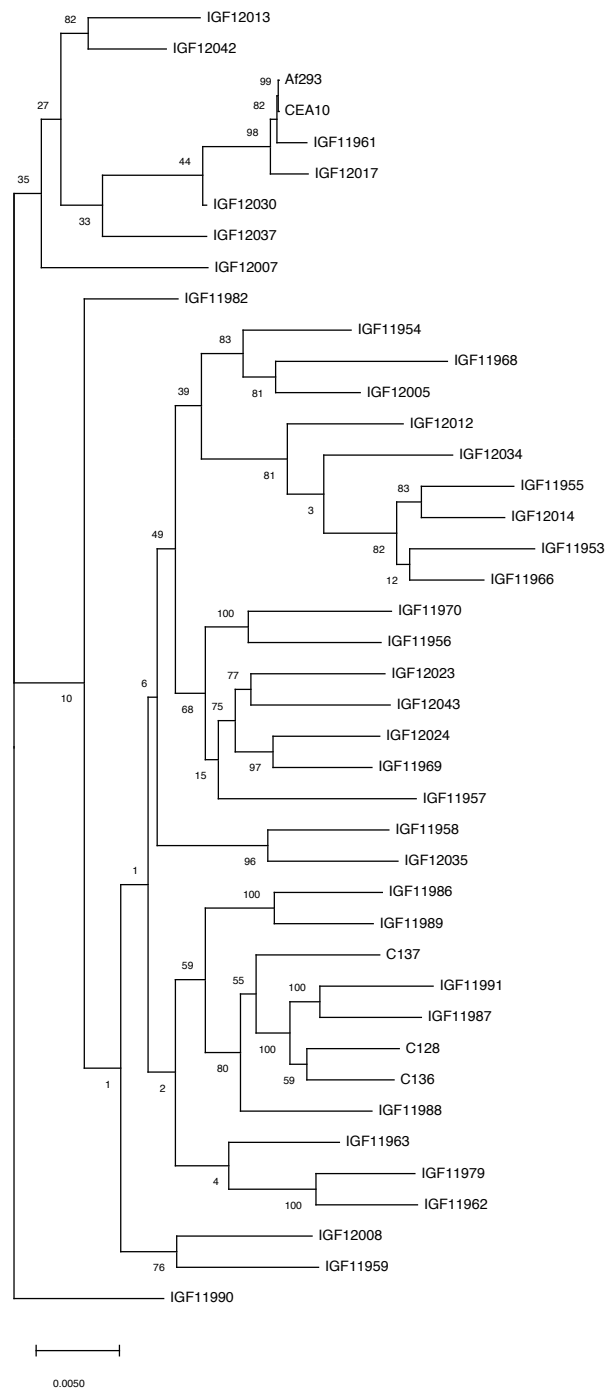


Figure 10A: Phylogenetic analysis of all 42 *A. fumigatus* isolates was generated by GWAS analysis on WGS SNP data.

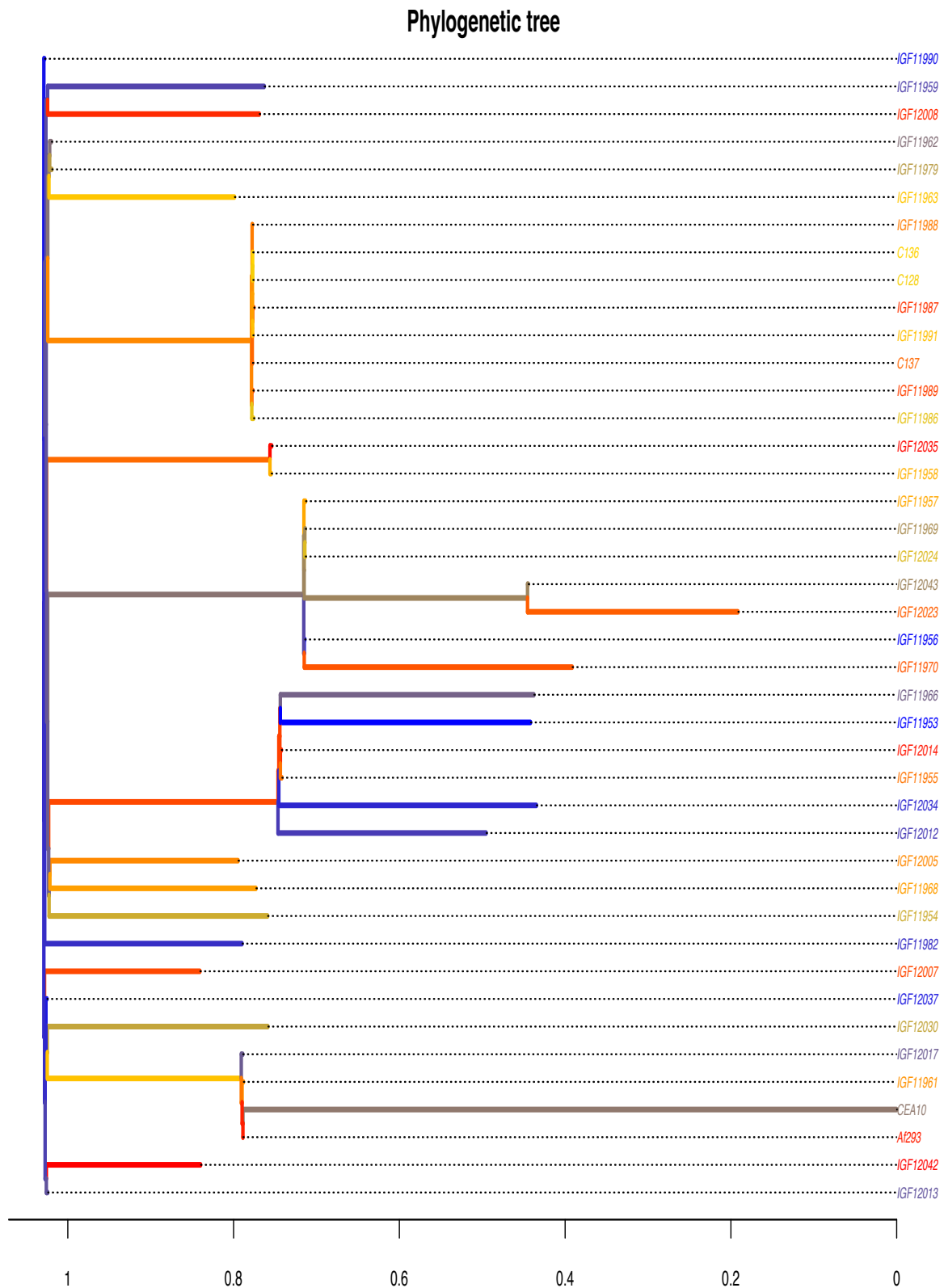


Table 6A: Descriptions of genes associated with the histamine phenotype, along with relevant Gene Ontology (GO) term analysis, revealed enrichment in significant biological processes. These identified genes were sourced from FungiDB (Basenko et al., 2018)

GeneID	Product Description	Gene Name or Symbol	Computed GO Components	Computed GO Functions	Computed GO Processes	Curated GO Functions	Curated GO Processes
AFUA_1G01910	Ortholog of A. nidulans FGSC A4 : AN0367, A. niger CBS 513.88 : An01g06350, <i>Aspergillus wentii</i> : Aspwe1_0025686, <i>Aspergillus sydowii</i> : Aspsy1_0027946 and <i>Aspergillus terreus</i> NIH2624 : ATET_04904	N/A	Golgi membrane; integral component of membrane	glycosyltransferase activity	GPI anchor biosynthetic process	molecular function	biological process
AFUA_1G01960	Has domain (s) with predicted RNA polymerase II transcription factor activity, sequence-specific DNA binding, transcription factor activity,	N/A	host cell nucleus	DNA-binding transcription factor activity, RNA polymerase II-specific; zinc ion binding	regulation of transcription, DNA-templated	N/A	N/A

	sequence-specific DNA binding, zinc ion binding activity						
AFUA_1G02060	Ortholog (s) have ATPase activity, role in meiotic DNA double-strand break formation, meiotic recombination checkpoint, reciprocal meiotic recombination and nucleolus localization	N/A	nucleolus	ATP binding;ATP hydrolysis activity	meiotic DNA double-strand break formation; meiotic recombination checkpoint signaling;reciprocal meiotic recombination	N/A	meiotic recombination checkpoint signaling;reciprocal meiotic recombination
AFUA_1G02140	Putative glycogen debranching enzyme	N/A	cytoplasm	4-alpha-glucanotransferase activity;amylase activity;alpha-1,6-glucosidase activity; catalytic activity	glycogen catabolic process	4-alpha-glucanotransferase activity;amylase activity;alpha-1,6-glucosidase activity	glycogen catabolic process
AFUA_1G05860	Ortholog of A. nidulans FGSC A4 : AN4104, A. niger CBS 513.88 : An18g03590, A. oryzae RIB40 :	N/A	N/A	N/A	N/A	molecular function	biological process

