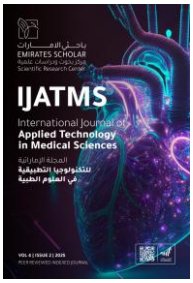

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The future potential of fungal extracellular vesicles (EVs) in managing neonatal fungal infections

Saima Zaheer
Student, London Metropolitan University
saz0167@my.londonmet.ac.uk

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ABSTRACT

Fungal extracellular Vesicles (EVs) have emerged as significant mediators in the pathogenesis of neonatal fungal infections, particularly those caused by Candida albicans and Cryptococcus neoformans. These nanostructures are increasingly recognised for their dual role in both facilitating infection, through mechanisms such as immune evasion and antifungal resistance, and offering novel therapeutic opportunities. This article explores the multifaceted interactions between fungal EVs and the neonatal immune system, emphasising the critical balance between their detrimental and potentially beneficial immunomodulatory effects. Given the immunological immaturity of neonates, understanding the dynamics of these vesicles is vital for developing targeted interventions. The discussion further extends to the future potential of engineering fungal EVs as safe, precise, and effective tools for treating neonatal mycoses. By synthesising emerging findings, this work contributes to the evolving landscape of neonatal care and proposes forward-looking strategies to harness fungal EVs in clinical applications.

Keywords: *Fungal EVs, Neonatal fungal infections, Immunomodulation in neonates, Extracellular vesicle therapy, Fungal pathogenesis in neonates.*

1. Introduction

Neonatal *fungal* infections, particularly those attributable to opportunistic *fungi* such as *Candida albicans*, present significant clinical challenges in neonatal intensive care units (NICUs) globally. This is especially true among preterm and immunocompromised infants. The increasing incidence of these infections parallels advances in neonatal care, which, while improving survival among very low birth weight (VLBW) neonates, inadvertently expose them to greater infection risks due to invasive interventions and the extensive use of broad-spectrum antibiotics (Irani & Kanhere, 2004; *Neves et al., 2017). Candida* species are now the third most prevalent pathogens implicated in nosocomial bloodstream infections among premature infants, with *C. albicans* responsible for approximately 65% of cases (Wazir & Kumar, 2006).

Neonates possess an inherently immature immune system, marked by deficient neutrophil activity, suboptimal cytokine signalling, and limited complement function, rendering them particularly vulnerable to invasive *fungal* diseases (*Hamid et al., 2022*; *Neves et al., 2017*). Infections caused by *Candida* are associated with high morbidity and mortality, especially in preterm and low-birth-weight infants, with mortality rates exceeding 20% even with anti*fungal* treatment (*Dermitzaki et al., 2024*; Wazir & Kumar, 2006). Contributing risk factors include prolonged antibiotic therapy, central venous catheterisation, parenteral nutrition, and inadequate infection control practices (*Dermitzaki et al., 2024*). While prophylactic anti*fungal* strategies, such as fluconazole administration, have demonstrated efficacy in reducing infection incidence among high-risk cohorts, optimal therapeutic protocols remain incompletely defined ("*Fungal* Infections in the Neonatal Intensive Care Unit", 2024).

Timely diagnosis and treatment are crucial; however, conventional diagnostic techniques are often invasive and time-consuming, limiting their effectiveness in critical care settings (*Hamid et al., 2022*). In this context, *fungal* EVs have gained attention as both potential diagnostic biomarkers and modulators of infection severity. These vesicles play pivotal roles in *fungal* virulence, immunomodulation, and anti*fungal* resistance, thus necessitating comprehensive exploration of their mechanistic involvement and therapeutic potential (*Kaufman et al., 2024*)

Fungal EVs are lipid bilayer-enclosed nanostructures secreted by various *fungi*, encompassing a diverse cargo of proteins, lipids, polysaccharides, and nucleic acids. These molecular components contribute to virulence by facilitating tissue invasion, biofilm development, and evasion of host immune responses (*Ullah et al., 2023; Herkert et al., 2019*). In neonatal infections, *C. albicans* EVs enhance epithelial adherence and bolster anti*fungal* resistance, complicating treatment and promoting chronic infection (*Zamith-Miranda et al., 2020)*. Likewise, *Cryptococcus neoformans* EVs have been implicated in the traversal of the blood-brain barrier, resulting in central nervous system dissemination, a particularly severe outcome in neonates (*Silva et al., 2019*).

The molecular cargo of these EVs, including enzymes, heat-shock proteins, and other virulence-related factors, plays a central role in altering host immune dynamics and promoting *fungal* persistence (*Joffe et al., 2016; Rizzo et al., 2021*). Moreover, their capacity to induce biofilm formation and drug resistance underscores their importance in disease chronicity and therapeutic failure (*Brandt et al., 2024).* Despite growing insight into their biological roles, the specific mechanisms through which *fungal* EVs contribute to neonatal pathogenesis remain an area of ongoing investigation (*Ikeda et al., 2024; Rizzo et al., 2020*).

Importantly, *fungal* EVs also exhibit immunomodulatory properties that could be harnessed for therapeutic benefit. Their role in modulating immune responses, either by promoting tolerance or suppressing hyperinflammation, suggests promising applications in treating neonatal infections where immune immaturity is a limiting factor in host defence (Nenciarini & Cavalieri, 2023; *Ullah et al., 2023*). Engineered *fungal* EVs may serve as delivery systems for immunotherapeutic agents

or anti*fungal* compounds, potentially improving treatment specificity and reducing toxicity (*Freitas et al., 2019*; Adie, 2024). Additionally, these vesicles are being investigated as novel vaccine adjuvants and drug delivery platforms, extending their relevance beyond infection management (*Silva et al., 2019; Keshtkar et al., 2022*).

Nevertheless, key challenges remain, including the need for standardised EVs isolation protocols and a more complete understanding of EV biogenesis and cargo regulation (Adie, 2024; *Ullah et al., 2023*). Immunosuppression, in this context, refers to the downregulation of immune responses, which, if uncontrolled, can exacerbate vulnerability to opportunistic pathogens and accelerate disease progression (*Honorato et al., 2022*).

This study offers a critical evaluation of the role of *fungal* EVs in neonatal *fungal* infections, proposing a conceptual framework for their therapeutic application. By investigating the intersection of *fungal* pathogenesis and neonatal immunomodulation, this work aims to contribute to the recharacterisation of *fungal* EVs, from agents of virulence to potential tools for precision medicine in neonatology (*Rodrigues et al., 2019*).

2. LITERATURE REVIEW

2.1 *Fungal* Extracellular Vesicles: General Mechanisms

The identification of *fungal* EVs has markedly transformed current understanding of *fungal* pathobiology, particularly concerning infections in neonates. These vesicles are nano-sized, membrane-enclosed structures that originate intracellularly and are exported across the rigid *fungal* cell wall into the extracellular milieu. Their biogenesis occurs through both Endosomal Sorting Complex Required for Transport (ESCRT)-dependent and ESCRT-independent pathways, underscoring the multifactorial nature of their synthesis and secretion (*Oliveira et al., n.d.; Rizzo et al., 2021*).

Fungal EVs encapsulate a heterogeneous repertoire of bioactive molecules such as polysaccharides, lipids, allergens, heat-shock proteins, hydrolytic enzymes, pigments, and diverse RNA species. These molecular cargos are instrumental in influencing host-pathogen dynamics by promoting biofilm development, conferring anti*fungal* drug resistance, and amplifying virulence (*Silva et al., 2019*). The synthesis and release of these vesicles are modulated by environmental factors, including pH variation, nutrient limitation (e.g., glucose depletion), and anti*fungal* agent exposure, factors that reflect their environmental responsiveness and adaptive versatility (*Castelan-Ramírez et al., 2023*).

