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Extracellular vesicles of human pathogenic fungi

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Extracellular vesicles play a significant role in many aspects of cellular life including cell-to-cell communication, pathogenesis and cancer progression. However very little is known about their role in fungi and we are just at the beginning of understanding their influence on fungal pathophysiology and host–pathogen interactions. Recent findings have revealed a role for fungal vesicles in triggering anti-microbial activities as well as in modulating virulence strategies, suggesting potential new avenues for antifungal therapies. In this review, we summarize our current understanding of fungal extracellular vesicles, including their biogenesis, secretion and size variation, and discuss how they may influence the human immune response and some key questions that remain unanswered.

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Introduction

Sensing and understanding the environment is critical for the survival of most organisms. In recent years, it has become clear that one essential route by which cells communicate with the surrounding environment is through the release of extracellular vesicles (EVs). These nano-sized bubbles are filled with membrane and cytosolic proteins, lipids, nucleic acids such as mRNAs and noncoding RNAs, polysaccharides, toxins, allergens and pigments surrounded by a lipid bilayer. These spherical and stable structures provide a durable mechanism by which to send 'messages' to other cells, either of the same species or others. In 2007, Rodrigues *et al.* provided the first evidence from a human pathogenic fungus, *Cryptococcus neoformans*, that its major virulence factor, extracellular capsular polysaccharide, is synthesized inside the cell and exported outside within nanovesicles during both *in vitro* growth and during *in vivo* infection [1]. Since then, the presence of EVs in fungal culture supernatants and from body fluids has been shown in other human pathogenic fungi including *Histoplasma capsulatum* [2], *Sporothrix schenckii* [2] and *Sporothrix brasiliensis* [3], *Candida albicans* [2] and *Candida parapsilosis* [2], *Malassezia sympodialis* [4], *Paracoccidioides brasiliensis* [5], *Alternaria infectoria* [6], *Cryptococcus gattii* [7••] and *Trichophyton interdigitale* [8].

There is fast-growing evidence that EVs isolated from human pathogenic fungi can be recognized by the host and its immune system and that EVs can modify immune function. This is of special interest as fungi are associated with more than 1 000 000 000 infections and more than 1 000 000 deaths each year, representing a major threat to human's health [9]. Novel strategies to target EVs may represent powerful new ways to treat fungal infections and deal with the emergence of acquired resistance to antifungal drugs [10].

This review will briefly summarize recent progress from the field of human pathogenic fungal EVs and highlight challenges for the future study of fungal EVs.

Biogenesis, size, sorting and release of fungal EVs

The role of EVs released by human pathogenic fungi has been extended recently from simple 'trash bags', discarding misfolded proteins and toxins outside the cell, to virulence messengers associated with pathological progression in the host [3,4,7••,8,11]. An explosion of recent research has shown that fungal EVs contain virulence proteins [2,3,6,11–19] and different classes of RNA [20,21••,22]. Nevertheless, little is known about mechanisms of their biogenesis, sorting and release by fungi, especially in the context of infection.

A major complexity for the field is that every EV is unique. Electron microscopy-based studies revealed wide heterogeneity in fungal EVs. They differ in size (see Table 1), electron-density, pigmentation or even the presence of additional internal vesicles [2,4–6,11–13,23]. The newest classification of EVs divides them into two main groups based on their size [24••]: small EVs (sEVs, and EVs of endosomal origin called 'exosomes'; up to 150 nm) and medium/large EVs (m/lEVs or microvesicles; bigger than 150 nm), something that appears to be true for EVs isolated from *C. neoformans* [16,23,25–27], *C. albicans* [18,28••], *H. capsulatum* [19,29] and *S. brasiliensis* [3].