	AO09000900035 4, <i>Aspergillus</i> wentii : Aspwe1_0026045 and <i>Aspergillus</i> sydowii : Aspsy1_0089091						
AFUA_1G17200	Fusarinine C non- ribosomal peptide synthetase (NRPS), putative	sidC	N/A	catalytic activity; ligase activity; phosphopantet heine binding	cellular response to oxidative stress; ferrichrome biosynthetic process; intracellular sequestering of iron ion; nonribosomal peptide biosynthetic process; obsolete pathogenesis	phosphopan tetheine binding	amino acid activation for nonribosomal peptide biosynthetic process; cellular iron ion homeostasis; cellular response to iron ion starvation; ferrichr ome biosynthetic process; ferricroci n biosynthetic process; nonribosomal peptide biosynthetic process; secondar y metabolic process; secondary metabolite bios
AFUA_2G00730	Has domain (s) with predicted	N/A	N/A	FAD binding; flavin adenine	obsolete oxidation- reduction process	N/A	N/A

	UDP-N-acetylmuramate dehydrogenase activity, flavin adenine dinucleotide binding activity and role in oxidation-reduction process			dinucleotide binding; oxidoreductase activity			
AFUA_2G04140	Ortholog of A. nidulans FGSC A4 : AN5221, A. oryzae RIB40 : AO090005001543, <i>Aspergillus wentii</i> : Aspwe1_0172392, <i>Aspergillus sydowii</i> : Aspsyl_0047769 and <i>Aspergillus terreus</i> NIH2624 : ATET_03670	N/A	N/A	N/A	N/A	molecular function	biological process
AFUA_2G04290	Ortholog (s) have role in steroid metabolic process	N/A	integral component of membrane	heme binding; iron ion binding; monooxygenase activity; oxidoreductase activity, acting	obsolete oxidation-reduction process; steroid metabolic process	N/A	N/A

				on paired donors, with incorporation or reduction of molecular oxygen			
AFUA_2G04330	Has domain (s) with predicted FAD binding, oxidoreductase activity and role in metabolic process	N/A	integral component of membrane	FAD binding; salicylate 1-monooxygenase activity	N/A	monooxygenase activity	N/A
AFUA_2G04390	Ortholog of A. nidulans FGSC A4 : AN10247, A. oryzae RIB40 : AO09000300022 5, <i>Aspergillus wentii</i> : Aspwe1_0046123 and <i>Aspergillus terreus</i> NIH2624 : ATET_06072	N/A	N/A	N/A	N/A	molecular function	biological_process; maintenance of protein localization in endoplasmic reticulum
AFUA_2G04420	Ortholog of A. nidulans FGSC A4 : AN5126, <i>A. fumigatus</i> Af293 : Afu1g07490, A. niger CBS 513.88 : An11g03310, An07g10050, A.	N/A	integral component of membrane	N/A	N/A	molecular function	biological process

	oryzae RIB40 : AO09001200099 9 and <i>Aspergillus</i> wentii : Aspwe1_0046371						
AFUA_2G04840	Ortholog of A. nidulans FGSC A4 : AN1864, A. niger CBS 513.88 : An11g03760, A. oryzae RIB40 : AO09000300024 6, <i>Aspergillus</i> wentii : Aspwe1_0055095 and <i>Aspergillus</i> sydowii : Aspsyl_0084294	N/A	N/A	N/A	N/A	molecular function	biological process
AFUA_2G04535	Has domain (s) with predicted oxidoreductase activity and role in metabolic process	N/A	N/A	N/A	N/A	N/A	N/A
AFUA_2G04560	Has domain (s) with predicted nucleobase transmembrane transporter activity, role in nucleobase transport,	N/A	integral component of membrane; membrane	transmembrane transporter activity	transmembrane transport	transmembrane transporter activity	N/A

	transmembrane transport and membrane localization						
AFUA_2G04690	Ortholog (s) have ubiquitin-specific protease activity	N/A	endosome	thiol-dependent deubiquitinase	ATP export; cellular response to lithium ion;endocytosis;free ubiquitin chain depolymerization; intralumenal vesicle formation; protein deubiquitination;regulation of DNA replication; ubiquitin recycling; ubiquitin-dependent protein catabolic process; ubiquitin-dependent protein catabolic process via the	N/A	N/A
AFUA_2G04720	Vacuolar protein sorting-associated protein 27, putative	vps27	ESCRT-0 complex; endosome membrane;fungus-type vacuole membrane;p	metal ion binding;phosphatidylinositol-3-phosphate binding	ascospore-type prospore assembly; cytoplasm to vacuole transport by the NVT pathway;endocytosis;intracellular protein transport;	phosphatidylinositol-3-phosphate binding; ubiquitin binding	late endosome to vacuole transport; protein targeting to vacuole;protein transport to vacuole involved in ubiquitin-dependent protein

			rospore membrane		late endosome to vacuole transport; protein transport to vacuole involved in ubiquitin- dependent protein catabolic process via the multivesicular body sorting pathway		catabolic process via the multivesicular body sorting pathway
AFUA_2G04740	Ortholog of A. nidulans FGSC A4 : AN2070, A. niger CBS 513.88 : An11g04410, A. oryzae RIB40 : AO09000300027 8, <i>Aspergillus</i> wentii : Aspwe1_0718370 and <i>Aspergillus</i> sydowii : Aspsy1_0142154	N/A	N/A	N/A	N/A	molecular function	biological process
AFUA_2G04750	Ortholog (s) have Cul3-RING ubiquitin ligase complex localization	N/A	Cul3-RING ubiquitin ligase complex; ubiquitin ligase complex	protein binding	N/A	molecular function	biological process

AFUA_2G04760	Ortholog (s) have role in autophagy, protein secretion	N/A	Atg1/ULK1 kinase complex	N/A	autophagosome assembly;autophagy;protein secretion	molecular function	N/A
AFUA_2G04770	Has domain (s) with predicted RNA polymerase II transcription cofactor activity, role in regulation of transcription from RNA polymerase II promoter and mediator complex localization	N/A	mediator complex	transcription coregulator activity	regulation of transcription by RNA polymerase II	N/A	N/A
AFUA_2G04790	Ortholog of A. nidulans FGSC A4 : AN2082, A. niger CBS 513.88 : An11g04530, A. oryzae RIB40 : AO090003000287, <i>Aspergillus wentii</i> : Aspwe1_0120888 and <i>Aspergillus sydowii</i> : Aspsy1_0127836	N/A	N/A	N/A	N/A	molecular function	biological process
AFUA_2G04850	Ortholog of A. nidulans FGSC A4 : AN2083, A. niger CBS 513.88	N/A	N/A	N/A	N/A	molecular function	biological process

	: An11g04540, <i>A. oryzae</i> RIB40 : AO090003000288, <i>Aspergillus wentii</i> : Aspwe1_0046199 and <i>Aspergillus sydowii</i> : Aspsy1_0039236						
AFUA_2G04860	Ortholog (s) have role in mismatch repair, nucleotide-excision repair and mitotic spindle, nucleus localization	N/A	nucleus	DNA binding; damaged DNA binding	mismatch repair; nucleotide-excision repair	N/A	N/A
AFUA_2G04880	Ortholog of <i>A. nidulans</i> FGSC A4 : AN10243, <i>A. fumigatus</i> Af293 : Afu4g09900, <i>A. oryzae</i> RIB40 : AO090003001243, <i>Aspergillus wentii</i> : Aspwe1_0072976, Aspwe1_0166814 and <i>Aspergillus sydowii</i> : Aspsy1_0141212	N/A	N/A	N/A	N/A	molecular function	biological process