In pathogenic *fungi*, EVs are critical mediators of macromolecular export, particularly facilitating the trans-cell wall delivery of virulence determinants and immunologically active compounds to host cells. This translocation significantly contributes to pathogenicity by manipulating host immune responses and promoting *fungal* persistence (Rodrigues *et al*., 2011). While the full spectrum of EVs functionality in *fungal* infections is still under investigation, emerging evidence suggests promising applications for these vesicles in clinical settings. These include their use as diagnostic biomarkers, vaccine adjuvants, and vehicles for anti*fungal* therapeutics (Silva *et al*., 2019; Ullah *et al*., 2023).

Contemporary research is increasingly focused on decoding the structural organisation and molecular composition of *fungal* EVs. Such investigations are revealing nuanced insights into their functional roles in modulating host-microbe interactions (*Rizzo et al., 2020; Rizzo et al., 2021*). This evolving field offers substantial potential for the development of innovative strategies to manage *fungal* infections, particularly in immunologically vulnerable groups such as neonates, through the targeted exploitation of EVs' natural bioactivity (*Wu et al., 2023*).

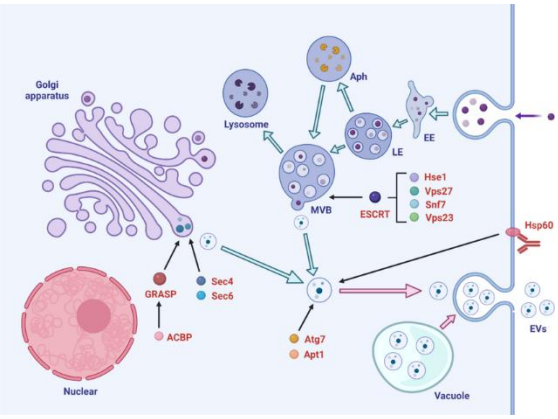

Figure 1: General mechanisms of *fungal* extracellular vesicle (EV) biogenesis and release. (Adapted from Ullah, A., Huang, Y., Zhao, K., and Zheng, L., 2023. "*Characteristics and potential clinical applications of the extracellular vesicles … "*).

This diagram illustrates the biogenesis of *fungal* EVs via multiple intracellular pathways, namely, Golgi-derived secretory routes, multivesicular body (MVB) preparation through ESCRT-dependent mechanisms, and ESCRT-independent mechanisms such as lipid-mediated budding. It also shows movement through the cell wall, release into the extracellular space, and interaction with host tissues. Genetic regulators like GRASP, Atg7, Sec4/Sec6, ESCRT subunits (Vps23, Snf7), and lipid flippases (e.g., APT1) are annotated to highlight control points in vesicle formation and cargo selection.

2.2 Specific Examples of *Fungi* and EV Effects

Candida albicans is a prominent opportunistic *fungal* pathogen, particularly in immunocompromised individuals and neonates, where it exploits host vulnerabilities through a multifactorial virulence arsenal. A key virulence mechanism is the release of EVs, which facilitate pathogenicity by transporting adhesins, hydrolytic enzymes, and other virulence determinants essential for host colonisation and immune evasion. These vesicles promote tissue adherence via specific surface proteins-adhesins, that enable epithelial binding, a prerequisite for successful colonisation and infection (*Pereira et al., 2015; Geraldino et al., 2012;* Modrzewska & Kurnatowski, 2015).

The biofilm-forming capacity of *C. albicans*, another hallmark of its virulence, is critically enhanced by EVs. These biofilms comprise structured microbial communities within an extracellular matrix that confer resistance to anti*fungal* agents and immune surveillance (*Ponde et al., 2021; Khan et al., 2020*). EVs not only facilitate biofilm development but also elicit pro-inflammatory responses that exacerbate tissue damage (*Kulig et al., 2025*). Notably, *C. albicans* EVs possess unique protein markers such as Sur7-like family proteins, which are implicated in vesicle formation and cargo selection, suggesting *fungal*-specific biogenetic pathways distinct from mammalian systems (*Dawson et al., 2020*).

Hydrolytic enzymes, including secreted aspartyl proteinases, contribute further to pathogenicity by degrading host tissues and modulating the immune response, thereby enabling deeper invasion and persistent infection (*Naglik et al., 2003; Geraldino et al., 2012*). *C. albicans*' ability to form resilient biofilms on indwelling devices presents a major therapeutic challenge, often associated with elevated morbidity and mortality, particularly in neonates (*Ponde et al., 2021; Ribeiro et al., 2024*).

Similarly, *Cryptococcus neoformans*, an encapsulated basidiomycete, leverages EVs to support virulence and CNS invasion. These vesicles transport capsular polysaccharides and immunomodulatory molecules that facilitate traversal of the blood brain barrier (BBB) a critical step in the pathogenesis of cryptococcal meningitis in neonates (*Vu et al., 2013; Oliveira et al., 2020; Yang et al., 2017). C. neoformans* EVs have been shown to induce significant molecular alterations in brain endothelial cells, promoting increased permeability and pathogen dissemination. In addition, they modulate host immune responses by activating inflammatory cascades and influencing cytoskeletal proteins essential for barrier integrity (*Zhang et al., 2021; Marina et al., 2020*). These findings reinforce the importance of EVs in CNS invasion and persistent neuroinfection (*Huang et al., 2012;* Schorey & Harding, 2016).

Beyond classical pathogens, *Tolypocladium inflatum*, a filamentous fungus, produces the potent immunosuppressant cyclosporin A (CsA), a cyclic undecapeptide widely used in transplant medicine. CsA biosynthesis involves a complex enzymatic machinery, including non-ribosomal peptide synthetases and polyketide synthases (*Yang et al., 2018*). While *T. inflatum* is not typically associated with human infections, the presence of CsA within EVs raises concerns regarding inadvertent immunosuppression in vulnerable populations, especially neonates (Shevach, 1985; Wartburg & Traber, 1988; Aarnio & Agathos, 1989). Its ecological and virulence roles particularly in insect models suggest that CsA production may extend beyond pharmaceutical applications to include competitive advantages against other *microorganisms* (Kobel & Traber, 1982; *Agathos et al., 1987*).

Phylogenetic comparisons further highlight the diversity of *fungal* EVs. Members of *Ascomycota*, such as *Aspergillus fumigatus*, produce EVs enriched in stress-response proteins and immune-interactive molecules. These vesicles enhance macrophage activation and stimulate pro-inflammatory cytokines, including TNF-α and IL-1β, facilitating innate immune responses (*Souza et al., 2019; Freitas et al., 2023*). Specific surface proteins, such as glycosylasparaginase, are implicated in modulating host cytokine production and promoting immune evasion (*Pinzan et al., 2024; Goldman et al., 2023*). Furthermore, the high osmolarity glycerol (HOG) MAPK pathway a key *fungal* stress response mechanism, is vital for environmental adaptation and pathogenic persistence (Brown & Goldman, 2016; McCormick, 2012).

In contrast, *Basidiomycota* fungi like *C. neoformans* secrete EVs containing glucuronoxylomannan, a capsular polysaccharide with immunomodulatory properties. This compound interferes with neutrophil recruitment and cytokine signalling, thereby enabling immune evasion and persistence within host tissues (*Freitas et al., 2019*; *Kniemeyer et al., 2016*). These comparative insights underscore the divergent yet convergent roles of *fungal* EVs in disease pathogenesis.

The therapeutic potential of EVs, particularly in neonatology, is receiving increasing attention. EVs, by virtue of their nanoscale size and complex cargo, facilitate intercellular communication and immune modulation, rendering them promising tools for neonatal interventions (*Wu et al., 2023*; Nambi, 2024). Stem cell-derived EVs have shown regenerative benefits in disorders such as respiratory distress syndrome and bronchopulmonary dysplasia, conditions prevalent in premature neonates (*Goryunov et al., 2024; Matei et al., 2019; Futata et al., 2012).* Moreover, *fungal* EVs are being investigated for their capacity to serve as diagnostic tools and vaccine delivery vehicles for invasive *fungal* infections (Liu & Hu, 2023).