Table 1

Characteristics of fungal EVs

Fungal species	Features and diseases caused	EVs size and the method of analysis
Cryptococcus neoformans	Basidiomycota encapsulated yeast. Human opportunistic pathogen. Causes cryptococcosis mainly in immunocompromised patients. May disseminate from the lungs to brain and other organs.	• 60–300 nm — EM [1] • 20–40 nm and majority 160 to 260 nm — QELS (melanized vesicles: 321–380 nm, non-melanized vesicles: 160 to 260 nm) [23] • 50–300 nm — EM [75] • 100–200 nm — EM [38] • 10–150 nm and 400–1000 nm — DLS [26] • 20–2000 nm (15% EVs: 80 nm; 50.6%: larger than 90 nm) — EM [15] • ~40 to ~80 and ~120 to ~230 nm — DLS [16] • 40–60 nm and 100–200 nm — DLS [25] • 40–60 nm and 250–350 — DLS [76] • 50 to ~800 nm and majority 50 to 250 nm — NTA [22] • Mean 150 nm — DLS [54] • 40–60 nm and 170 and 250 nm — DLS [19] • ~20 to ~120 and ~200 to ~400 nm — DLS [27]
Cryptococcus gattii	Basidiomycota encapsulated yeast. Human primary pathogen. Causes cryptococcosis mainly in the lungs. Rare.	• 26.3–397 nm (median: 108 nm) — NTA [7••]
Histoplasma capsulatum	Dimorphic fungus in the division Ascomycota and a systemic pathogen. In immunocompromised individuals may disseminate from the lungs to other organs.	• 40 and 60 nm and 170 and 250 nm — DLS [19] • 10–350 nm with majority 7 to 50 nm — EM [2] • ~50 nm and 175–250 nm — DLS [29]
Sporothrix schenckii	Dimorphic fungus in the division Ascomycota. The causative agent of sporotrichosis, commonly known as 'rose handler's disease'—enters the body through broken skin. In immunocompromised individuals may disseminate to joints, brain and spine.	• 50–100 nm (11% EVs) – EM [2]
Sporothrix brasiliensis	Dimorphic fungus in the division Ascomycota. The causative agent of sporotrichosis. In immunocompromised and immunocompetent individuals may disseminate to other organs and develop hypersensitivity reactions.	• 50–350 nm with majority 50–100 nm — NTA [3]
Malassezia sympodialis	Yeast in the division Basidiomycota. Skin commensal of healthy and diseased individuals associated with dandruff and atopic dermatitis.	• 50–200 nm with an average of approximately 100 nm — EM [4] • 50–600 nm with a mean around 200 nm — NTA; when yeasts cultured in mDixon broth pH 5.5: 193.9 ± 9.9 nm (mean ± SD); when yeasts cultured in mDixon broth pH 6.1: 213.2 ± 12.0 (mean ± SD) [20] • 171 ± 12 nm (mean ± SD) – NTA (yeasts cultured in RPMI medium for 48 hour) and 245 ± 10.9 nm (when yeast cultured for 72 hour in mDixon broth) [11]
Candida albicans	Dimorphic fungus in the division Ascomycota and human opportunistic pathogen, the most common fungal pathogen in humans.	• 50–100 nm (13% EVs) and larger than 100 nm (1% EVs) — EM [2] • ~50 to ~200 (mean: 60–150 nm) — EM [17] • 50–100 nm and 350–850 nm — EM and DLS [18] • 50–100 nm with a smaller percentage of EVs bigger than 200 nm — EM [12] • 50 to 100 nm (mean: 68.9 nm) — DLS [77] • Biofilm EVs: 30–200 nm — DLS; planktonic EVs: 30 to 200 nm and 200 to 1000 nm — DLS [28••]
Candida parapsilosis	Dimorphic fungus in the division Ascomycota opportunistic pathogen of human, animals and insects. A causative agent of sepsis.	• 50–100 nm (36% EVs) and larger than 100 nm (18% EVs) — EM [2]
Paracoccidioides brasiliensis	Dimorphic fungus in the division Ascomycota. Systemic pathogen that can cause paracoccidioidomycosis in immunocompromised and immunocompetent patients.	• Smaller than 100 nm — EM [5]
Alternaria infectoria	Filamentous fungus in the division Ascomycota. Plant pathogen and an opportunistic human pathogen.	• 20–40 nm (a mean size of 28.36 nm) — EM; 50 nm and 100 nm — DLS [6]
Trichophyton interdigitale	Dermatophyte in the division Ascomycota. Skin pathogen of humans and animals.	• 20–380 nm (a mean size of 110 nm) — NTA [8]