AFUA_2G04920	Ortholog (s) have nuclear periphery localization	N/A	nuclear periphery; nuclear pore cytoplasmic filaments	protein binding; structural constituent of nuclear pore	poly (A)+ mRNA export from nucleus; ribosomal large subunit export from nucleus; ribosomal small subunit export from nucleus	molecular_f unction;tran scription corepressor activity	biological process
AFUA_2G04950	Ortholog of A. nidulans FGSC A4 : AN2088, A. niger CBS 513.88 : An11g04660, A. oryzae RIB40 : AO090003000298, <i>Aspergillus wentii</i> : Aspwe1_0177253 and <i>Aspergillus sydowii</i> : Aspsy1_0054006	N/A	nucleus	N/A	kinetochore assembly	molecular function	biological processes;mitotic sister chromatid segregation
AFUA_2G04960	Ortholog (s) have role in double-strand break repair, post replication repair, production of siRNA involved in RNA interference	N/A	N/A	3'-5' DNA helicase activity;ATP binding; catalytic activity;helicase activity;nucleic acid binding;	DNA recombination; DNA repair;DNA replication;cellular metabolic process; double-strand break repair;postreplication repair;posttranscrip	N/A	N/A

				nucleotide binding	tional gene silencing; production of siRNA involved in RNA interference		
AFUA_2G04970	Ortholog (s) have cytoplasm localization	N/A	N/A	protein binding	N/A	molecular function	biological process
AFUA_2G05000	Ortholog (s) have aminopeptidase activity, role in proteolysis involved in cellular protein catabolic process and extracellular region localization	N/A	extracellular region	aminopeptidase activity; peptidase activity	proteolysis; proteolysis involved in cellular protein catabolic process	N/A	N/A
AFUA_2G05010	Ortholog (s) have Rab guanyl-nucleotide exchange factor activity, phosphatidylinositol-3-phosphate binding, phosphatidylinositol-5-phosphate binding, phosphatidylserine binding activity	N/A	Mon1-Ccz1 complex;cytosol;endosome;fungus-type vacuole membrane	Rab guanyl-nucleotide exchange factor activity;phosphatidylinositol-3-phosphate binding;phosphatidylinositol-5-phosphate binding; phosphatidylserine binding	autophagy; cytoplasm to vacuole transport by the Cvt pathway;macroautophagy;positive regulation of GTPase activity; protein targeting to vacuolar membrane; protein targeting to vacuole;vesicle docking	N/A	N/A

AFUA_2G05010	Ortholog (s) have role in Golgi to endosome transport, cell morphogenesis, cleistothecium development, conidium formation and endoplasmic reticulum organization, more	N/A	endosome; trans-Golgi network	N/A	Golgi to endosome transport; cell morphogenesis;cleistothecium development;conidium formation;positive regulation of cleistothecium development; positive regulation of conidium formation	molecular function	N/A
AFUA_2G05020	Putative glycosphosphatidylinositol (GPI)-anchored cell wall protein	mp2	extracellular region	N/A	N/A	molecular function	biological process
AFUA_2G05021	Has domain (s) with predicted nucleic acid binding, transcription factor activity, sequence-specific DNA binding, zinc ion binding activity, role in regulation of transcription, DNA-templated	N/A	nucleus	DNA-binding transcription factor activity; helicase activity;metal ion binding;zinc ion binding	regulation of transcription, DNA-templated	RNA binding	heterochromatin assembly by small RNA;pericentric heterochromatin assembly

	and nucleus localization						
AFUA_2G05150	Has domain (s) with predicted substrate-specific transmembrane transporter activity, transmembrane transporter activity, role in transmembrane transport and integral component of membrane, membrane localization	N/A	integral component of membrane; membrane; plasma membrane	transmembrane transporter activity	transmembrane transport	carbohydrate:proton symporter activity	glucose import
AFUA_2G05180	Has domain (s) with predicted oxidoreductase activity and role in oxidation-reduction process	N/A	N/A	oxidoreductase activity	obsolete oxidation-reduction process	N/A	N/A
AFUA_2G05190	Ortholog of A. nidulans FGSC A4 : AN2111, A. fumigatus Af293 : Afu6g03190, A. niger CBS 513.88 : An11g05190, A. oryzae RIB40 :	N/A	N/A	N/A	N/A	molecular function	biological process

	AO09001000062 8, AO09000300033 2 and <i>Aspergillus</i> <i>wentii</i> : Aspwe1_0055162						
AFUA_2G05220	putative transcription factor	rfeD	N/A	N/A	N/A	molecular function	biological process
AFUA_2G05230	protein of unknown function	N/A	N/A	protein binding	N/A	molecular function	biological process
AFUA_2G05250	Has domain (s) with predicted role in transmembrane transport and integral component of membrane localization	N/A	integral component of membrane	N/A	transmembrane transport	molecular function	N/A
AFUA_2G05280	Has domain (s) with predicted DNA binding, RNA polymerase II transcription factor activity, sequence-specific DNA binding, zinc ion binding activity and role in regulation of transcription,	N/A	host cell nucleus; nucleus	DNA binding; DNA-binding transcription factor activity, RNA polymerase II- specific; zinc ion binding	regulation of transcription, DNA-templated; transcription, DNA-templated	N/A	N/A

	DNA-templated, transcription, DNA-templated						
AFUA_2G05330	Vacuolar membrane-associated protein iml1, putative	iml1	GATOR1 complex; fungal-type vacuole membrane	GTPase activator activity	cellular response to nitrogen starvation; intracellular signal transduction;negative regulation of TORC1 signalling	N/A	cellular response to amino acid starvation; negative regulation of TOR signaling;regulation of autophagy
AFUA_2G05380	Ortholog (s) have nucleolus localization	N/A	nucleus	N/A	rRNA processing	molecular function	biological process
AFUA_2G05390	Mitochondrial NADH dehydrogenase involved in oxidative phosphorylation	N/A	intrinsic component of mitochondrial inner membrane	NADPH dehydrogenase activity; calcium ion binding;oxidoreductase activity	obsolete oxidation-reduction process	N/A	N/A
AFUA_2G05420	Ortholog (s) have role in attachment of mitotic spindle microtubules to kinetochore, cohesin localization to chromatin, mitotic sister chromatid cohesion and negative	N/A	SMC loading complex;nuclear chromosome	chromatin binding; double-stranded DNA binding	mitotic cohesin loading; mitotic cohesin ssDNA (lagging strand) loading; mitotic sister chromatid segregation;positive regulation of maintenance of mitotic sister chromatid	chromatin binding	double-strand break repair; establishment of mitotic sister chromatid cohesion; establishment of protein localization to chromatin; mitotic

	regulation of G0 to G1 transition, more				cohesion; positive regulation of mitotic cohesin loading; regulation of gene expression		chromosome condensation; mitotic sister chromatid cohesion; obsolete transcriptional activation by promoter-enhancer looping; positive regulation of mitotic cohesin 1
AFUA_2G05450	Ortholog (s) have histone binding activity, role in histone exchange and Swr1 complex, cytoplasm localization	N/A	Swr1 complex; nucleus	histone binding	chromatin remodeling; histone exchange; regulation of transcription, DNA-templated	N/A	histone exchange
AFUA_2G05470	Ortholog of A. nidulans AN7482	N/A	N/A	N/A	N/A	molecular function	biological process
AFUA_2G05620	Ortholog (s) have mRNA guanylyltransferase activity, role in 7-methylguanosine RNA capping and nucleus localization	N/A	mRNA cap methyltransferase complex	GTP binding; mRNA guanylyltransferase activity	7-methylguanosine RNA capping; 7-methylguanosine mRNA capping	mRNA guanylyltransferase activity	7-methylguanosine mRNA capping; secondary metabolite biosynthetic process