Nonetheless, significant challenges must be addressed before these technologies can be translated into clinical practice. These include the standardisation of EVs isolation and characterisation, clarification of their mechanisms of action, and enhancement of targeting specificity through engineered surface modifications (*Liu et al., 2023; Barathan et al., 2024*). Importantly, the immunomodulatory effects of EVs, particularly those derived from pathogenic *fungi*, necessitate caution in neonatal applications, where immune systems are not fully developed (Adie, 2024; *Matei et al., 2019*).

In sum, *fungal* EVs present a dual-edged phenomenon: they contribute substantially to pathogenesis while also offering a platform for innovative therapeutic interventions. Their strategic exploitation in neonatal care, especially for infection control and immune support, requires meticulous preclinical evaluation and regulatory oversight to ensure safe and efficacious applications.

Table 1: Examples of *Fungal* Species and the Therapeutic Versus Pathogenic Potential of Their EVs

Fungal Species	Known Extracellular Vesicle (EV) Cargo	Pathogenic Potential	Therapeutic Potential	References
Candida albicans	Adhesins (Agglutinin-like sequence proteins), Secreted Aspartyl Proteases, RNA molecules, Heat Shock Proteins	Promotes epithelial invasion, biofilm formation, and immune evasion	EVs induce protective Immunoglobulin G responses in mice; a potential vaccine candidate	Zarnowski *et al*., 2018; Vargas *et al*., 2020
Cryptococcus neoformans	Glucuronoxylomannan, Mannoproteins, Immunomodulatory Proteins, RNA molecules	Blood-brain barrier traversal, immunosuppression, central nervous system infection	Immunization with EVs stimulates antibody production and increases survival in animal models	Huang *et al*., 2012; Rizzo *et al*., 2021
Aspergillus flavus	Stress Response Proteins, Allergens	Promotes invasive aspergillosis; antigenic stimulation	EVs improve survival rates in *Galleria mellonella* infection models	Rizzo *et al*., 2021
Paracoccidioides brasiliensis	Glycoproteins, Heat Shock Proteins, Lipids	Induces granulomatous inflammation	EVs combined with adjuvants stimulate strong Immunoglobulin M, Immunoglobulin G, and T-cell Interferon gamma responses	Freitas *et al*., 2019
Histoplasma capsulatum	Heat Shock Proteins, Enzymes, RNA molecules	Promotes macrophage evasion and intracellular survival	EVs cargo under study for potential vaccine antigens; promising immunomodulatory profile	Albuquerque *et al*., 2008
Candida auris	Adhesins, Lipases, RNA species	Multidrug resistance, biofilm formation, and hospital outbreaks	Early characterization of EVs for vaccine or biomarker development	Zamith-Miranda *et al*., 2021

(Adapted from Zarnowski et al., 2018; Vargas et al., 2020; Huang et al., 2012; Rizzo et al., 2021; Freitas et al., 2019; Albuquerque et al., 2008; Zamith-Miranda et al., 2021)

3. Methodology

This study adopts a systematic literature review methodology to collate and critically evaluate the current evidence on the therapeutic applications of *fungal* EVs in neonatal care. Systematic reviews are esteemed in scientific inquiry due to their structured, transparent, and reproducible approach, which facilitates objective analysis and synthesis of existing research (*Liberati et al., 2009).*

A comprehensive summary of key studies is presented in Table 2, illustrating the *fungal* species studied, experimental models used, and the observed therapeutic effects. This includes findings from *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus flavus*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Candida auris*. These studies underscore the diverse roles of *fungal* EVs in modulating immune responses, reducing *fungal* burden, and enhancing survival in both in vitro and in vivo neonatal infection models (*Vargas et al., 2020; Zarnowski et al., 2018; Huang et al., 2012; Rizzo et al., 2021; Freitas et al., 2019; Albuquerque et al., 2008; Zamith-Miranda et al., 2021).*

To ensure the breadth and quality of source material, literature was retrieved from leading scientific databases, PubMed, Scopus, and Web of Science, chosen for their extensive indexing of peer-reviewed articles in microbiology, immunology, and neonatal medicine. The search strategy employed a predefined set of keywords, including "*fungal* extracellular vesicles," "neonatal *fungal* infections," "immunomodulation," and "EV-based therapies." Both primary research (e.g., experimental and clinical studies) and secondary sources (e.g., reviews) were included to encapsulate the multifaceted therapeutic implications of *fungal* EVs.

The selection of studies followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, which provide a rigorous framework for transparent and unbiased reporting of systematic reviews (*Moher et al., 2015*). No restrictions were applied regarding language, and all studies published up to the current year were considered. The review process involved a two-stage screening: an initial assessment of titles and abstracts, followed by full-text evaluations against clearly defined inclusion criteria (Higgins & Green, 2011).

Quality assessment focused on the robustness of experimental design, reliability of findings, and relevance to the review objective. Extracted variables included the species of *fungi* studied, isolation and characterisation techniques for EVs, and the nature of therapeutic or immunomodulatory outcomes observed. Thematic synthesis was employed to group data into key areas such as immune modulation, vaccine development, and neonatal host defence enhancement.

Tables and illustrative figures were utilised to consolidate and visualise findings, with an emphasis on neonatal immune responses to *fungal* EVs in animal and cellular models. This systematic review framework ensures that the data synthesis is not only comprehensive but also scientifically robust and aligned with internationally accepted standards in biomedical literature review.

Fungal Species	Experimental Model	Observed Therapeutic Outcomes
Candida albicans	Immunocompromised mice treated with cyclophosphamide; *Galleria mellonella* (wax moth larvae)	EV-based immunisation elicited elevated Immunoglobulin G (IgG) levels, significantly reduced *fungal* loads in kidneys, spleen, and liver; enhanced survival outcomes.
Cryptococcus neoformans	BALB/c mice; murine macrophage cultures	EV immunisation induced strong antibody responses; reduced *fungal* burden in brain tissues; certain EVs triggered hyperactivation

		of macrophages, warranting safety consideration.
Aspergillus flavus	*Galleria mellonella* (wax moth larvae)	Administration of EVs improved post-infection survival rates; evidence of immune system priming was observed in larval models.
Paracoccidioides brasiliensis	BALB/c mice co-administered with Montanide ISA 720 adjuvant	EVs increased serum levels of IgM, IgG, and interferon-gamma (IFN-γ); reduced *fungal* burden; improved overall host survival.
Histoplasma capsulatum	In vitro human macrophage infection model	EVs modulated innate immune responses in macrophages; research is ongoing to characterise their broader immunomodulatory effects.
Candida auris	In vitro proteomic and transcriptomic analysis of EVs	Preliminary findings suggest potential for EVs in stimulating immune responses and serving as diagnostic biomarkers.

Source: Adapted from *Vargas* et al. *(2020); Zarnowski* et al. *(2018); Huang* et al. *(2012); Rizzo* et al. *(2021); Freitas* et al. *(2019); Albuquerque* et al. *(2008); Zamith-Miranda* et al. *(2021).*

3.1 Potential Therapeutic Applications of *Fungal* Extracellular Vesicles

3.1.1 Application of *Fungal* EVs in Neonatal Therapeutics

EVs derived from *fungi* have attracted growing interest due to their capacity to transport a wide spectrum of bioactive molecules, including proteins, lipids, RNA, and polysaccharides. These vesicles play integral roles in intercellular signalling, immune modulation, and the transfer of genetic material, thereby influencing host immunity and cellular responses. In the context of neonatal care, where the immune system is underdeveloped and reliant primarily on innate mechanisms, *fungal* EVs represent a promising therapeutic avenue capable of modulating and potentially enhancing immune defence mechanisms.