Abbreviations: DLS — dynamic light scattering (also known as QELS — quasi-elastic light scattering); EM — electron microscopy; NTA — nanoparticle tracking analysis.

EVs may originate from endosomes and multivesicular bodies (MVB) or from the plasma membrane (reviewed already in Refs. [30–33]) and are only released by living cells [1,5,8]. *C. albicans* biofilm EVs contain endosomal sorting complexes required for transport (ESCRT) subunits Hse1 and Vps27 and are created by the ESCRT pathway. Among 21 *C. albicans* mutants lacking different ESCRT subunits, 76% of them revealed decreased EV production including components of polyprotein complexes belonging to ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and ESCRT-DS [28••]. There is also evidence for secretory autophagy contributing to EV formation in human pathogenic fungi, since *C. neoformans* mutants lacking Golgi reassembly and stacking protein (GRASP) and the autophagy regulator Atg7 showed changes in EV size, producing only sEVs [22], suggesting that stress-induced unconventional route may bypass the Golgi through autophagosomal structures and ESCRT/MVB pathway as shown in mammals [34].

Additionally, it has been suggested that some proteins might be released via multiple secretion routes [35•]. In fact *P. brasiliensis* heat shock protein 70 (Hsp70) and enolase can be found in EVs and in non-vesicular fractions [14], similarly to the cryptococcal virulence factor glucuronoxylomannan (GXM), which can be found in post-Golgi secretory vesicles [36], and in some EVs, but not in all of them [25]. Importantly, some proteins are synthesized in stages. Studies on endosomal sorting nexin in *Aspergillus fumigatus* and *Aspergillus nidulans* revealed that melanin cell wall deposition depends on two routes of biosynthesis: the early step of melanin biosynthesis takes place in endosomes where all the enzymes necessary in the process are delivered through a non-conventional secretory pathway, while the late steps of melanin synthesis occur in the cell wall, where all the enzymes required are accumulated [37].

