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Review

Proteomic Analysis of *Aspergillus fumigatus* – Clinical Applications.

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Abstract

Introduction: Aspergillus fumigatus is a ubiquitous saprophytic fungus capable of producing small airborne spores, which are frequently inhaled by humans. In healthy individuals, the fungus is rapidly cleared by innate mechanisms, including immune cells. However, in individuals with impaired lung function or immunosuppression the spores can germinate and prompt severe allergic responses, and disease with limited or extensive invasiveness. *Areas Covered:* The traits that make *A. fumigatus* a successful colonizer and pathogen of humans are multi-factorial. Thus, a global investigative approach is required to elucidate the mechanisms utilized by the fungus to cause disease. *Expert Commentary:* In doing so, a better understanding of disease pathology can be achieved with improved therapeutic/ diagnostic solutions,

thereby improving patient outcome. Proteomic analysis permits such investigations and recent work has yielded insight into these mechanisms.

Key words

Aspergillus fumigatus, proteome, immunoproteome, infection, secondary metabolism, development, biomarkers, antifungal, adhesion, fungal proteomics

1. Introduction

Aspergillus fumigatus is one of the most prevalent airborne fungal pathogens identified to date. It is a saprophytic mould capable of producing small (2-3 µm) hydrophobic spores. It is estimated that humans inhale several hundred spores per day; however manifestation of disease following inhalation is restricted to certain risk groups [1,2]. Host status represents the main factor in determining the pathogenicity of *A. fumigatus.* In healthy individuals, inhaled conidia are rapidly cleared by the mucociliary escalator. Any conidia that escape this initial defence are subsequently cleared by alveolar macrophages, neutrophils and a T cell-mediated response [2,3]. However, when lung function or the immune system are compromised in some way, *A. fumigatus* can manifest itself in various forms of disease within the host [1,4]. In individuals with impaired lung function, including cystic fibrosis (CF) and asthma, *A. fumigatus* can invoke a hypersensitive allergic response and cause allergic bronchopulmonary aspergillosis (ABPA). Development of ABPA is associated with conidial germination and *A. fumigatus* colonisation. It is characterised by reduced lung function, *Aspergillus* skin reactivity, high IgE levels, and fungal infiltrates [5]. *A. fumigatus* can also cause hypersensitivity reactions in chronic obstructive pulmonary disease (COPD) patients. Non-invasive colonisation of the lung with aspergilloma, also termed “fungus-ball”, can occur in pre-existing lung cavities, for example those resulting from tuberculosis infection. The aspergilloma comprises a mass of hyphae embedded in an extracellular matrix with proteins and host constituents. Surgical removal is often necessary [1,4]. The most notorious manifestation of *A. fumigatus* disease is invasive aspergillosis (IA). IA carries a mortality rate of up to 90% in high-risk patient groups. Risk factors for IA include: neutropenia, COPD, organ or haematopoietic stem-cell transplantation, corticosteroid therapy, haematological malignancy, and AIDS [6,7]. In such cases

inhaled *A. fumigatus* conidia germinate forming hyphae, which can invade the blood vessels and disseminate throughout the host. Lack of early diagnostic methods and limited treatment options are major factors underlying the morbidity of *A. fumigatus* related diseases. Thus, there is a requirement for novel strategies in diagnosing and treating disease caused by *A. fumigatus*.

The virulence of *A. fumigatus* is multifactorial. Survival in its niche environment, where nutrient starvation, temperature fluctuations, and various stresses are encountered, has conferred it with a sophisticated adaptability. This adaptability to harsh environments has translated into survival in susceptible hosts [8]. The nature of its virulence means that a global investigation of the multi-faceted phenotype of *A. fumigatus* is necessary to understand it as a pathogen. Since the sequencing of the *A. fumigatus* genome, "omic" technologies have permitted this insight [9,10]. By analysing all of the proteins in an organism (proteome), mass spectrometry (MS)-based proteomic analysis can inform on its phenotype. A bottom-up approach is predominantly applied for the identification of fungal proteins [11]. In this approach proteins are digested into peptide fragments that are detected by MS (Figure 1). The sequences of these peptides are aligned to *in silico* peptide sequences in databases, to identify their cognate protein. Traditionally protein mixtures are separated by molecular mass alone (1-DE; 1-dimensional gel electrophoresis), or by their isoelectric point and molecular mass (2-DE; 2-dimensional gel electrophoresis), prior to in-gel digestion. However, recent advances in shotgun proteomics allows for analysis of total protein content in a complex peptide mixture. If necessary, pre-fractionation of proteins or peptides can be carried out to reduce sample complexity and improve limits of detection [12–14]. Fractionation according to specific properties can provide further insight into a proteome. Shotgun proteomics has facilitated analysis of proteins previously intractable to 2-DE, such as membrane proteins through gel-free approaches [15,16]. However, 2-DE still plays an important role in analysing post-translational modifications and the immunoproteome [17–24].

Since early global analysis of the *A. fumigatus* proteome provided reference maps [25–28], MS-based proteomics has yielded significant insight into the phenotype of

the organism under a variety of conditions (Figure 1). This review focuses on recent work that has utilised proteomics to provide new information on *A. fumigatus* relevant to infection.

1.1. Understanding the *A. fumigatus* proteome in establishing infection

1.1.1. The dynamic proteome of early development

Characterisation of the proteome of *A. fumigatus* conidia at different stages of early development has revealed it to be highly dynamic (Figure 2) [29–32]. Early work comparing resting conidia with mycelia by 2-DE with LC-MS/MS or MALDI-ToF/ToF identification provided the first global insight into the conidial proteome [25,29]; however the limitations of 2-DE in detecting low abundance proteins and resolution can make this work challenging and cumbersome. Recent work has used quantitative shotgun proteomics to provide a detailed insight into stage-specific proteomic events relevant to early host colonisation [30,31]. Cagas *et al.* used iTRAQ labelling with MALDI-ToF/ToF detection, while in a subsequent study, Suh *et al.* used label-free quantitative analysis with LC-MS/MS to detect proteins with differential abundance over the course of early development.

Suh *et al.* identified 375 different proteins in developing conidia across several time points (0 h, 4 h, 6 h, and 8 h post-inoculation), including several proteins previously not identified in conidia [19,25,29,30]. 143 proteins were detected in all time points, many of which are conserved in other fungi. Proteins enriched in dormant conidia included Pep2 (AFUA_3G11400) and ConJ (AFUA_6G03210). Pep2 is a surface associated endopeptidase with increased abundance under hypoxia, it is also transcriptionally up-regulated following contact of conidia with neutrophils [25,28,29,33,34]. ConJ is transcriptionally up-regulated during early murine infection [35]. The function of ConJ is not yet elucidated in *A. fumigatus*; however a homolog in *Aspergillus nidulans* has been implicated along with another Con protein in germination [36]. Several heat shock proteins (HSPs) were also enriched in resting conidia including Scf1 (AFUA_1G17370). In accordance with Teutschbein *et al.* SodB,

a superoxide dismutase (AFUA_4G11580), was also abundant in dormant conidia [29]. In expanding conidia (4 h), 85 proteins showed increased abundance compared with dormant conidia including several immunoreactive proteins: Wos2/ Sba1 (AFUA_5G13920), as well as allergens, Asp F8 (AFUA_2G10100) and Asp F3 (AFUA_6G02280) [5,18]. Cell wall proteins were also enriched, indicative of the functional cell wall remodelling during early germination. Proteins included the antigenic β-1,3-endoglucanase EglC (AFUA_3G00270) [21], and Ecm33 (AFUA_4G06820) which has roles in anti-fungal tolerance and germination [37]. CipC (AFUA_5G09330), which has been described as a hyphal-specific protein [38], was also enriched in expanding conidia. Its presence in expanding conidia was therefore surprising; however any analysis which identified it as such compared hyphae with germlings at either 6 h or 8 h of growth [32,38]. This work did not detect CipC at 6 h or 8 h and it is possible the protein also functions within early expanding conidia. In hyphae with early germ tubes (6 h), 127 proteins showed increased abundance compared with dormant conidia including proteins shown to be transcriptionally up-regulated during early stages of murine infection: thiazole biosynthetic protein ThiF (AFUA_6G08360), pyridoxine biosynthetic protein PyroA (AFUA_5G08090) and a pyruvate carboxylase (AFUA_4G07710) [35]. Many of the proteins enriched at 6 h were also detected at 8 h after inoculation, including superoxide dismutase SodA (AFUA_5G09240) and glucanase BtgE (AFUA_8G05610), and are also transcriptionally up-regulated during murine infection [35]. Collectively, recent work has indicated the proteome of *A. fumigatus* during early development to be extremely dynamic with significant modifications occurring within the first 8 h. The enrichment of cell wall and stress proteins illustrates the dynamic remodelling and robust stress tolerance required of *A. fumigatus* conidia in the early stages of development [29,31]. The relevance of this proteome to infection is apparent based on the presence of proteins implicated in host recognition and virulence.