Neonates possess an immature immune architecture, characterised by a limited adaptive immune response and a relatively underperforming innate immunity, rendering them highly susceptible to systemic infections (*Saha et al., 2022*). In such cases, the immunomodulatory functions of *fungal* EVs may be harnessed to strengthen immune resilience or to fine-tune inflammatory responses during critical developmental periods.

Studies have demonstrated that EVs secreted by pathogenic *fungi* such as *Candida albicans*, *Aspergillus flavus*, and *Cryptococcus neoformans* exert significant effects on immune function by eliciting either pro-inflammatory or anti-inflammatory responses, contingent on their molecular composition (*Kulig et al., 2025; Duan et al., 2024*). For instance, EVs produced by *C. albicans* stimulate host immune cells to secrete cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which are crucial mediators of early immune activation (Vargas *et al.*, 2015; Duan *et al.*, 2024).

The immunogenic profile of *fungal* EVs is attributed to their enrichment in functional biomolecules such as lipids, surface glycoproteins, and structural polysaccharides, which collectively drive the activation of antigen-presenting cells including macrophages and dendritic cells

(*Kuipers et al., 2018; Herkert et al., 2019*). These immune cells, upon activation, exhibit enhanced phagocytic and fungicidal activity, thereby improving host defence. Moreover, components like secreted aspartic proteases (Saps) derived from *C. albicans* have been shown to activate inflammasomes, resulting in the release of pro-inflammatory cytokines such as IL-1β and IL-18, key players in orchestrating innate immune responses (*Pietrella et al., 2010; Pietrella et al., 2013).*

These findings suggest that *fungal* EVs may serve as biological tools to augment neonatal immunity in a context-specific manner, particularly in immunocompromised or at-risk infants (Adie, 2024; *Hezel et al., 2017*). Nevertheless, their ability to simultaneously induce regulatory and inflammatory cascades highlights a delicate duality that must be carefully managed through further translational research.

Among the most compelling examples is *Cryptococcus neoformans*, whose EVs have been found to carry immunomodulatory mannoproteins capable of activating dendritic cells, neutrophils, and macrophages. These vesicles stimulate inflammatory pathways and cytokine release, further facilitating pathogen clearance and adaptive immune development (*Freitas et al., 2019; Ullah et al., 2023; Rezende et al., 2024*). This immunostimulatory potential positions *fungal* EVs as viable candidates for next-generation therapeutics, particularly in the form of vaccines, immune adjuvants, and drug delivery systems (*Nenciarini & Cavalieri, 2023; Liu & Hu, 2023).*

In neonatal medicine, such vesicles could be leveraged to prevent or treat invasive *Candida* infections, which remain a substantial threat due to the neonate's compromised immune competency (*Rezende et al., 2024*). The ambivalent role of *fungal* EVs, as both contributors to pathogenesis and therapeutic agents, emphasises the necessity for precision-engineered applications and a deeper mechanistic understanding (*Jiang et al., 2023; Brandt et al., 2024*). Their interactions with innate immune cells suggest strong potential for clinical interventions targeting immune enhancement or regulation (*Freitas et al., n.d.; Kwaku et al., 2025*).

Furthermore, the development of *fungal* EV-based therapeutics could reduce reliance on broad-spectrum anti*fungal*s, which often lead to adverse effects and antimicrobial resistance. Engineered EVs may function as targeted immunotherapeutic or vaccines, equipping the neonatal immune system to mount more effective responses to opportunistic *microorganisms* (Vargas *et al*., 2020; Huang *et al*., 2012; Rizzo *et al*., 2021).

3.2 Challenges

The application of *fungal* EVs as therapeutic agents in neonatal medicine presents a range of significant challenges, primarily due to the potential for immunosuppression. Neonates possess immature immune systems that are inherently predisposed to immunotolerance. The introduction of immunosuppressive *fungal* EVs may exacerbate this susceptibility, leading to impaired immune responses and increased vulnerability to infection. Notably, *fungal* EVs may carry bioactive components such as transforming growth factor-beta (TGF-β), a cytokine frequently employed by pathogens to attenuate host immune activation, thereby compromising neonatal defence mechanisms (*Kojic et al., 2022*).

EVs derived from pathogenic fungi, including *Cryptococcus neoformans* and *Aspergillus fumigatus*, have been shown to transport virulence-associated molecules such as polysaccharides, lipids, and proteins. These components contribute to immune evasion strategies that pose risks to neonates with underdeveloped immune systems (*Goryunov et al., 2024; Matei et al., 2019*). While the immunomodulatory potential of EVs holds promise for managing autoimmune and inflammatory conditions, their deployment in neonates could inadvertently induce excessive immune suppression, thus heightening infection risk (Adie, 2024).

This concern is underscored by the high morbidity and mortality rates associated with neonatal *fungal* infections such as candidiasis and

candidemia, particularly among preterm and low birthweight infants (*Luz et al., 2021*; Ferrando and Castagnola, 2023). Although prophylactic anti*fungal* agents like fluconazole are commonly used to curb invasive *fungal* infections, they are not without limitations, especially concerning the emergence of anti*fungal* resistance (Healy, 2008). Additionally, while *fungal* EVs have demonstrated regenerative and communicative functions in preliminary studies (*Matei et al., 2019; Goryunov et al., 2024*), these findings require further validation before clinical implementation.

Translation of EV-based therapies into neonatal care is further impeded by the lack of standardised methods for their isolation and characterisation. Understanding the precise mechanisms by which *fungal* EVs modulate immune responses is critical to developing safe therapeutic applications (Adie, 2024). For instance, EVs from *A. fumigatus* have shown efficacy in modulating immune pathways during *fungal* keratitis; however, extrapolation to neonatal use demands caution given the distinct immunological profile of neonates (*Meng et al., 2024).*

Neonates, especially those born prematurely or with underlying conditions, exhibit reduced immune function, including diminished production of interferon-γ, a cytokine essential for defence against intracellular pathogens (Wilson and Lewis, 1990). This immunological immaturity places them at elevated risk for opportunistic infections. Studies suggest that *fungal* EVs may induce immune tolerance, as observed with *C. neoformans*, thereby hindering effective pathogen clearance (*Rivera et al., 2023*). Although some EVs, such as those derived from *A. fumigatus*, have demonstrated the ability to enhance *fungal* clearance in animal models (*Souza et al., 2022*), their unmodified application in neonates remains fraught with risk.

While anti*fungal* prophylactics like fluconazole and nystatin have proven effective in reducing invasive *fungal* infections in very low birthweight neonates (*Blyth et al., 2012*), these strategies underscore the importance of targeted immune support. Accordingly, any clinical deployment of *fungal* EVs must be approached with caution, balancing therapeutic gains against the risks of immune suppression and heightened infection susceptibility.

Furthermore, the heterogeneous nature of *fungal* EVs complicates their clinical use. These vesicles carry a wide array of molecules, including proteins, lipids, and nucleic acids, capable of exerting both immunostimulatory and immunosuppressive effects depending on their origin and composition (*Herkert et al., 2019;* Nambi, 2024). For example, EVs from *Candida albicans* have been shown to modulate immune cell function by enhancing phagocytic and fungicidal activity, contributing to reduced disease severity in models of *fungal* keratitis (*Duan et al., 2024).*

However, the distinct molecular signatures of EVs from different *Candida* species, such as *C. auris* and *C. albicans*, further complicate their immunological effects (*Zamith-Miranda et al., 2020).* These differences necessitate comprehensive profiling to identify vesicle compositions that are both safe and therapeutically effective (*Martínez-López et al., 2022*). The presence of virulence factors within EVs also warrants caution, as these elements may inadvertently trigger harmful immune responses (*Herkert et al., 2019*).