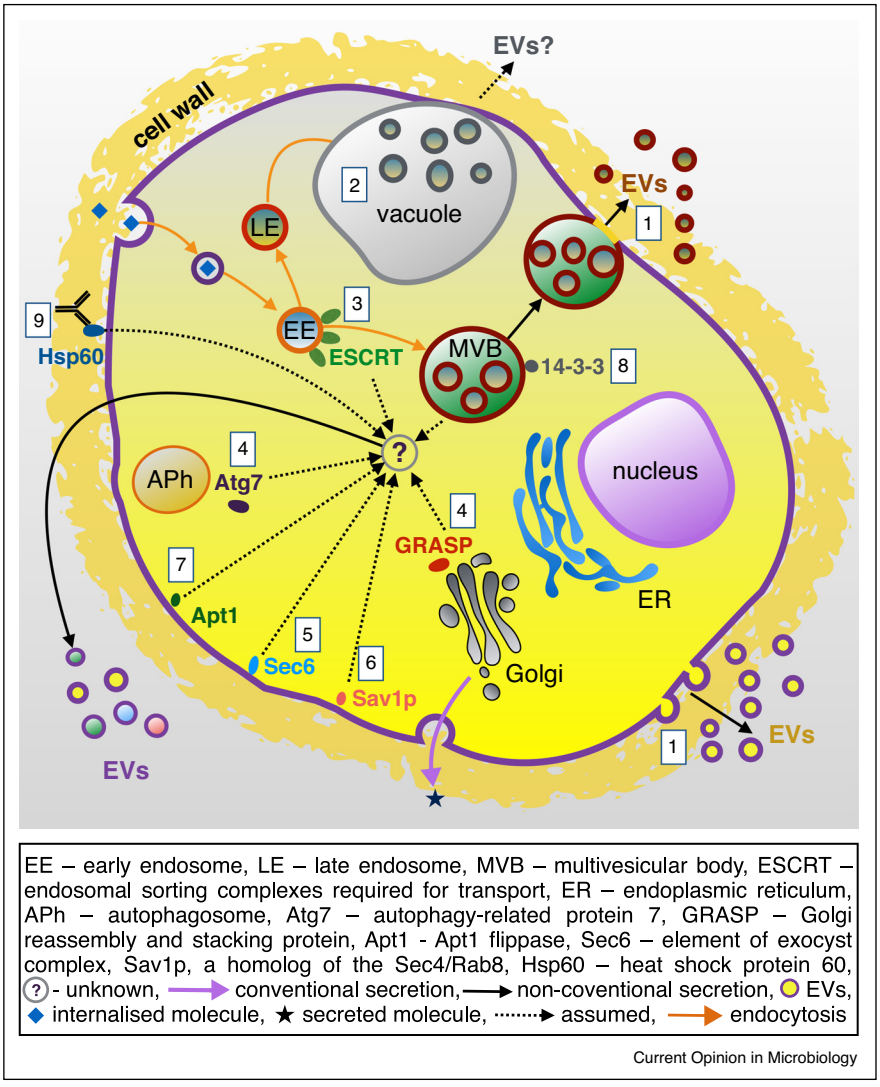
EV dimensions may be connected with specialised functions contained in their protein or RNA content. For instance, cryptococcal EVs become bigger upon melanization [16,23] and bigger cryptococcal EVs (100–200 nm in diameter) are more stable in solution, than smaller EVs [25]. Interestingly, it has been shown that the size of EVs may affect their release. In *C. neoformans* EVs larger than 100 nm are only released in multiple vesicle events (where a group of vesicles is released at the same time), while smaller vesicles can leave the cell in multiple or single events [16]. This suggests that EVs larger than 100 nm may have an endosomal origin, whilst smaller EVs may originate also from other pathways including both conventional and unconventional secretory routes [33]. In fact, many mutants (from pathogenic and non-pathogenic species) deficient in several Golgi proteins or proteins involved in transport of Golgi-originated vesicles to the plasma membrane show defects in the formation and release of fungal EVs (see Figure 1) [22,26,36,38,39].

Another poorly understood aspect is how the specificity of cargo sorting into fungal EVs is regulated. A *C. neoformans* mutant deficient in GRASP showed alterations in mRNA content of the vesicles [22], although levels of pigmentation and urease activity were not affected [40]. Indirect evidence of sorting characteristics in EV-protein sequence was provided from studies on laccase mislocalisation in *C. neoformans* cells, where typical cell wall localization of the enzyme was altered to cytoplasmic vesicles after deletion of the C-termini of the protein [41], suggesting the presence of specific posttranslational modifications that might influence protein sorting to EVs and their release. Indeed, a recent study in mice showed that ubiquitin-like 3 (UBL3)-dependant modification regulates protein (but not microRNA) sorting to MVBs and sEVs [42].

Furthermore, vesicle characteristics may be affected by environmental stimuli like nutrient or oxidative stresses. For instance, EVs derived from *C. albicans* biofilm or *C. neoformans* melanized cells are composed of a single size population, but EVs derived from planktonic *C. albicans* yeast or *C. neoformans* non-melanized cells are more diverse in size with the presence of two populations [23,28••].