Given their proximity, the cell surface proteome of *A. fumigatus* during early development is particularly relevant to host interaction (Figure 2). In a study analysing changes in the cell surface during morphogenesis, the surface proteome of germlings (6 h) was compared with hyphae (72 h) [32]. Using a mild DTT treatment,

proteins transiently linked to the cell wall or via disulphide bonds were extracted. Viability and cell wall integrity was monitored to ensure membrane leakage and intracellular material from dead cells did not account for proteins in extracts. Biotinylation of cell surface proteins prior to extraction and Western blot comparison with SDS-PAGE indicated no additional non-biotinylated intracellular proteins were present, thereby validating the methodology employed. Using 2D-DIGE with MALDI-ToF/ToF, 39 differentially abundant proteins were identified including proteins involved in metabolism, translation, defense and transport. Translation elongation factor, eEF-3 (AFUA_7G05660) was found to be abundant on the surface of germlings but not hyphae. In *Cryptococcus neoformans* eEF-3 interacts with the calcium channel, Cch1 at the plasma membrane [39]. As calcium is important for several cellular activities and eEF-3 is not present in mammalian cells, eEF-3 was proposed as a possible drug target. Interestingly, eEF-3 was also identified as antigenic exclusively in patients with proven aspergillosis so it may represent a biomarker of early infection [23]. The authors also proposed CipC as a drug target, after detection exclusively in hyphae; however previous investigations detected CipC in early expanding conidia, indicating this protein may not exclusively function in hyphae [25,31]. Two putative adhesins, enolase/ Asp F22 (AFUA_6G06770) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; AFUA_5G01970), were identified from the cell-surface proteome using the fungal adhesion prediction algorithm FungalRV [40]. Both proteins are glycolytic enzymes and showed increased abundance in hyphae. GAPDH has been implicated in host adhesion in other species [41,42] while enolase is an allergen and has been identified among antigens expressed during IA [43]. Despite an intracellular function, surface presentation of enolase has recently been confirmed in several fungi including *A. fumigatus* using immunostaining [44]. Lacking a signal peptide, its non-classical secretion mechanism awaits elucidation. However, export of enolase in *Saccharomyces cerevisiae* is dependent on a N-terminal sequence including residues which are highly conserved in other fungi and thus may underlie export [44]. Comprehensive profiling of the cell-surface proteome during development has yet to be achieved, since the extraction method by Kubitschek-Barreira *et al*. did not release proteins covalently linked to carbohydrates in the cell wall, such as GPI-anchored proteins [32]. Also, despite

using an alkali extraction method, Suh *et al.* were not successful in enriching for cell wall-associated proteins based on the abundance of intracellular proteins [31].

The dramatic remodelling undertaken by conidia during early development is concomitant with de-masking of cell wall hydrophobin, RodA (AFUA_ 5G09580), and exposing pattern associated molecular patterns (PAMPs) such as β-(1,3)-glucan, which are recognised by the host [45,46]. Polysaccharides, including glucans such as β-(1,3)-glucan and α-(1,3)-glucan, account for a large proportion of the conidial and hyphal cell wall. Deletion of the genes (*Δags1Δags2Δags3 /Δags*) responsible for α-(1,3)-glucan synthesis resulted in severely attenuated virulence *in vivo* in an immune-competent and immunocompromised murine model [47]. *Δags* conidia were also phagocytosed more efficiently by murine alveolar macrophages *in vitro*. Atomic force microscopy indicated the presence of an amorphous material covering *Δags* conidia; however similar levels of RodA protein were isolated from *Δags* and wild-type. Interestingly, SDS-PAGE and 2-DE with MALDI-ToF/ToF analysis identified a layer of glycoproteins exclusively in *Δags* conidial NaCl extracts. Exposure of alveolar macrophages to this extract stimulated TNFα production. In addition, there was increased expression of polysaccharide PAMPS on the surface of swollen *Δags* conidia. Thus, the authors concluded that deletion of *ags1, ags2* and *ags3* caused significant structural rearrangements of the cell wall resulting in the matrix of glycoproteins masking the rodlet layer in the resting conidia and increased PAMP exposure in germinating conidia. This in turn, led to rapid immunological detection and clearance of *Δags in vivo*. This work highlights the importance of cell wall carbohydrates in the conidial surface proteome and their role in evading early immune recognition during host colonisation.

1.1.2. Fungal adhesin proteins

Adhesion to host cells represents an important point in the initiation and maintenance of infection. Inhaled conidia can adhere to the mucus lining, respiratory epithelium and alveolar macrophages. Following evasion of macrophage

killing and germination, invasive hyphae continue to adhere to host proteins and surface constituents (Figure 2). The fungal carbohydrates and proteins at the cell wall that mediate this process are referred to as adhesins [48–50]. Most fungal adhesin proteins contain a C-terminal glycosylphosphatidylinositol (GPI) anchor to the plasma membrane, a glycosylated serine/ threonine rich domain and a N-terminal domain that mediates ligand binding [40,50]. Characterising host adherence mechanisms in *A. fumigatus* is warranted, as impeding this process represents a potential therapeutic strategy to prevent and/or treat infection.

Using bioinformatic analyses, Upadhyay *et al.* identified putative protein adhesins in *A. fumigatus* based on their amino acid sequence [51]. Proteins (n = 82) were assigned a 'probability of being adhesin' (P_{ad}) value of greater than 0.9. Cell wall protein AfCalAp (AFUA_3G09690), with a P_{ad} = 0.94, was selected for further study. AfCalAp was found to be present on the surface of swollen conidia and to have affinity for laminin and murine lung cells [51]. In a subsequent study, Upadhyay *et al.* showed that supernatant proteins could increase binding of conidia to the extracellular matrix (ECM) [52]. Proteins were analysed by 2-DE, and Western blot using biotinylated fibrinogen, with MALDI-ToF/ToF identification. Seven proteins with fibrinogen binding activity were identified using this approach. Proteins included pectate lyase A (PlyA AFUA_2G00760), which had not previously been implicated in adhesion, and predicted cell wall protein β-glucosidase A (BglA AFUA_1G05770) [52]. Using a similar approach with the aim of a therapeutic strategy, *A. fumigatus* supernatant proteins were extracted for murine immunisations and monoclonal antibody (mAb) development [53]. Interestingly, the isolated mAb showed binding to several supernatant proteins by Western blot. After de-glycosylation of protein extracts, no binding was observed indicating the mAb was active against a glycoprotein carbohydrate moiety. Immunofluorescence indicated the presence of the mAb target antigen on the surface of conidia and hyphae. Importantly, the mAb had anti-adhesive properties *in vitro*, reducing adhesion of *A. fumigatus* conidia to fibronectin-coated plates by 70%. The efficacy of this mAb as a therapeutic strategy remains to be elucidated *in vivo*, but the

inhibition of conidial host adhesion represents a plausible clinical application and promising product of an investigation of the adhesin proteome of *A. fumigatus.*

Fucose-specific lectin (FleA; AFUA_5G14740) was detected in the immunosecretome of *A. fumigatus* supernatant proteins probed with ABPA patient sera [20]. FleA was subsequently identified in protein extracts of an *A. fumigatus* strain isolated from a cancer patient based on hemagglutination activity against rat erythrocytes [54]. Houser *et al.* characterised FleA in detail and showed the protein to have strong binding affinity for the host carbohydrate, fucose. Immunostaining indicated the presence of FleA predominantly on the surface of conidia and not hyphae. Fucose is found on the surface of many human cells and therefore, FleA was postulated to aid initial conidial attachment to lung epithelium [55]. Recently, Kerr *et al.* showed that binding to mucin and macrophages is FleA-dependent. Deletion of FleA resulted in reduced macrophage phagocytosis and increased lung invasion in an immune-competent murine model. Hence, FleA is in fact likely not a virulence factor but a PAMP important in early host recognition of inhaled conidia [56].