AFUA_2G05690	Transcription activator of gluconeogenesis acuK, putative	acuK	host cell nucleus; nucleus	DNA binding; DNA-binding transcription factor activity, RNA polymerase II-specific; DNA-binding transcription repressor activity, RNA polymerase II-specific; RNA polymerase II transcription regulatory region sequence-specific DNA binding; protein heterodimerization activity; sequence-specific DNA binding	cellular response to starvation; filamentous growth; filamentous growth of a population of unicellular organisms in response to biotic stimulus; filamentous growth of a population of unicellular organisms in response to starvation; negative regulation of transcription by RNA polymerase II; negative regulator	N/A	carbon utilization; obsolete pathogenesis; regulation of transcription, DNA-templated; secondary metabolite biosynthetic process
AFUA_2G05780	Putative MFS multidrug transporter	N/A	N/A	transmembrane transporter activity	transmembrane transport	molecular function; xenobiotic transmembrane	secondary metabolite biosynthetic process; xenobiotic transmembrane transport

						transporter activity	
AFUA_2G05830	Putative golgi apparatus Ca ²⁺ /Mn ²⁺ P-type ATPase	pmr1	Golgi membrane; endoplasmic reticulum membrane; integral component of membrane; plasma membrane	ABC-type manganese transporter activity; ATP binding; P-type calcium transporter activity; calcium ion binding; nucleotide binding	calcium ion import; calcium ion transmembrane transport; cellular calcium ion homeostasis; cellular manganese ion homeostasis; cellular response to drug; conidiophore development; conidium formation; hyphal growth; macroautophagy; manganese ion transmembrane transport; manganese ion transport; regulation of gr	ABC-type manganese transporter activity; ATP hydrolysis activity; P-type calcium transporter activity; P-type proton-exporting transporter activity	calcium ion transmembrane transport; calcium ion transport; cellular calcium ion homeostasis; fungal-type cell wall organization; manganese ion transport
AFUA_2G05840	Ortholog (s) have small-subunit processome localization	N/A	small-subunit processome; viral nucleocapsid	protein binding	N/A	molecular_function	biological_processes
AFUA_2G05860	Ortholog (s) have fungal-type vacuole	N/A	integral component of membrane	N/A	N/A	molecular_function	biological_processes

	membrane localization						
AFUA_2G05930	Ortholog (s) have U3 snoRNA binding, U4 snRNA binding activity and role in mRNA splicing, via spliceosome, maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	N/A	U4/U6 x U5 tri-snRNP complex;box C/D RNP complex;nucleolus;ribonucleoprotein complex;small-subunit processome	RNA binding;U3 snoRNA binding;U4 snRNA binding	mRNA splicing, via spliceosome;maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA);ribosome biogenesis	RNA binding	mRNA splicing, via spliceosome;maturation of LSU-rRNA;maturation of SSU-rRNA
AFUA_2G05940	JmjC domain-containing histone demethylation protein 1, putative	jhd1	host cell nucleus;nucleus	DNA-binding transcription factor activity, RNA polymerase II-specific;histone H3-methyl-lysine-36 demethylase activity;zinc ion binding	regulation of transcription, DNA-templated	N/A	biological_processes
AFUA_2G05950	Ortholog of <i>S. cerevisiae</i> : TDA6, <i>A. nidulans</i> FGSC	N/A	N/A	N/A	N/A	molecular_function	biological_processes

	A4 : AN7454, <i>A. niger</i> CBS 513.88 : An02g14280, <i>A. oryzae</i> RIB40 : AO090001000716 and <i>Aspergillus wentii</i> : Aspwe1 0055229						
AFUA_2G05970	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_023340	N/A	N/A	N/A	N/A	molecular_function	biological_processes
AFUA_2G05980	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_023350	N/A	integral component of membrane	N/A	N/A	molecular_function	biological_processes
AFUA_2G06260	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_023340	N/A	N/A	N/A	N/A	molecular_function	biological_processes
AFUA_2G06270	Ortholog of <i>Aspergillus fumigatus</i> A1163 : AFUB_023350	N/A	integral component of membrane	N/A	N/A	molecular_function	biological_processes
AFUA_2G06320	Ortholog (s) have single-stranded DNA binding activity and role in DNA repair, mitotic DNA replication, premeiotic DNA	N/A	DNA replication factor A complex;nuclear chromatin;nuclear chromosom	DNA binding;nucleic acid binding;single-stranded DNA binding	DNA recombination;DNA repair;DNA replication;DNA unwinding involved in DNA replication;mitotic DNA	N/A	N/A

	replication, telomere maintenance		e, telomeric region;nucle us;site of DNA damage;site of double- strand break		replication;negative regulation of mitotic cohesin ssDNA (lagging strand) loading;positive regulation of telomere maintenance via telomerase;premeio tic DNA replication;telomer e maintenance		
AFUA_2G06330	Putative ubiquitin C-terminal hydrolase	N/A	N/A	protein binding;thiol- dependent deubiquitinase; zinc ion binding	protein deubiquitination;ub iquitin-dependent protein catabolic process	N/A	N/A
AFUA_2G07640	Ortholog of A. nidulans FGSC A4 : AN5826, A. niger CBS 513.88 : An05g00180, Neosartorya fischeri NRRL 181 : NFIA_083370, <i>Aspergillus wentii</i> : Aspwe1_0043748 and <i>Aspergillus</i>	N/A	N/A	N/A	N/A	molecular_f unction	biological_proces s

	versicolor : Aspve1_0082576						
AFUA_2G07670	Ortholog (s) have palmitoyltransferase activity and role in protein palmitoylation, protein targeting to membrane, regulation of endocytosis, regulation of pheromone-dependent signal transduction involved in conjugation with cellular fusion	N/A	Golgi apparatus	G-protein beta/gamma-subunit complex binding;palmitoyltransferase activity;protein binding	negative regulation of pheromone-dependent signal transduction involved in conjugation with cellular fusion;protein palmitoylation;protein targeting to membrane;regulation of endocytosis	N/A	N/A
AFUA_2G07680	L-ornithine N5-oxygenase, first committed step in siderophore biosynthesis	sidA	N/A	N/A	cellular response to oxidative stress;ferrichrome biosynthetic process	N,N-dimethylamine monooxygenase activity;NADP+ binding;iron ion binding;monooxygenase activity;obsolete	cellular iron ion homeostasis;cellular response to iron ion starvation;ergosterol biosynthetic process;obsolete pathogenesis;secondary metabolite biosynthetic process;siderophore biosynthetic process;siderophore

						coenzyme binding	re-dependent iron import into cell
AFUA_2G08230	Has domain (s) with predicted role in transmembrane transport and integral component of membrane localization	N/A	N/A	transmembrane transporter activity	transmembrane transport	molecular_function;transmembrane transporter activity	transmembrane transport
AFUA_2G10080	Has domain (s) with predicted zinc ion binding activity	N/A	cytoplasm	protein binding	sporulation resulting in formation of a cellular spore	histone-lysine N-methyltransferase activity	biological processes;histone lysine methylation
AFUA_2G10090	40S ribosomal protein S15	N/A	ribosome;small ribosomal subunit	RNA binding;structural constituent of ribosome	rRNA export from nucleus;translation	structural constituent of ribosome	ribosomal small subunit assembly
AFUA_2G10130	Predicted adhesin-like protein, ortholog of A. nidulans AN5993	N/A	N/A	N/A	N/A	N/A	biological processes
AFUA_2G10590	Ortholog (s) have fungal-type vacuole localization	N/A	integral component of membrane; membrane	transmembrane transporter activity	transmembrane transport	molecular_function	biological processes
AFUA_2G10560	Ortholog (s) have protein disulfide	N/A	endoplasmic	protein disulfide	N/A	N/A	N/A

	isomerase activity, role in protein folding and Golgi apparatus, endoplasmic reticulum localization		reticulum;integral component of membrane	isomerase activity			
AFUA_2G12940	Ortholog (s) have role in double-strand break repair via homologous recombination, replication fork processing and nucleus localization	N/A	nucleus	N/A	DNA repair;cellular response to DNA damage stimulus;double-strand break repair via homologous recombination;replication fork processing	molecular_ function	double-strand break repair via homologous recombination;replication fork processing
AFUA_2G13860	Histone H4	N/A	host cell nucleus;nucleosome;nucleus;replication fork protection complex	DNA binding;protein heterodimerization activity	histone H3-K79 methylation;sexual sporulation resulting in formation of a cellular spore;transfer RNA gene-mediated silencing	DNA binding	nucleosome assembly
AFUA_2G18070	Has domain (s) with predicted metalloendopeptidase activity, metallopeptidase	N/A	extracellular region	metal ion binding;metalloendopeptidase activity;metall	proteolysis	N/A	N/A

	activity and role in proteolysis			opeptidase activity			
AFUA_3G03720	protein of unknown function	N/A	N/A	N/A	N/A	molecular_f unction	biological_proces s
AFUA_3G03760	protein of unknown function	N/A	N/A	N/A	N/A	molecular_f unction	biological_proces s
AFUA_3G12120	Putative fatty acid oxygenase	ppoC	N/A	N/A	asexual sporulation resulting in formation of a cellular spore;fatty acid metabolic process;hydrogen peroxide catabolic process;negative regulation of asexual sporulation resulting in formation of a cellular spore;negative regulation of sexual sporulation resulting in formation of a cellular spore;o	N/A	prostaglandin biosynthetic process
AFUA_4G14300	Has domain (s) with predicted GTP binding, GTPase activity	N/A	N/A	N/A	N/A	GTPase activity;mic rotubule binding	biological_proces s;dynamain family protein polymerization involved in mitochondrial fission;membrane