In the host context, binding of Hsp60 expressed on the *H. capsulatum* cell wall [43] by different classes of monoclonal antibodies (MAbs) leads to changes in EV release and their protein load [19,29]. This is of special interest as *H. capsulatum* Hsp60 is recognized by phagocytic cells [43] and monocytes [44]. These data indicate that the extracellular milieu/host may affect EV size, protein sorting to EVs and EV release. In fact, the *cis*-acting signals (higher-order oligomerization of cell-surface proteins induced e.g. by antibodies and/or plasma membrane association via an anchor like a myristoylation tag) have been shown to target proteins to EVs in human cells and by retroviruses [45,46]. Interestingly, vesicles isolated from MAb-treated *H. capsulatum* yeasts showed an increase in size, protein concentration and changes in protein profile. The most significant changes were observed in proteins associated with amino acid/protein metabolism, including changes in phosphatase and laccase activity but only a moderate reduction in activities of catalase and urease [19], suggesting again there might be different pathways for protein sorting into EVs, or different classes of EVs, depending on stimuli. EVs isolated from *M. sympodialis* growing at pH 5.5 and 6.1 (mimicking the pH of healthy skin and atopic eczema skin, respectively) did not differ in their size (50–600 nm with a mean around 200 nm), morphology, total number and their protein concentration [20]. Surprisingly, although those EVs contained several short non-coding RNAs predicted to be differentially expressed between the two pH conditions, levels of these short RNAs were similar [20]. pH also influences localization of laccase in *C. neoformans*

Figure 1



Schematic overview of biogenesis and release of EVs in fungi.
1. It is thought that some fungal EVs originate from endosome-derived MVB [16] and from the plasma membrane;
2. In addition, in *C. neoformans* EVs may also be derived from vesicle-containing vacuoles, which were found to fuse with the plasma membrane [13];
3. Mutations in the ESCRT machinery result in changes in EV production and EV-content sorting in *C. albicans* [28••];
4. EVs isolated from *C. neoformans* mutants lacking the autophagy regulator Atg7 or GRASP proteins were much smaller (around 100 nm) [22], suggesting that GRASP and Atg7, independently, are necessary for the formation of bigger m/lEVs. Additionally, GRASP mutants show changes in vesicular mRNA [22];
5. Mutants of *Sec6*, a protein belonging to an octameric exocyst complex responsible for polarized tethering of exocytic post-Golgi secretory vesicles to the plasma membrane, is highly impaired in EV release in *C. neoformans*. The vesicles were accumulated in the cytoplasm and the mutants showed changes in cell wall glucan and lower secretion of a soluble polysaccharide, laccase and urease with no EVs detected in the supernatant. Surprisingly, the capsule formation, phospholipase activity and growth at elevated temperatures were not affected, but the mutants demonstrated attenuated virulence in mice. It suggests that the exocyst protein Sec6 is necessary for sorting of EVs, their loading with laccase, urease and soluble exopolysaccharide and EV release [38];
6. *C. neoformans* mutants lacking Sav1p, a homolog of the Sec4/Rab8, involved in a conventional secretory processes accumulated ~100 nm vesicles containing GXM and other secreted proteins inside the cell and showed ~50% reduction in extracellular activity of acid phosphatase [36];
7. Reduction of the size range within the bigger vesicle population was also found in *C. neoformans Apt1* mutant [26], where lack of Apt1 flippase led to defects in Golgi morphology and in EV-based export of soluble GXM (but not capsular GXM) due to trapping pigmentous vesicles inside irregular vacuoles [73];
8. Decreased expression of a protein 14-3-3, a marker of cryptococcal microvesicles [50], leads to reduced GXM and protein content of EVs and lower activity of laccase and acid phosphatase [74];
9. Binding of Hsp60 by monoclonal antibodies in *H. capsulatum* leads to fluctuations in EVs size, their protein load and subsequent release [19,29].

cells, with the enzyme associating with cell wall at neutral pH but relocalizing to cytoplasmic vesicles at lower pH [41], probably as a prelude to release inside EVs. Interestingly, the low pH found within tumours increases EVs release and uptake by fusion [47].