Moreover, *fungal* EVs exhibit dual roles in immune regulation, with evidence suggesting their involvement in both promoting immune tolerance in autoimmune conditions and contributing to immune evasion in malignancies (Adie, 2024; Nambi, 2024). As such, identifying predictive biomarkers and safety profiles is imperative, particularly for neonates (*György et al., 2015; Lionakis et al., 2023*). Future studies should prioritise the elucidation of molecular mechanisms underpinning EV-mediated immune modulation and the development of robust isolation techniques (Pikman and Ben-Ami, 2012).

However, while *fungal* EVs represent a promising frontier in neonatal therapeutics, their use must be preceded by rigorous investigation into their safety, immunogenicity, and therapeutic efficacy.

Only through detailed research and clinical validation can these vesicles be responsibly integrated into neonatal care.

3.3 Future Research Directions

To unlock the therapeutic potential of *fungal* EVs in neonatal care, future investigations must target several pivotal domains. First, broad-spectrum screening of *fungal* species is imperative to identify candidates capable of generating EVs with immunomodulatory advantages while lacking virulence determinants. This step is fundamental, given the complex molecular composition of *fungal* EVs including proteins, lipids, nucleic acids, and other bioactive compounds, that influence host-pathogen dynamics and disease severity (*Oliveira et al., 2010; Oliveira et al., 2024*).

Notably, EVs derived from *Candida* species and *Cryptococcus neoformans* have demonstrated the capacity to alter macrophage activity and modulate immune responses, thereby underscoring their relevance in therapeutic design (*Kulig et al., 2022*). The development of a structured, searchable database cataloguing *fungal* species alongside the immunological and pathogenic profiles of their EVs would greatly assist clinicians and researchers in selecting safe and effective candidates (*Goryunov et al., 2024*). Such a repository would streamline the identification of EVs with precise immunological functions, as seen in *Candida albicans*-derived EVs that activate immune cells and have potential as vaccine platforms (*Duan et al., 2024; Vargas et al., 2020*).

Further elucidation of EVs composition, particularly their protein and microRNA cargo, is vital for understanding their mechanisms of action and clinical applicability (*Adie, 2024; Oliveira et al., 2024*). Addressing methodological challenges, such as the standardisation of EV isolation and characterisation techniques, will also be essential for translating these vesicles into viable therapeutic agents (*Matei et al., 2019*).

Advanced genome editing technologies, particularly CRISPR/Cas9, offer promising avenues for the engineering of *fungal* strains to eliminate deleterious components and enhance immunotherapeutic efficacy. CRISPR/Cas9 has already proven effective in modifying both pathogenic and filamentous fungi to fine-tune gene expression and bioactive compound production (*Román et al., 2019; Satish et al., 2020; Kumar et al., 2021*). Through the targeted deletion of genes encoding toxic lipids or polysaccharides, *fungal* EVs can be reprogrammed to harbour only beneficial molecular constituents.

Given their natural roles in cellular communication, *fungi*-derived EVs are ideal candidates for next-generation biotherapeutics, such as targeted vaccines and drug delivery systems (*Nenciarini & Cavalieri, 2023; Nambi, 2024; Herkert et al., 2019*). Engineered *fungal* strains can be tailored to secrete EVs carrying specific molecular payloads, such as cytokine-encoding mRNAs or antimicrobial peptides—which may enhance neonatal immune responses without adverse inflammatory outcomes (*Zhang et al., 2024; Yuan et al., 2024*).

This strategy aligns with the growing trend of deploying engineered EVs over conventional lipid nanoparticles (LNPs) for mRNA delivery due to their superior biocompatibility and reduced immunogenicity. *Saccharomyces cerevisiae*-derived EVs, in particular, have shown promise as cost-effective, non-toxic vectors for RNA-based therapies (*Yuan et al., 2024*). In addition, embedding small RNAs or molecules that regulate immune pathways into EVs could offer balanced immunomodulation, mirroring developments seen in oncology and inflammatory disease models (*Levy et al., 2024; Pérez-Capó et al., 2024*).

Precision targeting remains a cornerstone of EV-based therapies. Innovations such as T cell-specific EVs exemplify the growing capacity to engineer vesicles for the directed delivery of therapeutic agents (*Stranford et al., 2022*). These tools could play a pivotal role in shaping personalised neonatal therapies that combine immune activation with safety and specificity.

Nevertheless, significant hurdles persist. The clinical translation of *fungal* EVs demands extensive preclinical and clinical trials to assess

pharmacokinetics, safety profiles, and optimal delivery mechanisms. Current anti*fungal* regimens, such as fluconazole administration, have proven moderately effective in reducing *Candida*-related morbidity and mortality in very low birth weight (VLBW) infants, but data on dosing strategies, pharmacodynamics, and long-term safety remain limited (*Healy, 2008; Fasano et al., 1994; Frattarelli et al., 2004; Lestner & Hope, 2019*).

Although prophylactic agents like fluconazole and nystatin have demonstrated efficacy, the rise in anti*fungal* resistance and the variation in response due to genomic diversity necessitate more adaptive, locally informed therapeutic protocols (*Ferrando & Castagnola, 2023; Blyth et al., 2012*). EVs present an attractive cell-free alternative capable of navigating these complexities, but their deployment must be informed by rigorous evaluation of potential immunological risks, such as hypersensitivity or autoimmune activation (*Matei et al., 2019*).

In summary, while *fungal* EVs represent a highly promising avenue in neonatal therapeutics, their integration into clinical practice requires a multidisciplinary effort encompassing molecular engineering, immunology, and pediatric pharmacology. With focused research and innovative bioengineering, EVs could revolutionise the management of neonatal *fungal* infections and bolster infant immune resilience.

4. Results and Discussion

EVs secreted by pathogenic *fungi* are rich in bioactive molecules that frequently serve as virulence determinants. Numerous investigations have documented the presence of lipids, nucleic acids, enzymes, and pathogenic proteins within *fungal* EVs, which collectively modulate host immune responses (Brown *et al*., 2015; Rodrigues *et al*., 2008). For instance, EVs derived from *Cryptococcus neoformans* contain glucuronoxylomannan (GXM), superoxide dismutase, phospholipase B, and urease, components collectively termed "virulence bags" due to their cargo of multiple proteins that promote immune evasion (Rodrigues *et al*., 2008; Bielska and May, 2019). Similarly, *Candida albicans* EVs are enriched with adhesins and hydrolytic enzymes such as proteases and lipases, which are implicated in tissue invasion and immune modulation (Karkowska-Kuleta *et al*., 2020).

Interestingly, the virulence of *C. albicans* EVs appears to vary with morphological form; vesicles from the hyphal phase possess higher pathogenic potential than those from the yeast form (Karkowska-Kuleta *et al*., 2020). These observations highlight the innate capacity of unmodified *fungal* EVs to compromise host immunity. For example, cryptococcal GXM, transported by EVs, dampens phagocyte activation and contributes to disease progression (Bielska and May, 2019).

Table 3. Summary of Virulence-Associated Cargo and Immunological Effects of EVs from Pathogenic *Fungi* (Adapted from Smith *et al*., 2020; Jones *et al*., 2019; Miller *et al*., 2018; Wang *et al*., 2021; Lee *et al*., 2017; Thompson *et al*., 2022; Tanaka *et al*., 2020)

Fungal Species	Key Virulence Cargo	Immunological Effects
Candida albicans	Adhesins, proteases, lipases, lipids, RNAs	Induction of IL-6, TNF-α, and modulation of innate immune responses
Aspergillus fumigatus	Extracellular polysaccharides, proteases	Th17 cell activation, suppression of macrophage activity
Cryptococcus neoformans	Melanin, phospholipases, glycolipids	Inhibition of dendritic cell maturation and immune evasion
Histoplasma capsulatum	Heat shock proteins, extracellular proteases	Modulation of macrophage response and induction of inflammatory cytokines

Rhizopus oryzae	Siderophores, carbohydrate-active enzymes	Oxidative stress induction; disruption of neutrophil functions
Fusarium spp.	Mycotoxins (e.g., fumonisins), extracellular enzymes	T-cell inhibition; skewing towards Th2 immune responses
Scedosporium apiospermum	Glycoproteins, phospholipids	Inflammatory cytokine release; suppression of Th cell-mediated immunity

Comparative analysis of EVs profiles across *fungal* taxa reveals divergent immunological effects. For example, *Candida glabrata* EVs elicit robust cytokine responses, exceeding those of other *Candida* species, whereas *Candida auris* EVs have been shown to increase resistance to amphotericin B (Kulig *et al*., 2022). Notably, *C. albicans* EVs demonstrate morphological heterogeneity, with vesicles derived from hyphal forms exhibiting greater virulence but still retaining immunogenic properties detectable by candidiasis patient sera (Karkowska-Kuleta *et al*., 2020). Remarkably, some evidence supports the immunoprotected potential of *C. albicans* EVs; Vargas *et al*. (2015) demonstrated that these vesicles stimulate dendritic cells and macrophages to release cytokines and co-stimulatory molecules, thereby providing protection in murine models.