The role of proteins in the adhesion of *A. fumigatus* to host cells has been further revealed using proteomics as well as other analytical techniques. However, their position as virulence factors may not be so clear cut, as elucidation of the functionality of FleA has shown. Furthermore, post-translational modification for example, in the form of protein glycosylation, has emerged as an important factor in some adhesin functionality. Further dissection of early molecular events at the host:pathogen interface is warranted to fully understand the role of proteins in host adherence. Recent methodological advances in the recovery of membrane proteins presented by Ouyang *et al.* can help facilitate this characterisation by enabling more in-depth investigations of membrane-associated adhesins [15].

1.1.3. The secretome and proteases

Along with cell surface proteins, the secretome of *A. fumigatus* represents a focal point of the host:pathogen interface comprising various enzymes, adhesins and

toxins [4,57]. The relevance of this in host recognition is exemplified in immunoproteomic analysis indicating the presence of many proteins reactive with IgG and IgE from ABPA, IA and aspergilloma patients [17,20–22]. Using this approach, GliT (AFUA_ 6G09740) was identified as immunoreactive using IA patient sera [22]. Interestingly, GliT was first shown to be immunoreactive with IgG from normal human sera in 2010 [58]. Subsequently, an indirect ELISA for the detection of anti-GliT antibodies was shown to be effective for the diagnosis of IA in animal models [59]. Wartenberg *et al.* comprehensively described the secreted proteome of *A. fumigatus* grown on host-relevant substrates elastin, keratin and collagen [60]. Protease Alp1/ Asp F13 (AFUA_4G11800) was highly abundant on protein substrates and identified as a major secreted protease. In addition to other physiological roles, an ability to hydrolyse peptides means proteases are an important family of proteins in fungi with respect to substrate degradation [61]. Given the ease of extraction and their position at the host:pathogen interface, extracellular proteases are the best studied and many have proven to be allergenic [61]. The secretion of allergenic proteins is particularly important in the manifestation of ABPA. In an attempt to dissect the conditions regulating their secretion, Farnell *et al.* characterised the proteolytic response of *A. fumigatus* to growth on several substrates including casein, mucin and pig lung [62]. Supernatant proteins were separated by size exclusion chromatography, screened for proteolytic activity and analysed by SDS-PAGE with LC-MS/MS identification. Protein growth substrate used was found to impact the levels of protease activity, whereby growth with pig lung led to higher protease activity than that on casein, mucin or the medium control. The predominant class of protease also varied depending on the growth substrate. A proteolytic profile of predominantly metalloproteases including Lap1 (AFUA_3G00650) and Mep/ Asp F5 (AFUA_8G07080) was induced in casein. Conversely, growth in pig lung favoured serine proteases, including Alp1/ Asp F13 [62] which has recently been implicated in airway hyper-responsiveness in asthma patients [63,64]. Complement degrading activity for Alp1 has also been demonstrated, suggesting a role for proteases in infection [65]. By virtue of their ability to degrade macromolecules, a role for proteases in the virulence of pathogenic fungi seems plausible. However, their position as a virulence factor

remains controversial. Gene deletion studies do not indicate a role in virulence. For example, deletion of Alp1 or positive transcriptional regulator of secreted proteases PrtT did not attenuate virulence in a murine model [65–67]. A proteomic and transcriptomic characterisation of Δ*prtT* indicated reduced abundance of several proteases in the secretome [68]. Surprisingly, genes involved other functions such as secondary metabolism were also affected indicating involvement of PrtT in a more complex regulatory network. Moreover, with increased expression of genes and abundance of proteins not found in wild-type, such as polysaccharide degrading enzymes, the authors speculated that the lack of virulence attenuation in Δ*prtT* may be due to a compensatory phenotype in the mutant [68]. In addition, recent work demonstrated that enolase can bind proteolytic host protein, plasminogen, leading these authors to speculate that a commandeering of host proteolytic mechanisms may account for tissue invasion in the *prtT* null strain [44]. Thus, the role of secreted proteases in virulence still awaits unambiguous elucidation. Together, recent work has profiled the secreted proteome relevant to host colonisation and revealed mechanisms of regulation; however further work is required to improve treatment for the allergic responses associated with *A. fumigatus*.

1.1.4. The biofilm proteome

After conidial germination into hyphae, *A. fumigatus* produces an extracellular matrix (ECM), which surrounds hyphae growing in a biofilm. This ECM is composed of polysaccharides, melanin, proteins and extracellular DNA. Importantly, biofilm formation has been implicated in protection from host defences *in vivo* and significantly decreased susceptibility to antifungals [69]. An understanding of biofilm formation in *A. fumigatus* has only begun to emerge in recent years and proteomic analysis serves a major tool in investigating this growth form highly relevant to infection (Figure 2). Using an *in vitro* biofilm model, Bruns *et al.* carried out a proteomic and transcriptomic characterisation of *A. fumigatus* during biofilm development compared with planktonic growth [70]. *A. fumigatus* was grown for 24 h and 48 h and proteins were extracted from mycelia. Nine proteins with differential abundance between the two growth forms were identified using 2D-DIGE with

MALDI-ToF/ToF. In a similar study 25 proteins were identified with differential abundance between the two growth states [71]. Proteins involved in the TCA cycle, protein degradation and amino acid biosynthesis were among those with differential abundance. Interestingly, members of the gliotoxin biosynthetic gene cluster, glutathione *S*-transferase GliG (AFUA_6G09690) and gliotoxin oxidoreductase GliT showed increased abundance during biofilm-growth [70]. Induction of the gliotoxin cluster was verified with increased transcription of cluster members in microarray analysis and by RT-PCR. Additionally, HPLC and LC-MS analysis of supernatants showed significantly higher levels of gliotoxin during biofilm-growth. Gliotoxin is a well-characterised redox-active metabolite with significantly deleterious effects on mammalian cells [72]. It is regarded as an important factor contributing to the virulence of *A. fumigatus*; hence its increased production during biofilm-growth is highly relevant to infection. While, the proteomic and transcriptomic induction of the gliotoxin cluster correlated, overall there was little correlation between the two analytical formats. The authors speculated that biomolecule longevity and intrinsic differences in the analytical techniques might underlie this. Miscorrelation such as this also indicates more experimental investigation of these states is required. Moreover, given the clinical relevance of the results thus far, further investigation of biofilm growth is warranted.

1.2. Proteomic-based dissection of adaptations relevant to host infection

1.2.1. Natural product biochemistry: A case study on gliotoxin

Proteomics has proven to be an important means of dissecting biosynthetic gene clusters [73]. One of the key drivers for investigating microbial natural products has been their association with virulence against plant or animal hosts. Indeed, nowhere is this better exemplified than with the study of the fungal 'toxin', gliotoxin, produced by *A. fumigatus*. Biosynthesis of, and self-protection against, the redox-active metabolite gliotoxin is encoded by the *gli* gene cluster, composed of 13 co-expressed genes in *A. fumigatus*, and proteomics has played a major role in dissecting this hitherto obscure combination of cellular processes. Before discussing the specific role of proteomics in exploring gliotoxin biochemistry, it is important to

state that an alternative rationale for investigating gliotoxin biosynthesis and self-protection is that it can also uncover, directly and indirectly, new aspects of fungal systems biology [72].