							fusion;mitochondrial fission;organelle fission;peroxisome fission
AFUA_4G14310	Ortholog of A. nidulans FGSC A4 : AN6463, AN7976, A. fumigatus Af293 : Afu8g01590 and A. niger CBS 513.88 : An15g05580, An11g01710, An12g06830, An11g08100, An08g01130, An07g04970, An03g04950, An09g06370, An15g02610	N/A	N/A	N/A	N/A	molecular_function	biological_processes
AFUA_5G09260	Ortholog of A. nidulans FGSC A4 : AN0243, A. niger CBS 513.88 : An07g03750, A. oryzae RIB40 : AO090020000523, Aspergillus wentii : Aspwe1 0101961	N/A	N/A	N/A	N/A	molecular_function	biological_processes

	and <i>Aspergillus</i> sydowii : Aspsyl_0055067						
AFUA_5G06820	Ortholog of A. niger CBS 513.88 : An03g00400, A. oryzae RIB40 : AO090701000306, Neosartorya fischeri NRRL 181 : NFIA_036290 and <i>Aspergillus</i> niger ATCC 1015 : 45807-mRNA	N/A	N/A		N/A	N/A	molecular_function
AFUA_5G14340	Has domain (s) with predicted oxidoreductase activity and role in metabolic process	N/A	N/A		oxidoreductase activity	N/A	N/A
AFUA_5G14530	Ortholog (s) have hydrolase activity, hydrolyzing O-glycosyl compounds activity and role in cellulose catabolic process, positive regulation of cellulose	N/A	host cell nucleus;nucleus		DNA binding;DNA-binding transcription factor activity, RNA polymerase II-specific;hydrolase activity, hydrolyzing O-glycosyl	cellulose catabolic process;positive regulation of cellulose catabolic process;regulation of glycogen metabolic	N/A

	catabolic process, regulation of glycogen metabolic process				compounds;zinc ion binding	process;regulation of transcription, DNA-templated;transcription, DNA-templated	
AFUA_6G03020	Ortholog (s) have monooxygenase activity and role in sterigmatocystin biosynthetic process	N/A	N/A		N,N-dimethylaniline monooxygenase activity;NADP binding;flavin adenine dinucleotide binding;monooxygenase activity	obsolete oxidation-reduction process;sterigmatocystin biosynthetic process	N/A
AFUA_6G07310	Has domain (s) with predicted calcium ion binding, zinc ion binding activity	N/A	integral component of membrane		calcium ion binding;zinc ion binding	N/A	N/A
AFUA_6G07890	Ortholog (s) have role in ascospore formation, cellular response to calcium ion, fungal-type cell wall organization or biogenesis and cell cortex, cell division site, cell	N/A	cell cortex;cytoplasm;division septum		N/A	ascospore formation;cellular response to calcium ion;fungal-type cell wall organization	molecular_function

	septum, cytosol localization					or biogenesis	
AFUA_6G14640	Has domain (s) with predicted role in transmembrane transport and integral component of membrane localization	N/A	N/A		transmembrane transporter activity	transmembrane transport	molecular_function;transmembrane transporter activity
AFUA_7G08510	Putative alpha-1,3-glucanase	N/A	N/A		hydrolase activity	metabolic process	glucan endo-1,3-alpha-glucosidase activity

7 **Table 7A:** Summary of Quality Characteristics of the RNA-seq Data Obtained for *A. fumigatus* Grown Under Histamine and NaCl Conditions

- **Raw Reads:** The total count of reads obtained from the raw data, organized into four rows as a unit, along with statistics on the read counts for each sequencing run.
- **Raw Bases:** The total number of nucleotide bases generated from the raw sequencing data, calculated as the product of the number of raw reads and the sequence length, converted to gigabases (Gb). This quantity represents the sum of nucleotides (A, T, C, G).
- **Clean Reads:** The count of reads filtered from the raw data, reflecting the clean data set. The statistical method for clean reads is similar to that for raw reads. All subsequent analyses are based on clean data.
- **Clean Bases:** The total number of nucleotides bases post-filtering, calculated as the product of the number of clean reads and the sequence length, converted to gigabases (Gb).
- **Error Rate:** The rate of base errors occurring throughout the sequencing process.
- **Q20:** The percentage of total bases with Phred quality scores greater than 20. This is calculated as (Number of bases with Phred value > 20) / (Total number of bases) * 100.
- **Q30:** The percentage of total bases with Phred quality scores greater than 30. This is calculated as (Number of bases with Phred value > 30) / (Total number of bases) * 100.
- **GC_pct:** The percentage of guanine (G) and cytosine (C) bases relative to the total number of bases. This is calculated as (Number of G&C bases) / (Total number of bases) * 100.

Sample	raw_reads	raw_bases	clean_reads	clean_bases	error_rate	Q20	Q30	GC_pct
C32_C2	40608044	6.09G	40210322	6.03G	0.03	97.55	93.53	53.68
C32_C3	52415660	7.86G	51520776	7.73G	0.03	97.54	93.46	53.47
C32_HM1	45881812	6.88G	44243450	6.64G	0.03	96.92	92.21	51.47
C32_HM2	39997912	6G	39599574	5.94G	0.03	97.12	92.53	53.69
C32_HM3	44464230	6.67G	43569838	6.54G	0.03	97.45	93.22	53.39
C32_Na1	44718930	6.71G	44322946	6.65G	0.03	97.66	93.48	53.55
C32_Na2	54363930	8.15G	53285162	7.99G	0.03	97.05	92.3	53.42
IGF12048_C1	51530638	7.73G	48644528	7.3G	0.03	96.86	92.45	52.55