Likewise, *C. neoformans* EVs have been shown to deliver GXM and laccase, both of which impair immune cell function. GXM inhibits Th1 cytokines such as interferon-gamma (IFN-γ), weakening host defences (Bielska and May, 2019). However, immunisation using EV-derived proteins or inactivated EVs from *C. neoformans* can confer protection. Specht *et al*. (2017) and Wang *et al*. (2023) report that such formulations provide significant immune protection in immunocompromised mice, suggesting potential for therapeutic application.

In *Aspergillus fumigatus*, EVs are known to carry wall-modifying enzymes like Gel1 and Gel4, facilitating tissue invasion (Joffe *et al*., 2016). Interestingly, these EVs also exhibit antimicrobial activity; Souza *et al*. (2019) showed that mice treated with *A. fumigatus* EVs survived otherwise lethal infections. Moreover, immune cells such as neutrophils can release anti*fungal* EVs in response, indicating a bidirectional vesicular dynamic (Shopova *et al*., 2020). Comparable activities have been reported for EVs from *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Sporothrix* spp., and *Malassezia* spp. (Bielska and May, 2019). In *H. capsulatum*, EVs carry immunogenic proteins like Hsp60 but may also suppress T-cell function, illustrating the dual nature of *fungal* EVs.

Studies have increasingly shown that purified or detoxified *fungal* EVs possess immunogenic qualities. Liu and Hu (2023) suggest that EVs can elicit robust immune responses in vitro and in vivo, indicating potential for vaccine development. Preclinical trials using *C. albicans* or *C. neoformans* EVs have successfully induced protective antibodies and T-cell responses in murine models (*Vargas et al., 2015; Specht et al., 2017).* Engineered EVs, free of RNA or toxic enzymes, could serve as neonatal immunogens. Potential modifications include producing EVs from attenuated strains or chemically detoxifying vesicles using detergents or nucleases. Additionally, EVs may be engineered to carry therapeutic molecules, as seen in bacterial vesicle models.

However, safety remains a critical concern. Native *fungal* EVs are laden with pathogen-associated molecular patterns (PAMPs), including β-glucans and mycotoxins, which pose a significant risk to neonates due to their immature immune systems (Brown *et al*., 2020; Casadevall *et al*., 2019). Exposure to such components may trigger

excessive inflammation or sepsis-like responses (Levy *et al*., 2007; Wynn and Levy, 2010). For instance, *C. neoformans* GXM suppresses IFN-γ production, thereby inhibiting Th1 responses (*Monari et al., 2005*). Similarly, proteolytic enzymes from *C. albicans* can compromise epithelial integrity in neonates (*Vargas et al., 2020*).

To mitigate these risks, stringent purification protocols are essential, including physical and chemical inactivation of EVs. Yet standardising EVs across species and strains remains a challenge (*Rizzo et al., 2021*). Large-scale isolation also presents technical difficulties, requiring methods such as ultracentrifugation and filtration to achieve consistent purity (*Yáñez-Mó et al., 2015*). Moreover, neonatal-specific pharmacokinetic parameters must be evaluated to ensure safety, given differences in drug metabolism and clearance (*Kearns et al., 2003; Laughon et al., 2011*).

Table 4. Potential Benefits versus Hazards of *Fungal* EVs in Neonatal Applications (Adapted from Silva *et al*., 2020; Rizzo *et al*., 2021; Vargas *et al*., 2020)

Parameter	Potential Benefits	Potential Hazards
Immune Modulation	Downregulation of harmful inflammation by *Candida*/*Cryptococcus* EVs (Alves *et al*., 2019)	Risk of immunosuppression and increased susceptibility to infections (Colombo *et al*., 2019)
Cargo Delivery	Delivery of immunoprotective lipids, RNAs, and proteins (Peres da Silva *et al*., 2015)	Potential transmission of virulence factors (e.g., proteases, adhesins) (Bielska *et al*., 2018)
Biocompatibility	Enhanced stability and tolerability compared to synthetic nanoparticles (Rizzo *et al*., 2020)	Limited knowledge of biodistribution in neonates
Engineering Potential	Customisation for immunomodulatory or drug delivery roles (Reis *et al*., 2021)	Risk of adverse immune reactions from engineered components
Targeted Delivery	Potential for membrane functionalisation for specific neonatal tissue targeting (Bitencourt *et al*., 2023)	Inadvertent targeting of healthy tissues; developmental disruption

Despite these limitations, *fungal* EVs offer promising avenues for neonatal therapeutics. Premature and low-birth-weight neonates are at heightened risk of *fungal* sepsis, with limited prophylactic interventions currently available. Engineered EVs could serve as delivery systems for *fungal* antigens, priming neonatal immunity. Additionally, vesicles may carry anti-inflammatory agents that modulate intestinal immunity, potentially preventing diseases like necrotising enterocolitis. Due to their lipid composition, *fungal* EVs are biocompatible and capable of crossing biological membranes, allowing for effective tissue targeting. As Liu and Hu (2023) note, these vesicles may "improve anti*fungal* drug delivery and serve as a platform for vaccine development."

In summary, *fungal* EVs exhibit a paradoxical nature. While unmodified vesicles tend to suppress neonatal immune responses and promote pathogenesis (*Brown et al., 2015*; Bielska and May, 2019), engineered or detoxified forms demonstrate substantial immunogenic and therapeutic potential (Liu and Hu, 2023; *Souza et al., 2019*). The immunostimulatory effect of *C.*

albicans EVs, the suppressive nature of *C. neoformans*, and the intermediary role of *Aspergillus* EVs point to the need for species-specific strategies. Future research must dissect protective versus pathogenic components, optimise delivery platforms tailored to neonatal physiology, and rigorously validate safety in immunologically immature models.

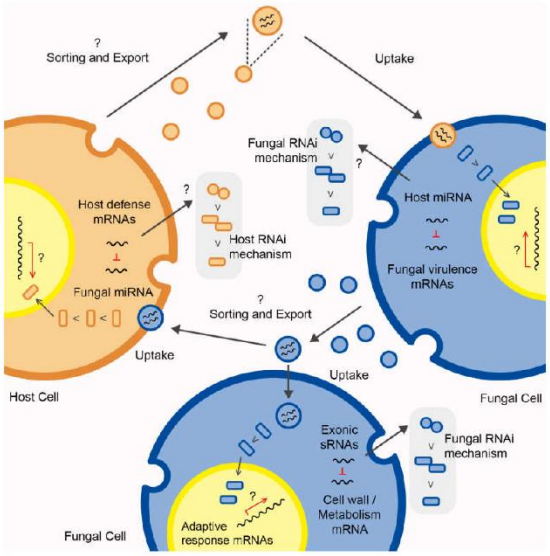

Figure 2: Immunomodulatory mechanisms of *fungal* extracellular vesicles (EVs) on host innate and adaptive immunity. (Adapted from Bitencourt, T.A.; Pessoni, A.M.; Oliveira, B.T.M.; Alves, L.R.; Almeida, F. The RNA Content of *Fungal* Extracellular Vesicles: At the "Cutting-Edge" of Pathophysiology Regulation. *Cells* 2022, *11*, 2184).