Using 2-DE and MALDI-ToF MS analysis, Schrettl *et al.* were the first to reveal that exposure of *A. fumigatus* wild-type to exogenous gliotoxin resulted in the increased abundance of the gliotoxin oxidoreductase GliT, and that *gliT* expression could also occur independently of *gli* cluster expression. In experimentation on the proteomic response in *A. fumigatus* to exogenously-added gliotoxin, under low gliotoxin producing conditions (0.27 $\pm$ 0.82 µg gliotoxin/mg hyphae (fifteen fold lower then optimal production)), a threefold increase in GliT abundance within 3 h of gliotoxin exposure was observed [58,74]. Specifically, the proteomic response to exogenous gliotoxin provided a snapshot of the effects of gliotoxin whereby, in addition to increased GliT presence, perturbation in the levels of at least 21 additional proteins (increased abundance (60 %); decreased abundance (40 %)) was identified. In particular, superoxide dismutase (SodA) and Asp F3 abundance was induced and increased, respectively, whereas catalase (Cat1; AFUA_3G02270) abundance was decreased- predicting elevated intracellular H_2O_2. Glutathione (GSH) levels were also significantly elevated ($p < 0.05$) in *A. fumigatus* Δ*gliT* compared to wild-type [74] which suggests that elevated GSH may potentiate the autotoxic effects of gliotoxin. Moreover, *A. fumigatus* Δ*gliT* was significantly more resistant to the effects of diamide, a GSH scavenger, than either wild-type or $gliT^{C}$ [74]. Furthermore, it was observed that *Saccharomyces cerevisiae* Δ*sod1* was hypersensitive to gliotoxin and that *S. cerevisiae* Δ*gsh1*, which contains significantly lower levels of intracellular GSH, was resistant to gliotoxin [74]. *S. cerevisiae*^{gliT} also exhibited increased resistance to gliotoxin [58]. Interestingly, although a threefold abundance in GliT levels was evident from these studies, subsequent Label-Free Quantitative (LFQ) proteomic analyses have revealed a more dramatic 75-fold increase in GliT abundance in response to gliotoxin exposure [75]. It was also observed by Schrettl *et al.* and Scharf *et al.* that *gliT* deletion from *A. fumigatus* resulted in the acquisition of a gliotoxin sensitive phenotype which positioned GliT, and disulphide bridge closure, as a key component of the mechanism which facilitates the biosynthesis of a highly reactive,

and growth-retarding, molecular species in eukaryotic cells (Figure 3) [58,76]. Subsequent work by Owens *et al.* has revealed that GliT-catalyzed conversion of dithiol gliotoxin to gliotoxin [75] is essential to facilitate subsequent gliotoxin efflux from *A. fumigatus* by the *gli* cluster-encoded MFS transporter GliA (Figure 3) [75].

Although *A. fumigatus ΔgliT* was shown to be highly sensitive to exogenous gliotoxin, strangely this mutant grew perfectly well in its absence. This inferred that either gliotoxin induction of *gli* cluster expression [58,77], endogenous dithiol gliotoxin formation or other unknown factors could contribute to the acquisition of the severe gliotoxin sensitivity in *A. fumigatus ΔgliT*. Amongst other altered protein abundances, LFQ proteomic analysis of *A. fumigatus* wild-type, *ΔgliT* and *ΔgliK* (deficient in a γ-glutamyl cyclotransferase), in response to exposure to exogenous gliotoxin, revealed the presence of a non-*gli* cluster encoded methyltransferase (AFUA_2G11120) which was uniquely present in *A. fumigatus* upon gliotoxin exposure (Figure 3). Subsequently, using proteomics-directed purification, allied to the development of a new MS-based enzyme activity assay [78], a novel enzyme gliotoxin bis-thiomethyltransferase GtmA, which sequentially converts dithiol gliotoxin to a bisthiomethylated derivative, was identified and characterized [78–80]. Dolan *et al.* demonstrated that deletion of *gtmA* did not result in acquisition of a gliotoxin sensitive phenotype, but instead – as revealed by LFQ proteomic analysis, resulted in increased abundance of key gliotoxin biosynthetic enzymes, including the non-ribosomal peptide synthetase GliP [81], and concomitant gliotoxin overproduction. Thus, GtmA is now categorized as a post-biosynthetic negative regulator of gliotoxin biosynthesis which competes with GliT for dithiol gliotoxin (Figure 3) [78]. Moreover, it is now clear that GliT converts dithiol gliotoxin to gliotoxin which is secreted via GliA, while GtmA converts it to bisthiomethylgliotoxin (BmGT), which requires two S-adenosylmethionine (SAM) molecules as the methyl donor, and effluxes from the cell via an unknown mechanism- possibly via passive diffusion (Figure 3) [78–80].

However, LFQ proteomic analysis, combined 2-DE and LC-MS/MS [75], and parallel RNAseq [82], allied to intracellular metabolite quantification, have uncovered

additional hidden interactions between gliotoxin/BmGT biosynthesis, self-protection and sulphur metabolism in *A. fumigatus*. In fact, it is now clear that GliT plays a pivotal role in preventing dysregulation of cellular sulphur metabolite homoeostasis during gliotoxin/BmGT biosynthesis. Specifically, in the absence of *A. fumigatus* GliT (or significantly attenuated abundance, as seen in *A. fumigatus ΔgliK*), gliotoxin addition activates *gli* cluster and *gtmA* expression. This in turn leads to overproduction of, dithiol gliotoxin initially, and then of BmGT with the consequent depletion of SAM and overproduction of S-adenosylhomocysteine (SAH) (Figure 3) [75]. Moreover, in *A. fumigatus* Δ*gliK*, combined 2-DE and LC-MS/MS revealed increased abundance of the cobalamin-independent methionine synthase MetH/D (AFUA_4G07360) (1.9- to 2.2-fold), and methylene tetrahydrofolate reductase MTHFR/MtrA (AFUA_ 2G11300) (1.8-fold), in response to gliotoxin exposure. These enzymes are essential to effect methionine biosynthesis [83,84] and function in the methyl/methionine cycle to ensure sufficient levels of intracellular SAM (Figure 3). Thus, proteomics has revealed a wealth of previously occluded interactions between primary and secondary metabolism, many of which have potential for exploitation in the clinical setting as anti-fungal drug targets. Indeed, given the essential nature of SAM for a plethora of cellular transmethylation reactions including, but not limited to, epigenetic regulation [85], and the potential inhibitory action of SAH on cellular methyltransferases [86], the metabolically catastrophic potential of dysregulating GliT-mediated control of gliotoxin/BmGT biosynthesis is only just becoming apparent. Further systems impacts of interfering with gliotoxin biosynthesis are only just emerging and, surprisingly, it appears that the biosynthesis of apparently unrelated natural products, like the antioxidant ergothioneine, is influenced either by gliotoxin [87,88] (and unpublished data), or specific reactions within its biosynthetic pathway [72]. So, the activity of gliotoxin against fungi and animal cells, often mediated by interference with redox homeostasis, is revealing new metabolic interactions within eukaryotic systems.

1.2.2. Deprivation of essential elements in the host

1.2.2.1. Hypoxia

The host can represent a resource-limited environment to the invading microorganism with respect to supply of elements essential for survival. Even in healthy individuals, oxygen levels drop from 21% in the atmosphere to 14% in lung alveoli and as low as 2% in tissue. Such limited oxygen means that during colonisation *A. fumigatus* encounters hypoxia in the host environment [89]. Risk groups for *A. fumigatus* colonisation such as COPD and CF patients can also experience further decreased oxygen levels in the lungs and alveolar hypoxia [7,90,91]. Moreover, during infection inflammation and necrosis further restrict oxygen availability. Hence adaptation to hypoxia is likely an important trait for human pathogens. Recently, significant efforts to characterise *A. fumigatus* in response to hypoxia have been made. Proteomic analysis via 2D-DIGE coupled with MALDI-ToF/ToF detection has served as a major analytical tool in these investigations and revealed adaptations relevant to the host pathogen interface. Proteins involved in glycolysis, the TCA cycle, secondary metabolism and respiration were among those with differential abundance under hypoxic stress [34,92].