An outstanding challenge for the field is to understand how EVs traverse the fungal cell wall. Several studies reported EVs crossing the cell wall [1,2,16], but how this transit within a dense polysaccharide/protein matrix is achieved remains mysterious. An elegant cryo-SEM study by Wolf *et al.* showed single and multiple vesicles crossing the cell wall, with no appearance of any channel or pore facilitating their passage [16]. A recent study implies that the fungal cell wall has viscoelastic properties that may lead to its prompt remodelling, which might allow crossing the cell wall by EVs. Liposomes used in the study had a diameter of 60–80 nm and their ability to penetrate *C. albicans* and *C. neoformans* cell walls in an intact form was dependent on their content [48••]. However, changes of the cell wall thickness accompanying melanization in cryptococci can lead to an increase in the number of vesicles seen in the area between the plasma membrane and cell wall [16].

EVs in host–pathogen interactions

The presence of diverse constituents within EVs raises the possibility of multiple interactions with the host (reviewed already by others [49]). Early reports presented evidence that EVs can enhance *C. neoformans* infection of the brain by boosting crossing of the blood-brain barrier [50] and modulate host anti-microbial activity by inducing release of increased levels of cytokines among other changes [4,18,51,52] (see also Table 2). Recent data from several systems indicate that fungal EVs can strongly impact on host immunity. EVs isolated from the skin disease-causing fungal pathogen *T. interdigitale* stimulated the release of nitrite (NO), TNF-α, IL-6 and IL-1β, but not IL-10, from mouse macrophages and human keratinocytes in a dose-dependent manner [8]. In addition, macrophages treated with *T. interdigitale* EVs showed increased iNOS, but not Ym1, mRNA levels, implying that *T. interdigitale* EVs may promote macrophage polarization towards a 'killing' M1 phenotype, similarly to EVs-derived from *P. brasiliensis* [52]. EVs from *M. sympodialis* (MalaEx) can interact with cells in human skin [11], stimulate inflammatory cytokine responses in human skin [11] and in PBMCs [4], although whether this effect was due to the presence of host vesicles containing pathogen

Table 2

The effects of fungal EVs on the host immune system

EVs from	Host	Pro-inflammatory cytokines								Anti-inflammatory cytokines				PI	Ref.
		IFNγ	TNFα	IL-1α	IL-1β	IL-6	IL-8	IL-12	NO	TGFβ	IL-4	IL-10	IL-13		
Cryptococcus neoformans	Murine macrophages RAW 264.7		↑						↑	↑		↑		↑	[51]
	In vivo Acanthamoeba castellanii model of infection													≅	[27]
Cryptococcus gattii	Murine macrophages J774A.1													≅	[7••]
Malassezia sympodialis	Human PBMCs (healthy donors)/CD14, CD34 depleted PBMCs (healthy or AE donors)		↑								↑				[4]
Trichophyton interdigitale	Mice BMDMs		↑		↑	↑			↑				≅	↑	
	Human keratinocytes (HaCaT)		↑		↑	↑	↑		↑						[8]
Sporothrix brasiliensis	Mice BMDDCs	↑	↑		≅	≅		↑			≅	≅		↑	
	In vivo murine skin model of infection		↑		↑										[3]
Candida albicans	Murine macrophages RAW 264.7/murine BMDMs/ murine BMDDCs		↑					↑		↑		↑			[18]
Paracoccidioides brasiliensis	Murine peritoneal macrophages		↑	↑	↑	↑		↑	↑					≅	
	Murine macrophages J774A.1		↑			↑		↑							[52]
Histoplasma capsulatum	Murine BMDMs													↓	
	Human macrophages (THP-1-derived macrophages)													≅	[29]

Abbreviations: PI — phagocytic index, PBMCs — peripheral blood mononuclear cells, AE — atopic eczema, BMDMs — bone marrow-derived macrophages; BMDDCs — bone marrow-derived dendritic cells, ↑ — increase, ↓ — decrease, ≅ — not changed.

associated molecular patterns (PAMPs) or a mixture of vesicles belonging to the host and to *M. sympodialis* remains unclear.