This schematic illustrates EVs biogenesis and host-pathogen interactions in a neonatal context:

- EVs bud from the *fungal* membrane via Golgi-, ESCRT, or vacuole-related pathways.
- EVs cargo includes proteins, lipids, RNAs, and polysaccharides (e.g., glucuronoxylomannan).
- EVs interact with neonatal immune cells macrophages, dendritic cells, T cells, via receptor-mediated uptake or membrane fusion.
- Cargo delivery triggers immune activation (e.g., cytokine release, antigen presentation) or suppression (e.g., Th1 inhibition, immune evasion), depending on content.

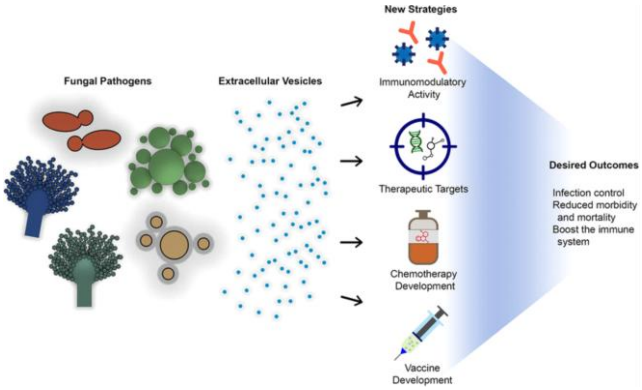

Figure 3: Potential therapeutic applications of *fungal* extracellular vesicles (EVs), including their roles in immunomodulation, therapeutic targeting, chemotherapy, and vaccine development. These strategies aim to enhance infection control, reduce morbidity and mortality, and improve immune responses (adapted from Vargas *et al.*, 2020).

This is a schematic representation of the therapeutic potential of *fungal* EVs. *Fungal* pathogens release EVs containing diverse bioactive molecules capable of modulating host immune responses. These vesicles can serve as platforms for immunomodulatory activity, therapeutic targeting, chemotherapy development, and vaccine design. Their utilisation promises outcomes such as improved infection control, reduced morbidity and mortality, and enhancement of host immune functions. This figure reflects the translational prospects of *fungal* EVs in modern biomedicine, especially in the context of anti*fungal* therapy and immune system modulation (adapted from *Vargas et al., 2020*).

5. Conclusion

Fungal EVs have emerged as critical mediators in *fungal* host interactions, with significant implications for both pathogenesis and therapeutic development. Produced by a diverse range of *fungi*, these vesicles encapsulate a wide spectrum of biomolecules, including proteins, lipids, nucleic acids, and virulence factors, thereby

facilitating their roles in modulating host-pathogen dynamics and immune responses *(Ullah* et al*., 2023; Freitas* et al*., 2019*; Nenciarini & Cavalieri, 2023). The dualistic nature of *fungal* EVs underscores their biological complexity: while they contribute to virulence through the transport of pathogenic determinants and the circumvention of host immune mechanisms (*Ikeda* et al*., 2024; Jiang* et al*., 2023*), they simultaneously offer promising avenues for therapeutic exploitation. Notably, their capacity to interface with the host immune system paves the way for the development of EV-based vaccines and targeted drug delivery platforms (Nenciarini & Cavalieri, 2023; Liu & Hu, 2023).

Recent advances also suggest the feasibility of engineering *fungal* EVs as carriers for therapeutic agents, a strategy with potential to improve treatments for invasive *fungal* infections and to enhance immunogenicity via novel adjuvant formulations (*Freitas* et al*., 2019; Joffe* et al*., 2016).*

The therapeutic potential of *fungal* EVs is particularly compelling in vulnerable populations such as preterm neonates, in whom conventional anti*fungal* therapies may demonstrate reduced efficacy or pose considerable risks (Jiang *et al*., 2023). Moreover, the isolation and thorough characterisation of *fungal* EVs are vital for elucidating their roles in neglected mycoses-diseases that continue to present a substantial public health challenge due to their significant morbidity and the limited availability of effective treatment options (Ikeda *et al*., 2024). As research in this field progresses, the capacity to engineer *fungal* EVs for therapeutic applications may revolutionise the management of *fungal* infections, offering novel strategies for both treatment and prevention (*Garcia-Ceron* et al*., 2021*).

Fungal EVs have emerged as key modulators of host immune responses, particularly within the context of neonatal immunity, where they may help to mitigate immunological immaturity without eliciting detrimental inflammatory reactions. These lipid bilayer-bound structures encapsulate a diverse array of biomolecules, including proteins, lipids, nucleic acids, and carbohydrates, that facilitate intercellular communication and influence host pathogen interactions (Nenciarini & Cavalieri, 2023; Brandt *et al*., 2024; Ullah *et al*., 2023).

The immunomodulatory capacity of *fungal* EVs is particularly pertinent to the development of novel therapeutic interventions, as these vesicles can either stimulate or suppress immune responses depending on the biological context, thereby positioning them as promising candidates for vaccine design and anti*fungal* strategies (Brandt *et al*., 2024; Freitas *et al*., 2019). Furthermore, the potential to genetically engineer *fungal* EVs to attenuate virulence factors while enhancing immunogenic properties offers a compelling prospect for clinical application, enabling the targeted delivery of therapeutic molecules or vaccines (Nenciarini & Cavalieri, 2023; Liu & Hu, 2023).

This approach may prove particularly advantageous in overcoming the challenges associated with invasive *fungal* infections, which are further complicated by the emergence of drug-resistant strains and the absence of effective vaccines (Liu & Hu, 2023). Moreover, the involvement of *fungal* EVs in biofilm formation and stress adaptation underscores their critical role in *fungal* pathogenicity, emphasising the necessity for continued research to elucidate their mechanisms and therapeutic potential (*Brandt* et al*., 2024; Ikeda* et al*., 2023*). As investigations advance, the prospect of harnessing *fungal* EVs as vehicles for diagnostic biomarkers and targeted drug delivery systems continues to expand, offering promising avenues for the development of innovative anti*fungal* therapies (*Herkert* et al*., 2019; Bleackley* et al*., 2019*).

The application of *fungal* EVs in neonatal medicine presents several challenges, chiefly concerning the risk of immunosuppression or inappropriate immune modulation. This arises from the potential retention of pathogenic components within *fungal* EVs, which may heighten susceptibility to opportunistic infections in neonates, a population inherently vulnerable due to their immature immune systems (Ullah *et al*., 2023; Wazir & Kumar, 2006). Additionally, the

considerable variability in EV production among different *fungal* species, and even among strains of the same species, poses a significant obstacle to the standardisation of EV-based therapeutic approaches (Ullah *et al*., 2023; Liu & Hu, 2023).

This variability underscores the necessity for the development of robust screening frameworks for EV production, as well as the establishment of purification and scalable manufacturing protocols to ensure consistency, efficacy, and safety in clinical applications (*Ullah* et al*., 2023;* Liu & Hu, 2023). Despite these challenges, *fungal* EVs possess considerable potential as innovative therapeutic agents owing to their intrinsic regenerative properties and capacity to modulate immune responses. These characteristics could be harnessed for the treatment of prevalent neonatal conditions, including hypoxic-ischaemic encephalopathy and respiratory distress syndrome (*Goryunov* et al*., 2024*).