During hypoxia, lack of oxygen as the terminal electron acceptor of the electron transport chain in aerobic respiration prompts many microorganisms to utilise fermentation for supply of NAD^+ to ATP generation via glycolysis. Hence, utilisation of fermentative strategies *in vivo* may be implemented when hypoxia is encountered. Under short-term exposure to hypoxia, increased abundance of proteins PdcA (AFUA_3G11070) and AlcA (AFUA_7G01010) was observed. These proteins are involved in the conversion of pyruvate to ethanol indicating that fermentation is utilised under hypoxia [92]. In contrast, there was no evidence for fermentation in proteomic analysis of *A. fumigatus* under long-term hypoxic stress [34]. However, the latter work used glucose-depleted chemostatic culture conditions which may account for this lack of fermentation [93]. PdcA is antigenic, indicating its synthesis at the host:pathogen interface [24]. Moreover, recovery of ethanol from the lungs of infected mice provides evidence for the use of fermentation *in vivo* [89].

While abrogation of ethanol production in *A. fumigatus* did not alter mortality in three immunologically distinct murine models, reduced fungal burden and increased inflammatory responses were observed in an alcohol dehydrogenase III (AlcC; AFUA _5G06240) deletion mutant. Thus, there is evidence to suggest the utilisation of fermentation *in vivo* during infection as a result of hypoxia.

Proteomic analysis has also revealed hypoxia-induced secondary metabolite production with members of the biosynthetic gene cluster for pseurotin A showing increased abundance under hypoxia [34]. This low oxygen-induced production was confirmed via RP-HPLC analysis of culture extracts and Northern blot. Additionally, qRT-PCR indicated transcription of the pseurotin A cluster genes in lungs of infected mice. Pseurotin A has been shown to inhibit IgE activity and possess low cytotoxicity activity against lung fibroblasts [94,95]. With induction *in vivo* during hypoxia, the immunomodulatory capacity of this secondary metabolite may aid the pathogenesis of *A. fumigatus.* Interestingly, in an example of secondary metabolite cross-talk, deletion of *gliT* resulted in decreased expression of pseurotin A biosynthetic enzymes upon exposure to exogenous gliotoxin, and reduced production of pseurotin A relative to the wild-type [82].

Hypoxia-induced oxidoreductase (HorA; AFUA_4G09810) was initially identified in the proteome *A. fumigatus* conidia and subsequently shown to be increased during hypoxia [29,92]. Recent characterisation showed HorA to be localised to mitochondria and suggested a role in coenzyme Q biosynthesis [96]. Decreased abundance of proteins involved in oxidative stress tolerance mechanisms was indicated by proteomic analysis of Δ*horA*. Impaired complex I activity as a result of coenzyme Q10 deficiency resulting in lower ROS generation may have accounted for this response. Interestingly, deletion of *horA* increased resistance to the antifungals voriconazole and amphotericin B, possibly due to impaired mitochondrial function partnered with induction of the multidrug resistance pathway. Δ*horA* also showed severely attenuated virulence in murine infection models. Importantly, HorA is a fungal-specific protein and therefore an intriguing target for novel antifungal therapy development [96]. Thus, oxygen deprivation *in vivo* has emerged as an important

factor within the host microenvironment. Recent proteomic analysis has shed light on the responses of *A. fumigatus* to hypoxia and revealed specific molecular adaptations undertaken by the fungus that are relevant to infection. Moreover, this insight has revealed putative drug targets specific to fungi.

1.2.2.2. Iron starvation

Embedded in many biological processes, iron is an essential element in all eukaryotes and similarly to oxygen its limited supply within the host represents a significant stress on invading microorganisms. By virtue of its ability to exist in two oxidative states iron can also prove deleterious in excess, through the generation of reactive oxygen species (ROS) via the Fenton reaction [97]. Hence, iron-requiring organisms have developed tight regulatory and uptake systems for maintaining iron homeostasis. Due to sequestration by host proteins to negate deleterious by-products, free iron levels in humans are very low. Moreover, upon microbial challenge the availability of free iron is further restricted due to an up-regulation of host sequestering mechanisms in a form of 'nutritional immunity' [98]. During iron starvation sophisticated regulatory mechanisms in *A. fumigatus* orchestrate an adaptive response including the implementation of high affinity iron acquisition strategies [99]. Low molecular mass iron (Fe^{3+}) chelators, termed siderophores produced by *A. fumigatus* are secreted *in vivo* and essential to virulence [100–103]. Uptake of ferri-siderophores is mediated by a family of fungal specific membrane transporters; siderophore iron transporters (SITs). Targeting siderophore uptake via SITs represents a promising anti-fungal strategy, but requires further dissection [104,105]. Recently, the iron-starved microsomal proteome of *A. fumigatus* has been characterized [16]. Mycelial lysates were successfully enriched for membrane proteins via cellular fractionation with ultracentrifugation [15]. By utilising LFQ proteomic analysis of the microsomal cellular fraction, issues with 2-DE resolution of membrane proteins were bypassed. Microsomal proteins (n = 231) with differential abundance were detected and 96 showed increased abundance. Among these proteins were several putative transmembrane transporters. Sera from healthy individuals showed higher IgG reactivity against microsomal proteins grown under

iron starvation, which highlights the frequency of normal host interaction with the *A. fumigatus* microsomal proteome and relevance of iron starvation therein. Many of the proteins identified in this study warrant further characterisation as siderophore transporters and therapeutic targets.

1.2.3. Investigating the host relevant proteome: emerging technology

To date, proteomic-based investigation of host-relevant adaptations in *A. fumigatus* has revealed considerable information on the phenotype of this organism during infection and provided the basis for therapeutic strategies. Dissection of natural product biochemistry has yielded insight into the mechanisms of secondary metabolism and the case study of gliotoxin exemplifies this in an important virulence determinant. The dramatic proteome remodelling observed in response to deprivation of iron and oxygen, has highlighted the importance of examining stresses reflective of the host environment. In addition, significant research has been conducted into other natural products and stress responses in *A. fumigatus* relevant to infection, such as dihydroxynaphthalene melanin and oxidative stress which are comprehensively reviewed elsewhere [106–108].

There is a demand on proteomic tools to provide more accurate and detailed insight into the phenotype of *A. fumigatus* during infection and emerging technology seeks to meet this demand. Activity-based protein profiling (ABPP) is a recent technology exploiting the use of activity-based chemical probes in combination with MS-based proteomic analysis, fluorescence microscopy or gel electrophoresis. Chemical probes comprise a reactive group that can react with target proteins based on their activity, a binding/ spacer group and a tag or site for later tag attachment, that facilitates specific detection (Figure 1) [109]. Using ABPP coupled with quantitative proteomic analysis, Wiedner *et al.* showed growth in human serum significantly alters the reactive enzyme proteome of *A. fumigatus* [110]. Exposure to human serum significantly lowered the overall enzyme reactivity of *A. fumigatus* lysates including proteins involved in secondary metabolism and stress tolerance. The authors speculated that this decreased stress was due to the ability of *A. fumigatus* to utilise

serum-derived nutrients. This work has shed light on the functional proteome of *A. fumigatus* relevant to the host environment. Moreover, ABPP represents a promising emerging technique with the potential to provide functional insight into metabolic and stress responses of *A. fumigatus* that make it a successful pathogen.