Fungal EVs also impact on host phagocytosis (see Table 2; PI). *T. interdigitale* EVs promoted the uptake of fungal conidia by macrophages by around 25% and reduced the number of viable intracellular fungi, suggesting that they enhance the fungicidal activity of macrophages and keratinocytes [8]. Loss of GRASP in *C. neoformans*, which reduces EV release, led to higher phagocytosis rate by macrophages [40]. In contrast, however, stimulation of the amoeba *Acanthamoeba castellanii* with *C. neoformans* EVs led to increased intracellular survival of *C. neoformans* yeast cells in a subsequent 24 hours infection assay, without accompanying changes in phagocytosis [27].

The uptake of EVs by recipient cells

There is a substantial body of evidence showing that many kinds of host cells are able to internalize fungal EVs [18,27,50,51]. Cryptococcal vesicles containing GXM can be found in the macrophage cytoplasm upon permeabilization of phagosomes by intracellularly replicating yeasts in macrophages after infection [53]. In addition, EVs isolated from macrophages infected with cryptococci showed additional peaks belonging to cryptococcal GXM in sucrose gradient fractions [1].

TEM pictures obtained from cross sections of the lung tissue of mice infected with *C. neoformans* revealed the presence of vesicles 2, 48 and 168 hours after infection [1]. Extracts of EVs from *H. capsulatum* or *C. neoformans* react with sera from patients with histoplasmosis [2] or cryptococcosis [13], respectively, showing that EV components contain immunogenic proteins. In addition, EVs were isolated from plasma of patients with *M. sympodialis*-associated atopic eczema [4] and from brains of mice infected with *C. neoformans* [50]. A very recent study from biofilms formed of *C. albicans* showed that the highest EVs production occurs 2 days after initial biofilm formation [28••].

The presence of vesicles in blood of mice 16 hours after infecting them with *C. neoformans* [50] suggests that the nano-size of EVs allow them to disseminate within the host, although we still do not know what distances vesicles can traverse. There is evidence from *in vitro* studies on cryptococcal EVs that human serum proteins like albumin [7••,25] and β-galactoside-binding protein galectin-3 [54] can lyse some vesicles within 60–90 s [55]. This suggests that a subpopulation of fungal EVs might be unstable in body fluids and that they may be able to act locally only.

One of the methods to study the uptake of fungal EVs is to use non-specific lipophilic dyes that become fluorescent after integration into lipid membranes. Indeed, 1,1′-Dioctadecyl-3,3,3′,3′- Tetramethylindocarbocyanine Perchlorate (DiI, Invitrogen) and its derivatives such as Vybrant DiI are the most popular chemicals used for fungal EV visualization, first described by Nicola *et al.* in 2009 [56]. EVs isolated from *M. sympodialis* and stained with 10 μg/ml of Vibrant DiI were actively bound and internalized by human primary keratinocytes and monocytes [11]. The uptake was not observed at low temperature, suggesting it was an active process. A longer incubation time of MalaEx with keratinocytes revealed strong perinuclear localization of the vesicles [11], similarly to the uptake pattern seen in macrophages or dendritic cells uptaking *C. albicans*-derived EVs [18]. Studies of EVs from *C. gattii* showed their relocalization from the cell periphery of J774 macrophages to the cell body in a time-dependent manner [7••], a rapid process with a half time of peak internalization of only 17.5 min.

Typically EVs of diverse origin can be taken up either by membrane fusion or via different endocytic routes such as lipid raft-dependent, clathrin-dependent or caveolin-dependent endocytosis, as well as macropinocytosis or phagocytosis [57,58]. At least in macrophages, 40 nm nanoparticles can be internalized via clathrin-mediated endocytosis, macropinocytosis and phagocytosis [59] and the uptake of *C. gattii* EVs by J774 macrophages was similarly blocked after pretreatment of phagocytes with lipid raft polymerisation and actin polymerisation inhibitors [7••]. Association between *C. albicans*-derived EVs and a macrophage also seems to be lipid raft-dependent [18].