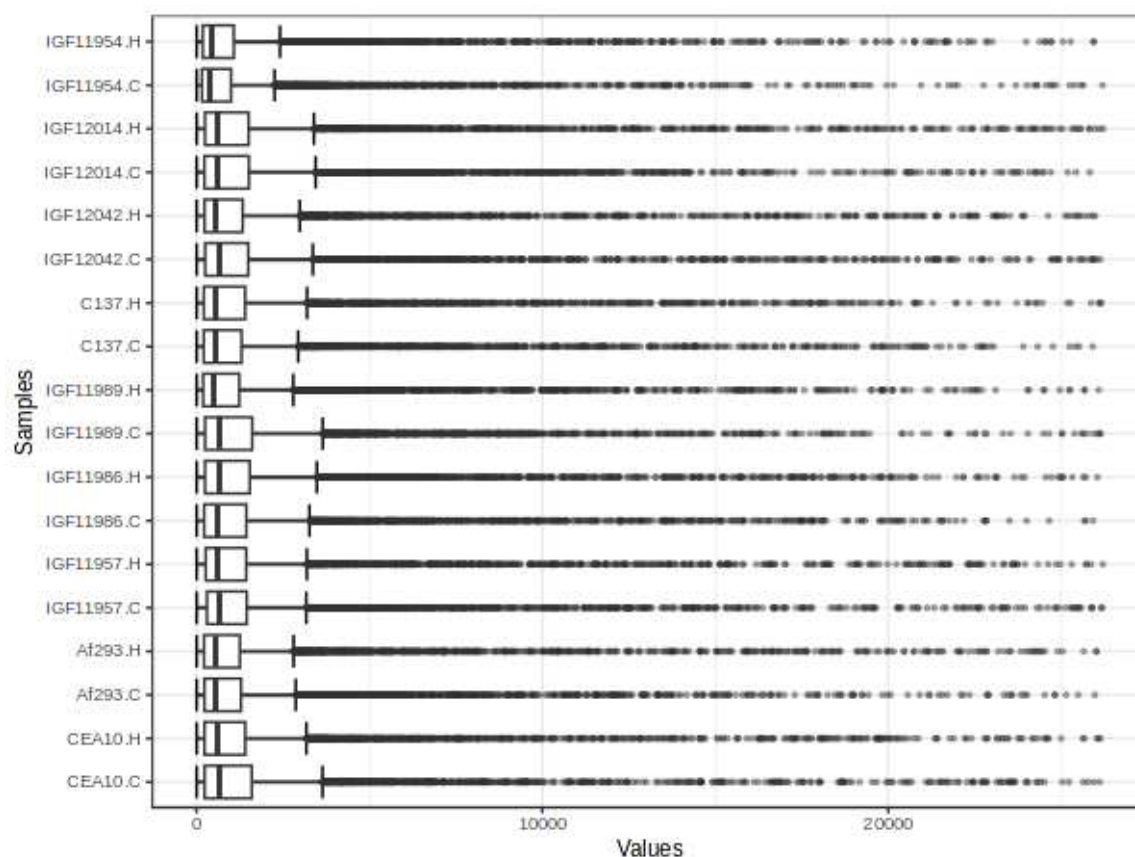
IGF12048_C2	47911982	7.19G	43244376	6.49G	0.03	96.77	92.17	51.73
IGF12048_C3	39522060	5.93G	37754460	5.66G	0.03	97.2	92.86	51.03
IGF12048_HM1	47652032	7.15G	47012134	7.05G	0.03	97.62	93.45	53.25
IGF12048_HM2	43995994	6.6G	42521226	6.38G	0.03	96.85	92.2	52.7
IGF12048_HM3	40412686	6.06G	38649794	5.8G	0.03	97.76	94.1	53.64
IGF12048_Na1	42142454	6.32G	41802054	6.27G	0.03	97.57	93.26	53.75
IGF12048_Na2	44380042	6.66G	43560558	6.53G	0.03	97.59	93.53	52.91
IGF12048_Na3	47504284	7.13G	45498380	6.82G	0.03	97.2	92.95	47.49
IGF11957_C1	48269172	7.24G	47711408	7.16G	0.03	97.4	93.01	52.72
IGF11957_C2	46498474	6.97G	45971996	6.9G	0.03	97.61	93.66	53.03
IGF11957_C3	45829468	6.87G	45216492	6.78G	0.03	97.4	93.17	52.61
IGF11957_HM1	46562994	6.98G	45352582	6.8G	0.03	97.08	92.5	52.7
IGF11957_HM2	44669138	6.7G	44069942	6.61G	0.03	97.25	92.84	53.09
IGF11957_HM3	45857618	6.88G	44921506	6.74G	0.03	97.44	93.33	52.79
IGF11957_Na1	35181866	5.28G	34370960	5.16G	0.03	97.42	92.99	52.28
IGF11957_Na2	42265824	6.34G	41810708	6.27G	0.03	97.53	93.46	52.83
IGF11957_Na3	47731740	7.16G	47118964	7.07G	0.03	97.28	92.83	53.17
IGF11986_C1	47670706	7.15G	47206322	7.08G	0.03	97.38	93.04	53.68
IGF11986_C2	45948684	6.89G	45238590	6.79G	0.03	97.46	93.26	52.88
IGF11986_HM1	38589612	5.79G	38128724	5.72G	0.03	97.19	92.41	53.37
IGF11986_HM2	49137490	7.37G	48666364	7.3G	0.03	97.56	93.48	53.6
IGF11986_HM3	42087276	6.31G	41263186	6.19G	0.03	97.51	93.42	53.24
IGF11986_Na1	42785022	6.42G	42262826	6.34G	0.03	97.5	93.41	52.73
IGF11986_Na2	43699550	6.55G	43316230	6.5G	0.03	97.5	93.37	53.72
IGF11986_Na3	46364706	6.95G	45504658	6.83G	0.03	97.61	93.61	53.28
IGF11989_C1	47317870	7.1G	46941898	7.04G	0.03	97.71	93.66	53.53
IGF11989_C2	42446618	6.37G	41838176	6.28G	0.03	97.53	93.5	52.81

IGF11989_C3	58171842	8.73G	56850740	8.53G	0.03	97.44	93.24	53.27
IGF11989_HM1	47267550	7.09G	46555216	6.98G	0.03	97.47	93.14	53.14
IGF11989_HM2	44525140	6.68G	44015970	6.6G	0.03	97.33	93.04	52.38
IGF11989_HM3	44023222	6.6G	43280720	6.49G	0.03	97.81	94.02	53.38
IGF11989_Na1	56630576	8.49G	56130482	8.42G	0.03	97.43	93.06	53.31
IGF11989_Na2	52805680	7.92G	52356280	7.85G	0.03	97.52	93.35	53.47
IGF11989_Na3	45512676	6.83G	44706048	6.71G	0.03	97.36	93.03	53.42
C137_C1	41048660	6.16G	39985026	6.0G	0.03	97.39	93.05	51.93
C137_C2	47412554	7.11G	46991532	7.05G	0.03	97.63	93.61	53.36
C137_C3	44449996	6.67G	43623032	6.54G	0.03	97.85	94.08	53.08
C137_HM1	46031664	6.9G	45672762	6.85G	0.03	97.58	93.23	53.36
C137_HM2	49501596	7.43G	48681088	7.3G	0.03	97.34	92.99	53.43
C137_HM3	43964194	6.59G	43239904	6.49G	0.03	97.56	93.47	52.98
C137_Na1	41230460	6.18G	40834746	6.13G	0.03	97.47	93.12	53.39
C137_Na2	51543784	7.73G	50696082	7.6G	0.03	97.43	93.16	53.49
C137_Na3	50056754	7.51G	49241602	7.39G	0.03	97.41	93.17	53.52
IGF12042_C1	50596054	7.59G	50177360	7.53G	0.03	97.34	92.85	53.66
IGF12042_C2	44427920	6.66G	43282198	6.49G	0.03	97.36	93.13	52.9
IGF12042_C3	42510250	6.38G	41974758	6.3G	0.03	97.26	92.77	53.6
IGF12042_HM1	46994048	7.05G	46507738	6.98G	0.03	97.42	93.04	53.48
IGF12042_HM2	40804024	6.12G	40092564	6.01G	0.03	97.49	93.4	53
IGF12042_HM3	55162910	8.27G	54285486	8.14G	0.03	97.35	93.05	52.61
IGF12042_Na1	45448850	6.82G	45098134	6.76G	0.03	97.54	93.23	53.67
IGF12042_Na2	51239928	7.69G	50336494	7.55G	0.03	97.46	93.29	52.97
IGF12042_Na3	44668126	6.7G	44006344	6.6G	0.03	97.58	93.55	53.56
IGF12014_C1	46994290	7.05G	45322312	6.8G	0.03	97.1	92.56	54.35
IGF12014_C2	40709372	6.11G	39886070	5.98G	0.03	97.52	93.42	53.4