1.3. Proteomics in advancing antifungal therapy

There are currently three classes of commonly administered antifungal drugs against *A. fumigatus*: polyenes, azoles, and echinocandins. These compounds target either ergosterol directly, or the biosynthesis of ergosterol or β-1,3-glucan [111]. Resistance and host toxicity represent significant challenges in the current use of these antifungals, and proteomic analysis provides a powerful tool in characterising the effects of existing and emerging antifungals to dissect their mode of action. Furthermore, proteomic analysis of resistant strains allows elucidation of the mechanisms of resistance and can provide biomarkers.

Caspofungin (CSF) is an echinocandin that targets the fungal cell wall via inhibition of glucan synthesis and in doing so diminishes the cell wall stability [111]. Cagas *et al.* characterised the proteomic responses of a susceptible and resistant strain of *A. fumigatus* to CSF [112]. The cell wall/ plasma membrane and secreted fractions at 24 h and 48 h were isolated as these fractions are highly exposed to the host during infection. An approach of gel-free based protein preparation with MALDI-ToF/ToF absolute quantification (iTRAQ) provided substantially better resolution than 2-DE based whole cell lysate protein analysis [112]. Using strict criteria, 122 proteins showing over a 2-fold change in response to CSF were identified in the susceptible strain. Proteins (n = 65) showed differential abundance in the cell wall/ plasma membrane fraction. Interestingly 21 out of the 26 proteins with increased abundance at 24 h in the cell wall/ plasma membrane were ribosomal proteins. The authors speculated that this represented a ribosomal reprogramming response to the antifungal. Importantly, this trend was not as striking in the resistant strain where only 4 out 19 proteins with increased abundance at 24 h were ribosomal proteins implicating ribosomal protein reshuffling in susceptibility to CSF. Chitinase

Chia1 (AFUA_5G03760) decreased over 12-fold at 24 h, which is in accordance with the increased chitin seen under exposure to CSF [113]. Cell stress proteins were also affected with decreased abundance of Hsp90 (AFUA_5G04170) and Hsp98 (AFUA_1G15270) as well as the Hsp90 co-chaperone (Wos2/ Sba1) in the susceptible strain. Repression of Hsp90 was subsequently shown to confer hypersensitivity to CSF [114]. In the secreted fraction, 57 proteins showed differential abundance. Levels of Asp F4 and two subunits of ATP citrate lyase; Acl (AFUA_6G10660) and subunit 1 (AFUA_6G10650) were increased in abundance at 24 h. Both subunits have previously been implicated in other stress responses. AFUA_6G10650 has been shown to increase during early responses to heat shock and in Δ*elfA* (AFUA_1G17120) under hydrogen peroxide stress when compared to wild-type [115,116]. While AFUA_6G10660, which has recently been identified as antigenic, decreases in response to gliotoxin exposure [24,74]. Importantly, out of the 122 proteins with differential abundance in the susceptible strain, 103 were unchanged in the resistant strain. Several proteins, which were relatively unaltered in the resistant strain, showed over a 12-fold change in abundance in the susceptible strain. These included Asp F1 (AFUA_ 5G02330), mitochondrial hypoxia response domain protein (AFUA_1G12250) and citrate lyase (Cit1/ McsA; AFUA_6G03590), which may represent promising biomarkers.

Amphotericin B (AMB) is a polyene that works by binding ergosterol in the cell membrane forming channels, thereby disrupting membrane function and leading to leakage of ions and cytoplasmic material as well as ROS accumulation [111]. Exposure of *A. fumigatus* to AMB resulted in differential abundance in 48 proteins when analysed by 2-DE with MALDI-ToF/ToF identification. Proteins (n = 44) showed increased abundance and 4 showing decreased abundance [117]. Ergosterol biosynthetic protein, Erg13 (AFUA_3G10660), was shown to increase in the proteome under exposure to AMB [117]. Ergosterol biosynthesis requires the input of heme as a co-factor [118]. Increased abundance of heme biosynthetic protein Hem13 (AFUA_1G07480) reflects this extra demand. The induction of oxidative stress defences was also observed in the proteomic analysis, with increased abundance of manganese superoxide dismutase (Mn-SOD; AFUA_1G14550), catalase

(Cat1) and Prx1/ LsfA (AFUA_4G08580). This supports the report that AMB driven cell membrane damage results in the generation of oxidative stress via ROS generation.

Itraconazole (ITC) is an azole that targets the biosynthesis of ergosterol and leads to the accumulation of toxic sterols [111]. Exposure of *A. fumigatus* to ITC resulted in differential abundance of 54 proteins detected by 2-DE with MALDI-ToF/ToF identification [119]. Analysis revealed 12 proteins with increased abundance and 42 decreasing in abundance, representing 44 unique proteins. Similar to AMB, increased abundance of several proteins in response to oxidative stress were also observed including catalase, Cat1. Interestingly, 26 of the proteins detected had previously shown differential abundance in *A. fumigatus* in response to the anti-malarial compound, artemisinin (ART) reported to also have antifungal activity. Proteomics also represents a useful and efficient tool in investigating novel antifungals such as ART. While administration of ART alone is unlikely to be a viable therapy, Gautam *et al.* demonstrated synergy with ITC, indicating it may prove effective in combination therapy [120]. Exposure of *A. fumigatus* to ART led to differential abundance of 85 proteins following 2-DE with MALDI-ToF/ToF identification. 29 showed increased abundance and 56 decreased. Decreased abundance of cell wall protein PhiA (AFUA_3G03060) indicated that cell wall remodelling was underway. Interestingly, in contrast to AMB, decreased abundance of Erg3 (AFUA_2G00320) and Hem13 (AFUA_1G07480) was also observed. Heme is required for the action of ART. Therefore, decreasing heme biosynthesis may represent a means of tolerance in the fungus, and decreasing the abundance of heme-dependent enzymes an adaptive response. Stress-related proteins were affected, with increased abundance of antioxidant peroxiredoxin (Prx1/ LsfA). Thus, pathways targeted by other antifungals including cell wall, ergosterol and stress were also targeted by artemisinin.

Proteomic analysis has also provided insight into other putative antifungals. Screening a range of coumarin derivatives, Gupta *et al.* identified a synthetic coumarin derivative termed SCD-1 with activity against *Aspergillus* species [121].

Subsequently, the proteome of *A. fumigatus* following exposure to SCD-1 was investigated to dissect its antifungal mechanism [122]. Cytosolic proteins were extracted from germlings and analysed by 2-DE with LC-MS/MS identification. One hundred and forty three proteins with differential abundance were detected; 96 increased and 30 decreased in abundance. In addition, 4 proteins were unique to the control and 13 were unique to the SCD-1 treatment. Proteins involved in riboflavin biosynthesis (AFUA_1G06240 and AFUA_2G05820) showed decreased abundance, indicating it as an important target of SCD-1. The absence of riboflavin biosynthesis in humans makes this pathway an ideal drug target, limiting host toxicity. Similar to CSF, there was increased abundance of ribosomal proteins, including 60S ribosomal protein P0 (AFUA_1G05080), 40S ribosomal protein S12 (AFUA_1G05500), and 40S ribosomal protein S3 (AFUA_1G05630), indicating a ribosomal reshuffling in response to exposure. There was also increased abundance of stress proteins including antioxidant Prx1/ Lsf1 and Hsps, Hsp90, Hsp88 (AFUA_1G12610) and Hsp30/ Hsp42 (AFUA_3G14540).

The proteomic analysis of *A. fumigatus* exposed to each of these antifungal compounds has indicated some targeting of common pathways. Coping mechanisms for oxidative stress, including the induction of antioxidant proteins is evident upon exposure to all compounds. Interestingly, some of these share proteins for example, Cat1 was increased upon exposure to AMB, ITC and ART while Prx1/ LsfA was increased during AMB, ART and SCB-1 exposure. Phosphoglycerate kinase PgkA (AFUA_1G10350) showed differential abundance in response to all antifungals. Allergen enolase/ Asp F22 was also differentially abundant upon exposure to many of these compounds; with increased abundance during exposure to AMB, ART, CMR but decreased abundance in ITC. Enolase is classified as an allergen with a predicted adhesin motif and its increased abundance during exposure to these antifungals is therefore relevant to treatment methods in patients with ABPA [5,40].