EVs and polymicrobial interactions

In the environment and in the human host, fungi encounter a huge diversity of other microbes, and mixed infection in the human host are common [60]. For instance, mixed cryptococcal infections occur in nearly 20% of patients diagnosed with cryptococcosis [61]. *In vitro* studies from our lab have shown that during infection between different species of the same genus, more virulent isolates may increase the survival of less pathogenic ones inside macrophages [62] and that the effect is transferred by EVs isolated from the virulent strain [7••]. This may contribute to poorer prognosis of the disease. Indeed, patients infected with several cryptococcal strains showed increased fungal dissemination and increased resistance to antifungal drugs, although mortality was reduced [61].

Similarly, the high density of cells and extracellular polymeric matrix in *C. albicans* biofilms leads to production of EVs that are distinct from those released by planktonic cells and which enhance drug resistance [28••].

Can fungi uptake other EVs? So far, the only direct evidence has come from studies on plant fungi. EVs isolated from extracellular fluids of sunflower seedlings were internalized by the phytopathogenic fungus *Sclerotinia sclerotiorum*, which affected fungal growth,

morphology and cell death of the spores [63••]. Also *Botrytis cinerea* internalizes *Arabidopsis* EVs filled with small RNAs which leads to silencing of the fungal genes involved in pathogenicity [64••]. Indirect evidence was also provided from studies on *C. gattii* where EVs isolated from highly virulent Pacific Northwest outbreak isolate colocalized inside the phagosomes with low-pathogenic *C. gattii* yeasts and increased their survival inside macrophages [7••]. Because of the fact that fungal EVs contain different classes of RNA [21••] including those that can modulate gene expression in recipient cells, it raises the possibility that those pathogenic fungi such as *C. gattii* and smut fungus *Ustilago maydis*, which lost their RNA interference machinery during evolution [65], gained some evolutionary advantage by reducing the opportunity to be hijacked by other organisms and their RNA moieties inside the EVs [66].

Conclusions and remaining questions

There are more than 300 fungi that are pathogenic to humans [67–69], but only in 11 fungal species, research about EVs has been performed to date (Table 1).

In order to study EV uptake, fungal vesicles need to be visible. Lipophilic dyes are one way to achieve this, although some (such as DiI) may increase EV size [27] or create false-positive signals from non EV-associated fluorescent nanoparticles [70]. In other organisms, EV-specific tetraspanins are useful biomarkers, but these appear to be absent in human fungal pathogens [71], making fungal EVs research field challenging. An additional problem is the variation in EV quantification methods used across groups, leading to the recommendation that the field adopts a universal protein:lipid ratio method [24••].

Finally, there are a number of outstanding questions for the field:

1 How many routes are associated with fungal EV biogenesis and how are these pathways affected by the host environment?
2 What influences EV loading with specific components and how are these processes controlled?
3 Does the fungal cell cycle influence EV biogenesis and release?
4 What are the differences in EVs after morphological switching in dimorphic fungi?
5 Why do senescent cells release more EVs than active cells [72]?
6 Why do fungal EVs 'prime' recipient cells?
7 What are the levels of EVs *in vivo* during infection?
8 What happens with EVs released by fungi inside host cells—are they destroyed by the host, kept inside or released to the extracellular environment?
9 Are all EVs serum-sensitive or do serum-resistant populations exist?
10 How do antifungal drugs influence biogenesis/sorting and release of fungal EVs?
11 Amphotericin B, a common antifungal, sequesters ergosterol from fungal membranes, including fungal EV membranes (E. Bielska, *unpublished*), so how does this treatment impact on EVs in the host?
12 Do fungal EVs influence antifungal resistance in 'recipient' fungi?
13 Do fungal EVs influence other microorganisms within the human body?

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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