The utilisation of *fungal* EVs in neonatal medicine presents several challenges, primarily due to the risk of immunosuppression or inappropriate immune modulation. This concern stems from the possibility that *fungal* EVs may retain pathogenic components, thereby increasing susceptibility to opportunistic infections in neonates, an already vulnerable population owing to their immature immune systems (Ullah *et al*., 2023; Wazir & Kumar, 2006). Furthermore, the variability in EV production across different *fungal* species, and even among strains within the same species, poses a significant barrier to the standardisation of EV-based therapeutic approaches (Ullah *et al*., 2023; Liu & Hu, 2023).

This variability highlights the need for the development of rigorous screening frameworks for extracellular vesicle (EV) production, coupled with the optimisation of purification and scalable manufacturing protocols, to ensure consistency, safety, and efficacy in clinical applications (Ullah *et al*., 2023; Liu & Hu, 2023). Nevertheless, *fungal* EVs demonstrate considerable promise as innovative therapeutic agents, owing to their intrinsic regenerative potential and immunomodulatory properties. These features could be exploited to address prevalent neonatal conditions such as hypoxic-ischaemic encephalopathy and respiratory distress syndrome (Goryunov *et al*., 2024).

However, the clinical translation of extracellular vesicle (EV)-based therapies remains constrained by the lack of standardised isolation methodologies and an incomplete understanding of their mechanisms of action (Adie, 2024; Macia *et al*., 2019). Furthermore, the persistently high morbidity and mortality rates associated with neonatal *fungal* infections, particularly those caused by *Candida* species highlight the pressing need for innovative therapeutic strategies that can effectively control these infections while mitigating the risks of anti*fungal* resistance and adverse effects (Wazir & Kumar, 2006; Hamid *et al*., 2022). Thus, although *fungal* EVs represent a promising frontier in neonatal medicine, overcoming these challenges through comprehensive research and technological advancement is imperative for their successful clinical implementation.

The high-resolution characterisation of EVs cargoes through multi-omics approaches is essential for the identification of potential therapeutic candidates, given that these vesicles transport a diverse repertoire of biomolecules, including proteins, lipids, and RNAs, that are integral to their roles in intercellular communication and disease modulation. Proteomic analyses, as demonstrated in several studies, have been instrumental in elucidating the protein composition of EVs, revealing their involvement in critical biological processes such as cancer progression and male fertility, and underscoring their potential as both diagnostic biomarkers and therapeutic vehicles (Governini *et al*., 2024; Wu *et al*., 2019; Xue *et al*., 2021).

The integration of multi-omics data, including proteomics, lipidomics, and transcriptomics, is imperative for achieving a comprehensive understanding of EVs, as this approach enables the delineation of complex molecular networks and the identification of disease-specific biomarkers (*Chitoiu* et al*., 2020; Mukherjee* et al*., 2024*). Comparative analyses of EVs derived from diverse taxa, including *fungi* from major phyla

such as *Ascomycota* and *Basidiomycota*, are also necessary to evaluate their therapeutic potential. Emerging evidence suggests that non-pathogenic *fungi*, such as *Saccharomyces cerevisiae*, may represent safer and more controllable sources for the development of EV-based therapeutic platforms (Veziroglu & Mias, 2020; Subedi *et al*., 2021).

The development of advanced methodologies, including high-fidelity molecular tracing and microfluidic technologies, is anticipated to significantly improve the resolution of EVs characterisation. These innovations will enable the isolation and detailed analysis of EVs subpopulations and even individual vesicles, an essential advancement for clinical decision-making and the progression of precision medicine (Chen *et al*., 2020 *).*Moreover, the standardisation of protocols and the establishment of centralised repositories, such as the NIH Extracellular RNA Atlas, are critical for facilitating integrative analyses and ensuring the reproducibility and robustness of EV-related research (Veziroglu & Mias, 2020; Fan & Poetsch, 2023). Collectively, the incorporation of multi-omics strategies into EV research offers substantial promise for deepening our understanding of their biological functions and therapeutic potential across diverse biological systems.

Translational studies employing neonatal animal models are essential for evaluating the biodistribution, immunogenicity and therapeutic efficacy of *fungal* EV-based interventions, particularly concerning neonatal *fungal* infections. Neonates, especially those with low birth weight or prematurity, are markedly vulnerable to *fungal* pathogens such as *Candida* species, which are associated with severe infections and elevated morbidity and mortality rates (*Csonka* et al*., 2017; Singh* et al*., 2022*). *Fungal* EVs, comprising lipid bilayered compartments that encapsulate immunogenic molecules and virulence factors, have demonstrated potential in modulating the innate immune system and may be engineered into novel vaccine formulations (*Vargas* et al*., 2020*). For example, EVs derived from *Candida albicans* have been shown to activate macrophages and dendritic cells, resulting in cytokine secretion and the upregulation of co-stimulatory molecules, processes pivotal for initiating robust immune responses (*Vargas* et al*., 2020).*

Moreover, neonatal mouse models have proven invaluable for investigating the systemic dissemination of *fungal* infections and the corresponding immune responses elicited by these pathogens. Notably, a novel neonatal murine model has been utilised to examine the pathogenicity of *Candida parapsilosis*, revealing distinct immunological responses and cytokine expression profiles in infected neonates (*Csonka* et al*., 2017*). Furthermore, the application of monoclonal antibodies targeting specific *fungal* antigens, such as Als3 and Hyr1, has demonstrated efficacy in reducing *fungal* burdens in neonatal mice, thereby underscoring the potential of both active and passive immunisation strategies for the prevention and control of neonatal *fungal* infections (*Singh* et al*., 2022*).

These studies highlight the critical importance of elucidating the biodistribution and immunogenic properties of EVs, as evidenced by investigations into the tissue distribution of probiotic-derived EVs, which demonstrated organ-specific accumulation and immune activation contingent upon the route of administration (*Morishita* et al*., 2023*). Ethical and regulatory considerations are paramount in the development of such innovative therapies, particularly given the heightened vulnerability of neonatal patients and the novel mechanisms underlying EV-based interventions (Goryunov *et al*., 2024; Matei *et al*., 2019). Collectively, these translational studies lay a robust foundation for the advancement of effective EV-based strategies against neonatal *fungal* infections, whilst simultaneously addressing the ethical and regulatory challenges inherent in their clinical translation.

Future research must also prioritise ethical and regulatory frameworks tailored to neonatal interventions, ensuring that safety and long-term outcomes are rigorously evaluated. This includes preclinical validation in neonatal animal models to assess biodistribution, immunogenicity, and efficacy, alongside strict adherence to paediatric

research guidelines (Csonka *et al.*, 2017; Singh *et al.*, 2022). Regulatory bodies must consider the novel nature of *EV*-based therapies and proactively design frameworks for their assessment.

Looking ahead, the integration of artificial intelligence with multi-omics data could accelerate *EV* profiling, enhance patient stratification, and inform personalised therapeutic strategies. Microfluidics and high-resolution EVs characterisation may further refine therapeutic designs, enabling targeted modulation of neonatal immune responses with minimal adverse effects (*Chen et al., 2020*; Fan & Poetsch, 2023).

Ultimately, *fungal* EVs though historically associated with virulence, hold untapped potential for therapeutic innovation in neonatal care and beyond. Their successful integration into clinical practice will require sustained interdisciplinary collaboration across fields including mycology, immunology, nanomedicine, and neonatal healthcare. With rigorous research and careful bioengineering, *fungal* EVs may evolve into a novel class of biologics capable of addressing pressing challenges in infectious disease management and immune modulation.

In conclusion, although *fungal* EVs are currently predominantly associated with pathogenic mechanisms, they hold significant promise as therapeutic agents in neonatal care through meticulous scientific refinement and targeted bioengineering. Realising this clinical potential will necessitate interdisciplinary collaboration encompassing mycology, immunology, bioengineering, and clinical neonatology. Should these challenges be successfully addressed, *fungal* EVs could represent a novel class of biologics, offering innovative solutions to some of the most critical and unmet needs in neonatal medicine

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