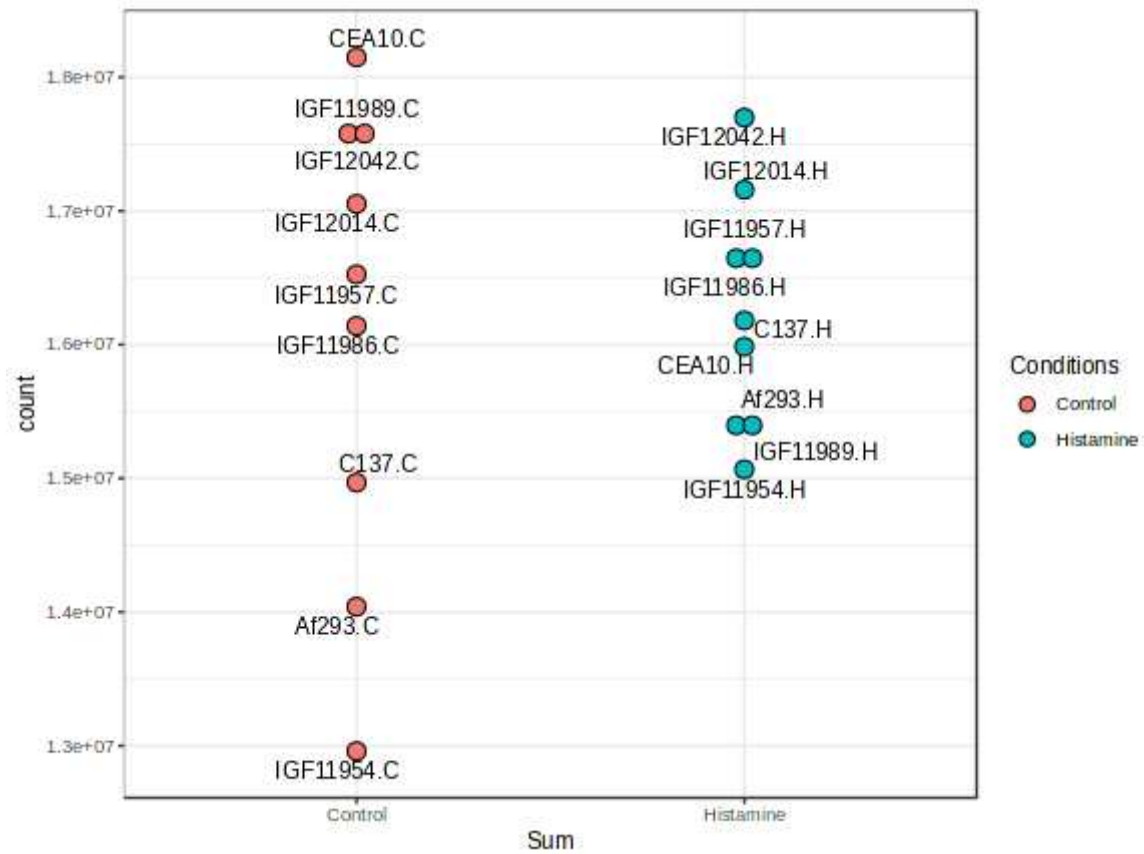
IGF12014_C3	46368342	6.96G	45816502	6.87G	0.03	97.78	94	53.53
IGF12014_HM1	48185852	7.23G	47708238	7.16G	0.03	97.6	93.61	53.68
IGF12014_HM2	41607404	6.24G	40715342	6.11G	0.03	97.35	93.05	53.25
IGF12014_HM3	43318940	6.5G	42665194	6.4G	0.03	97.4	93.13	53.61
IGF12014_Na1	43444638	6.52G	42923084	6.44G	0.03	96.93	92.14	53.4
IGF12014_Na2	46506758	6.98G	45820396	6.87G	0.03	97.29	92.88	53.46
IGF12014_NA3	39515238	5.93G	38868714	5.83G	0.03	97.13	92.38	53.28
IGF11954_C1	34837040	5.23G	32833226	4.92G	0.03	96.74	92.02	52.81
IGF11954_C2	48919870	7.34G	47989328	7.2G	0.03	97.41	93.31	52.56
IGF11954_C3	42931748	6.44G	39657684	5.95G	0.03	96.61	91.85	50.79
IGF11954_HM1	44414530	6.66G	42890686	6.43G	0.03	97.2	92.83	51.62
IGF11954_HM2	46883558	7.03G	45411536	6.81G	0.03	96.9	92.23	51.99
IGF11954_HM3	45407062	6.81G	44294216	6.64G	0.03	97.21	92.79	52.29
IGF11954_Na1	39716490	5.96G	38653070	5.8G	0.03	96.88	92.13	51.97
ICF11954_Na2	51005164	7.65G	50050936	7.51G	0.03	97.49	93.36	52.6

Figure 11A: RNA-seq QC normalisation deseq2 data for Histamine against the control condition. **A)** Normalized count distributions are displayed as box plots. **B)** Principal component analysis (PCA) showing the variance between the individual samples. **C)** QC counts plot the standard deviation of the transformed data, across samples, against the mean, using the shifted log transformation, the regularized log transformation and the variance stabilizing transformation. **D)** Density plot shows how all the samples have very similar gene expression profile what makes them comparable for the differential expression analysis.

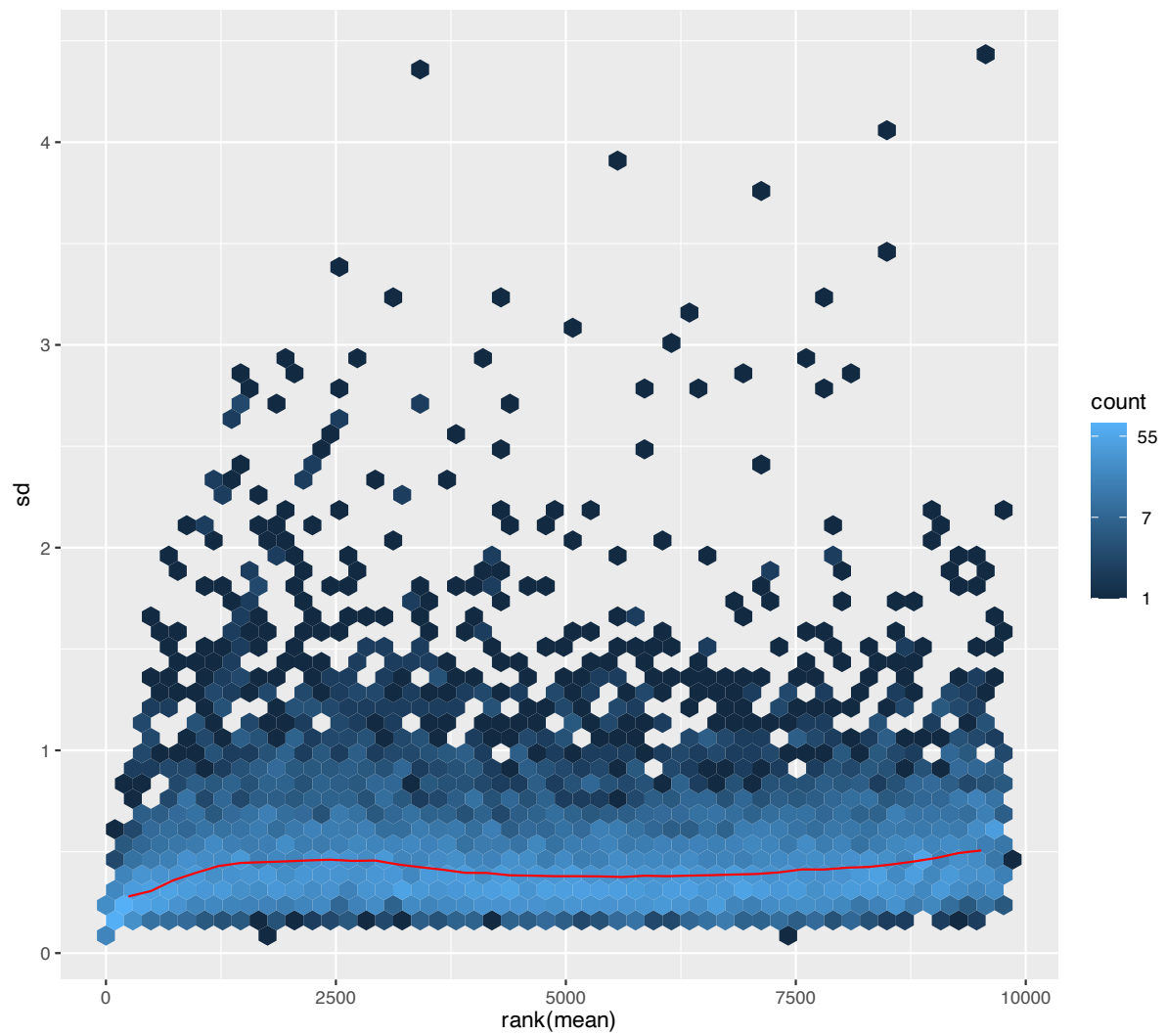
A) QC Bar plot of the transformed and normalized read count