1.4. Proteomic characterisation of *A. fumigatus* antigens

1.4.1. Vaccine development

Due to the issues with aforementioned challenges associated with antifungal therapy, the prospect of a vaccine against *A. fumigatus* is enticing [123,124]. Evidence for this strategy has emerged, for example with the allergen Asp F3. Immunisation of mice with crude hyphal extracts provided protection against invasive pulmonary aspergillosis under later immune suppression [125]. Using a combination of fractionation, Western blotting, and SDS-PAGE with MALDI-ToF, Asp F3 was identified as the predominant immunogen in the murine sera. Importantly, it was possible to remove the IgE binding capacity of Asp F3 while still retaining protection in mice [126]. Asp F3 has high homology to several other fungal proteins raising the basis of a pan-fungal vaccine [127]. In an attempt to identify pan-fungal protein-based vaccine candidates, Champer *et al.* performed a large-scale survey of the cytosolic, cell wall and secreted proteome of 13 fungal species. Using LFQ MS^E (mass spectrometry – elevated collision energy) several thousand proteins were detected [128]. The most abundant proteins were evaluated for interspecies homology and for homology with human proteins. Interestingly, the majority of the most abundant cytosolic proteins shared significant homology to human proteins. In contrast, many of the most abundant cell wall proteins shared little or no homology to human proteins. Due to their accessibility to immune constituents during infection, cell wall proteins serve as ideal vaccine targets. Thus cell wall proteins Gel1-4 (AFUA_2G01170, AFUA_6G11390, AFUA_2G12850 and AFUA_2G05340) and Bgt1 (AFUA_1G11460) were proposed as candidates as they have no homology to human proteins and were detected in high abundance in several fungi.

1.4.2. Immunoproteomic strategies: considerations

Many studies on the immunoproteome of *A. fumigatus* have utilised patient sera pools [17,19,21–23]; however there is also need to analyse individual patient serum to gain a robust insight into the individual biomarker frequency among patients. Using 2-DE and MALDI-ToF/ToF, Teutschbein *et al.* recently identified antigenic

proteins using sera from 43 individual patients undergoing chemotherapy including 22 with probable and 2 with proven IA [24]. 49 antigenic proteins were identified, 31 of which had previously been identified as antigenic or allergenic in individuals with IA or ABPA [19,22,23]. Consistently, proteins identified as antigenic in this study were represented among the most abundant in the *A. fumigatus* proteome as detected in other analyses [28,29,127]. This led the authors to suggest that proteins with higher abundance are more likely to provoke an immune response *in vivo* [24]. Supervised machine learning with a decision tree classification was used to identify proteins capable of predicting the outcome of infection. Using this approach, several proteins were shown to be associated with a positive (survival) and negative (fatal) outcome of infection including CpcB (AFUA_4G13170) and Shm2 (AFUA_3G09320), respectively. Both proteins were recombinantly expressed and exposed to PBMCs. Interestingly, Shm2 induced a pro-inflammatory response while CpcB did not. Thus, given Shm2 was associated with a poor patient outcome, it is possible that Shm2 can trigger a deleterious host inflammatory response [24]. The response associated with these proteins is in accordance with recent characterisation of $CD4^+$ T cell target antigens in healthy individuals. Based on the cytokine profile induced, Shm2 was considered immunogenic while CpcB lacked induction of an effector function [129]. Also apparent in work by Teutschbein *et al.* and others is the anti-*A. fumigatus* antibody response present in uninfected patients. This is indicative of the ubiquity of *A. fumigatus*; however it also illustrates the need to consider antigens specific to infection as biomarkers. In a recent study by Virgino *et al.*, sera from patients who presented with similar underlying disease but without fungal infection were used as controls in analysing the immunoproteome of germlings [23]. Ten proteins identified as antigenic were also previously proposed as possible biomarkers by others; however these were shown to cross-react with control sera indicating they may not be suitable in diagnosis. Furthermore, following reports of difficulty in distinguishing between different fungi in diagnosis, the proteome was also analysed with pooled sera from patients with several other invasive fungal infections. Several proteins (n = 22) exhibited antigenicity, indicating that they are also unsuitable in diagnosis. Fourteen proteins were exclusively detected in the proteome probed with sera from proven cases of aspergillosis, 4 of which were also immunoreactive with sera from

probable aspergillosis cases. Importantly, 4 proteins had not previously been proposed as possible diagnostic antigens. BLAST searches revealed that two of the proteins analysed had no homology with human proteins. This included eEF3, a fungal specific protein having previously been proposed as a drug target with overexpression in germlings [32] (above). eEF3 is involved in a fungal specific aspect of translation, and mutations in it can deleteriously affect growth and translation [130]. Hence, eEF3 has emerged as a promising diagnostic antigen and drug target. Work by Teutschbein *et al.* also revealed heterogeneity in the anti-*A. fumigatus* profile in sera of individual patients with probable or proven IA, which the authors reasoned indicates that one antigen alone is unlikely to be an effective prognostic or diagnostic marker of IA [24]. Further immunoproteomic analysis is warranted to reveal more antigens including those with immunostimulatory properties. Such studies can yield insight into to the pathology of aspergillosis and a relatively high-throughput means of surveying candidate drug targets.

2. Expert commentary

MS-based proteomic analysis has facilitated a much-needed recent global characterisation of *A. fumigatus*. Shotgun proteomic analysis has shown the proteome during early development to be highly dynamic with significant changes within the first 8 h. Many of the proteins observed during early development have been implicated in infection and represent potential biomarkers of early infection. Host constituent interactions with the cell surface and secreted proteome of conidia and growing hyphae represent an important interface. Dissection of proteins herein has revealed mechanisms of fungal adhesion and host recognition. It is important to consider and represent the *in vivo* phenotype at this interface during *in vitro* investigations. Biofilm growth has recently been revealed as clinically important and proteomics has provided a means of characterising this phenotype during *in vitro* modelling. The results have revealed altered secondary metabolism in the form of gliotoxin, which carries significant implications for infection. Comprehensive dissection of clinically important pathways, such as that of gliotoxin metabolism, has been expedited by the use of proteomics. The host environment also comprises

various stresses that affect the phenotype of *A. fumigatus.* The magnitude of proteomic remodelling under these host-relevant stresses is demonstrated under iron and oxygen starvation. It is imperative to consider such parameters to better represent the host environment when analysing gene functions and antifungals *in vitro*. The domain of antifungal therapy against *A. fumigatus* currently faces challenges in the form of limited drug choice and emerging resistance. Profiling the proteome of *A. fumigatus* in response to existing and novel compounds has permitted elucidation of the mode of action, and revealed potentially better approaches, for example in combination therapies. Moreover, analysis of susceptible and resistant strains provides important insight into the mechanisms and biomarkers of resistance. Immunoproteomic analysis has continually provided a wealth of knowledge on the antibody-mediated response against *A. fumigatus* during several manifestations of diseases. This has provided biomarkers, demonstrated to be effective in diagnosis, as well as information on the pathology of *A. fumigatus* infection.