B) QC PCA plot showing variance between the isolates under the same condition



C) QC Counts plot



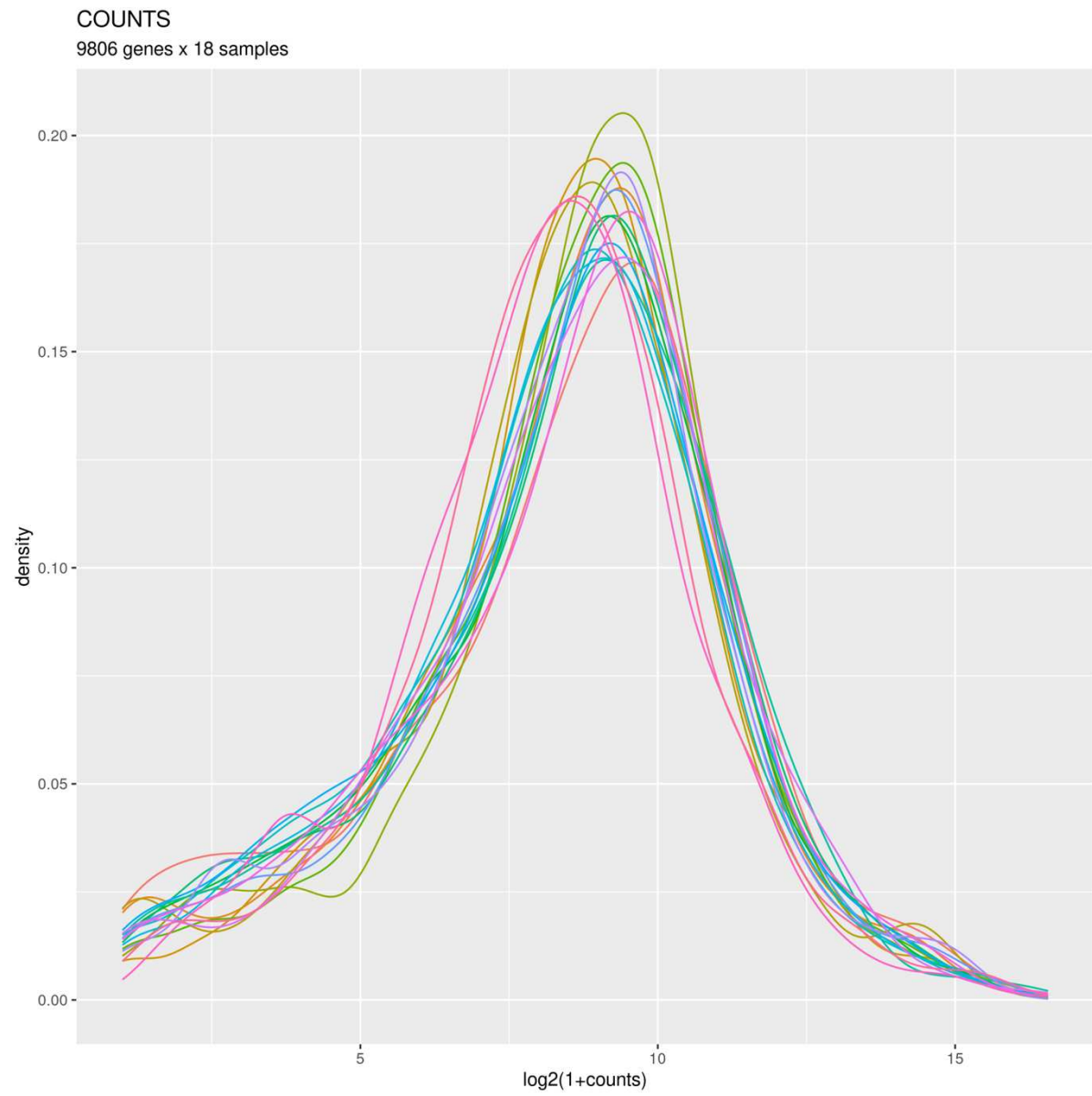
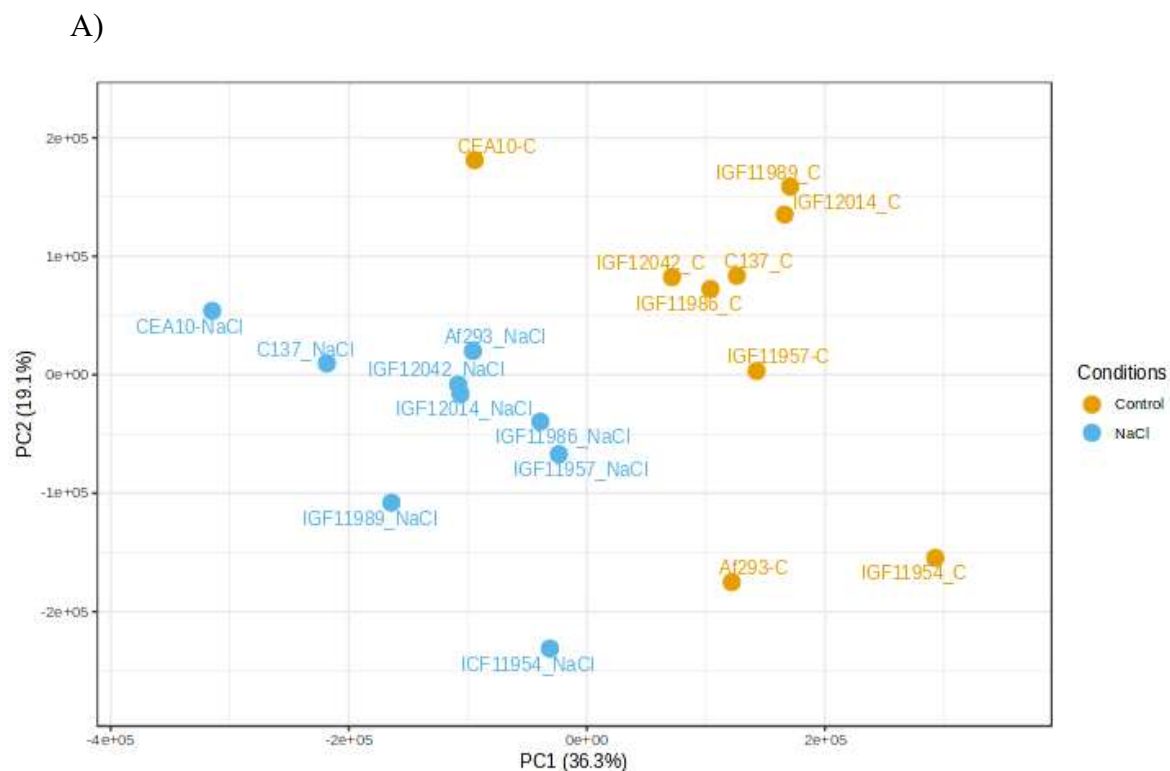
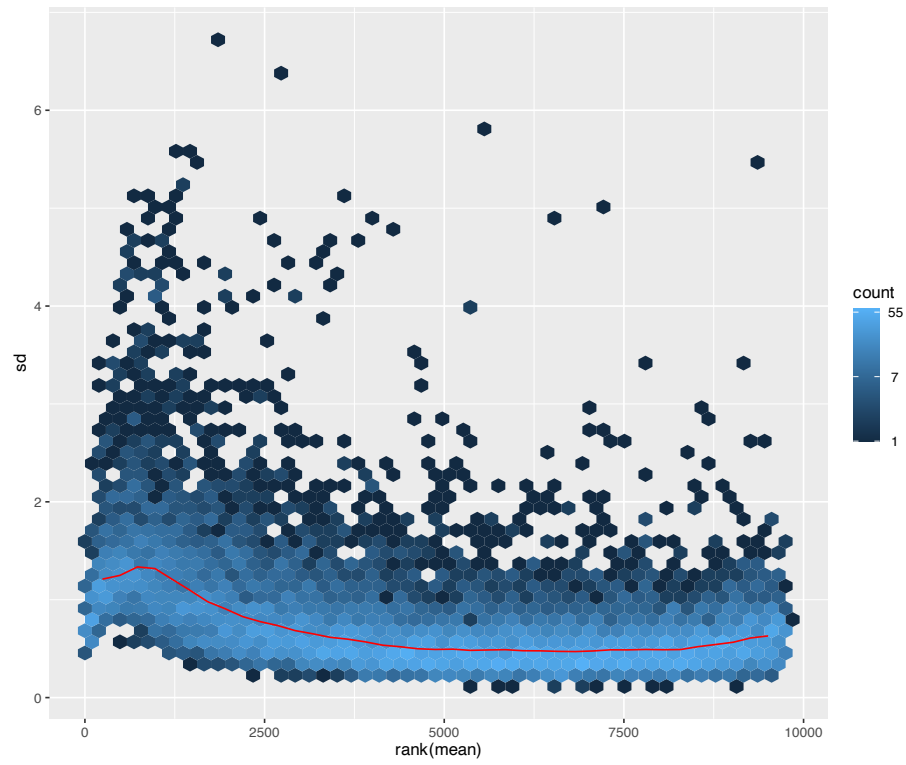
D) QC Density plot

Figure 12A: RNA-seq QC normalisation deseq2 data for NaCl against the control condition.

A) PCA showing the variance between the individual samples. The PCA showed a close clustering of the individual samples and a clear separation between the different conditions. Approximately 55.4% of the transcriptome variation among the compared samples could be explained in PC1 (36.3%) and PC2 (19.1%), indicating clear variation in transcriptome profiles among the two conditions. **B)** QC counts plot the standard deviation of the transformed data, across samples, against the mean, using the shifted log transformation, the regularized log transformation and the variance stabilizing transformation.

C) Normalized count distributions are displayed as box plots.



B)

D)