3. Five-year view

MS-based proteomic analysis has proven an important tool in dissecting the mechanisms of *A. fumigatus* pathogenicity. In recent years, advances in shotgun quantitative proteomic strategies have extended the capability of this tool. Continued research with such technology, plus enhanced detection of post-translational modifications, will further unveil the proteome of *A. fumigatus* informing on proteins previously somewhat intractable to analyses. Importantly, this cumbersome protein subset includes membrane proteins, which represent an important focal point of the host:pathogen interface. Insight into this domain will most likely expand the drug targets and may provide the basis for development of much needed novel antifungals. Immunoproteomic analysis has indicated a cell-mediated and humoral immune response against *A. fumigatus* in healthy individuals. Continued analysis herein will allow for a thorough dissection of the competent immune response and successful mechanisms of defence against this frequently encountered organism. Immunoproteomic analyses are close to yielding clinically

applicable biomarkers. Future work continuing the recent utilisation of non-biased classification strategies will further refine this biomarker candidate set. Implementation of a community-standardised method would expedite this and allow for a larger and directly comparable dataset. Advances in gel-free immunoproteomic strategies will permit better representation of the interactions therein. Recent evidence suggests MALDI-ToF based identification represents a robust, cost-effective and timely means of diagnosing *A. fumigatus* as well as other species. Strides in this area may see the implementation of this technique in a mainstream clinical setting. Recent large-scale proteome analysis has indicated the pursuit of a pan-fungal vaccine is plausible. Moreover, it is a worthwhile endeavour and tempting to speculate that developments in this area will progress rapidly in the coming years.

4. Key issues

- *A. fumigatus* remains a significant cause of morbidity and mortality in individuals with impaired lung function or compromised immune systems. A better understanding of the interactions between host and pathogen is required to improve patient outcome.
- MS-based proteomic analysis can provide a necessary global characterisation of the adaptations undertaken by *A. fumigatus* during host colonisation.
- Recent proteomic analyses have revealed the proteome during early development and attempts at host colonisation to be represent a robust and dynamic phenotype.
- Secreted and cell surface proteins have emerged as an important aspect of the host:pathogen interface of particular relevance to allergenic manifestations of the disease.
- Proteomic characterisation of biofilm growth has helped identify traits in this form relevant to virulence.
- External cues such as oxygen and iron limitation are representative of stresses within the host. Such cues significantly alter the proteome and their examination has revealed infection-relevant phenotypes and drug targets.

- Proteomic analyses have elucidated the mode of action of novel and existing antifungals as well as exposing the mechanisms utilised by resistant strains.
- In recent years, immunoproteomic analysis has revealed informative and promising biomarkers as well as vaccine candidates. Additionally the allergenic response against *A. fumigatus* has been further characterised. Interestingly, this work has also demonstrated the high frequency of *A. fumigatus* interactions with healthy individuals.

Declaration of Interest

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** of considerable interest

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Figure 1. Proteomic workflows used in recent *A. fumigatus* studies. *A. fumigatus* protein extracts can be obtained from mycelial lysates, culture supernatants, co-culture scenarios or following sub-cellular fractionation. Once protein extracts are in situ, they are subjected either to direct proteolysis (generally via trypsinization), or gel-based fractionation, prior to MS analysis. Label-free quantification (LFQ) or isotopic and isobaric labeling, without protein fractionation, enables assessment of differential protein abundance between comparative conditions for shotgun approaches. Image analysis of PAGE or DIGE gels facilitates detection of differential protein abundance followed by LC-or MALDI-MS protein identification.

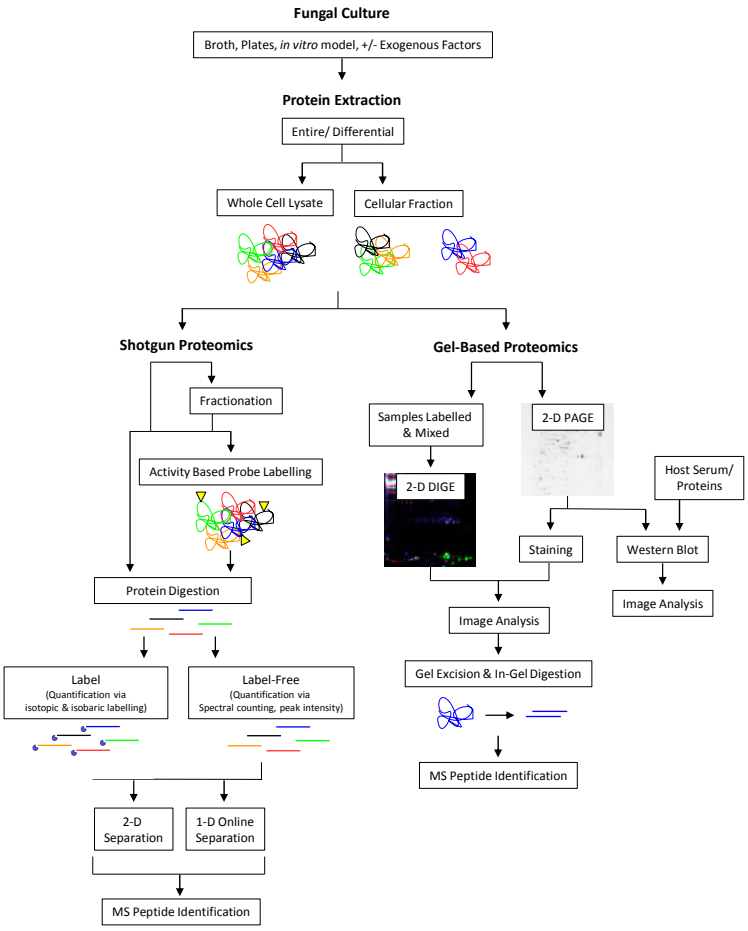

Figure 2. The role of the proteome in establishing host colonisation. Inhaled *A. fumigatus* conidia adhere to cells, extracellular matrix and host proteins. After conidia begin to germinate (conidial swelling) higher abundance of immunoreactive proteins can induce host recognition mechanisms. Germlings undergo significant morphological changes, growing into invasive hyphae. Secretion of immunoreactive proteins such as proteases can exacerbate allergenic responses. Hyphal growth in biofilms decreases antifungal susceptibility and increases deleterious secondary metabolite production. **Redrawn and adapted from Filler and Sheppard (2006) Fungal invasion of normally non-phagocytic host cells. *PLOS Pathog* 2(12): e129. doi:10.1371/journal.ppat.0020129.**

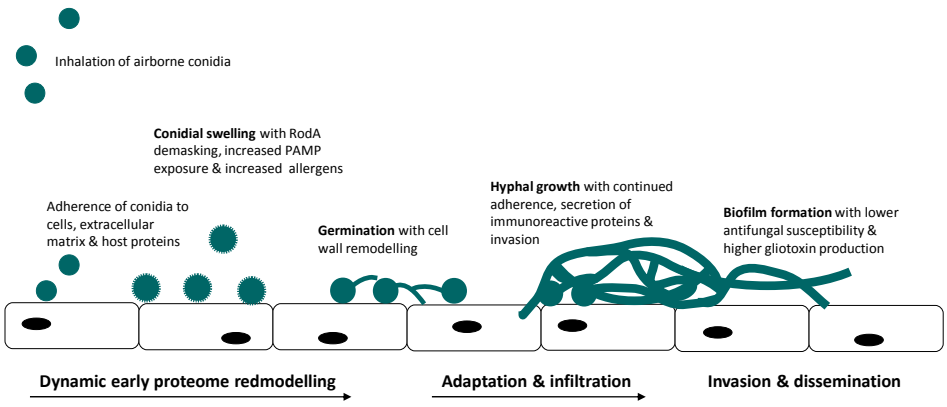

Figure 3. Proteomics reveals the interplay between gliotoxin biosynthesis, self-protection, regulation, and primary metabolism. Exogenously added, or endogenously produced, gliotoxin (GT-SS) can be converted to dithiol gliotoxin (GT-$(SH)_2$) in the absence of GliT activity. Consequentially, GtmA converts GT-$(SH)_2$ to bisthiomethylgliotoxin (BmGT) with simultaneous conversion of SAM to SAH (2 mol equivalents per mol BmGT). This in turn leads to depletion of SAM and activation of the methyl/methionine cycle, thereby revealing a cryptic link between biosynthetic gene cluster-enabled metabolite biosynthesis and primary metabolism. In effect, GliT functions to prevent dysregulation of the methyl/methionine cycle by forming gliotoxin which is subsequently effluxed from mycelia via GliA. Adapted from Owens *et al.* [75].